JOANA ANDREIA JOAQUIM DA SILVA

Characterization of the behavioural phenotype of calpain-knockout mice

UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

2016

JOANA ANDREIA JOAQUIM DA SILVA

Characterization of the behavioural phenotype of calpain-knockout mice

Tese de Mestrado em Ciências Biomédicas

Trabalho efectuado sob a orientação de:

Professora Doutora Inês Maria Pombinho de Araújo

UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

2016

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída,

(Joana Andreia J. da Silva)

Nas grandes batalhas da vida,

o primeiro passo para a vitória é o desejo de vencer!

(Mahatma Gandhi)

Ao papá, à mamã e à mana, Paulo, Cláudia e Carolina,

pelo encorajamento, dedicação e apoio que sempre me deram

e pelos valores que me transmitiram ao longo dos anos.

AGRADECIMENTOS

É com grande satisfação que agradeço profundamente a todos aqueles que contribuíram de alguma forma para a realização desta tese de mestrado que é mais uma etapa na minha formação académica.

Primeiramente, quero agradecer à Professora Doutora Inês Araújo, minha orientadora, por ter permitido a realização desta tese na minha área de interesse. Agradeço também por todo o apoio prestado ao longo desta etapa e por me permitir crescer academicamente.

A todas as minhas colegas de laboratório, Vanessa, Sofia, Adélia e Sónia que de alguma forma contribuíram para realização desta tese. Em especial à Vanessa por todo o conhecimento que me foi transmitido sobre as calpaínas e testes de comportamento, bem como o rigor em todas as experiências realizadas e pela disponibilidade em ajudar e dar apoio. Agradeço também à Sofia por me ter transmitido valores essenciais de trabalho em laboratório e pela disponibilidade em ajudar no que podia, mesmo não estando a trabalhar com calpaínas.

Aos alunos de monografia e de medicina por terem cativado em mim a vontade de transmitir o meu conhecimento aos outros.

Ao senhor Paulo Jorge pela disponibilidade e amabilidade em fazer os labirintos que foram utilizados nos testes de comportamento.

Aos senhores jardineiros que tiveram a amabilidade de nos ajudar, no início, a encher e a vazar a piscina destinada aos testes de comportamento com ratinhos.

Aos meus primos João e Vânia, pela dedicação e por todo o tempo despendido de volta da fantástica capa amovível, para que ficasse exactamente como idealizei.

O meu mais profundo agradecimento é dirigido aos meus pais e à minha irmã, por todo o apoio incondicional que me deram ao longo desta caminhada, bem como em toda a minha vida. Pelo encorajamento e aconselhamento em dias difíceis, mas também por toda a alegria e amor que me transmitiram e continuam a transmitir.

ABBREVIATIONS

C

Capn1 KO ……..……..Calpain1 knockout

Capn2 KO ……..……..Calpain2 conditional knockout

cDKO ……..……..Conditional double knockout for calpain1 and calpain2

Cre Ctrl ……..……Mouse with Cre control

D

DCX ……..Doublecortin

E

EdU ……5-ethynyl-2'-deoxyuridine

G

GZ ……...Granular zone

N

NHS ……..Normal Horse Serum

P

PBS ……..……..Phosphate Buffer Solution

PFA ……Paraformaldehyde

T

TAE Buffer ……..……Tris-acetate-EDTA Buffer

S

SGZ ……Subgranular zone

V

V-SVZ ……..Ventricular-subventricular zone

W

WT ……..……Wild Type

TABLE OF CONTENTS

ABSTRACT

Adult neurogenesis consists of the production of new neurons in specific brain regions, or neurogenic niches. The most relevant niches in rodents are the subventricular zone lining the lateral ventricles and the dentate gyrus of the hippocampus. Neurogenesis has been shown to influence cognitive function dependent on these regions. Evidence from the literature suggests that calpains are able to influence neurogenesis. However, little information is available regarding their participation in neurological functions. We have evaluated the involvement of calpains in cognitive and emotional behaviour by evaluating these functions in mice genetically modified to lack calpain 1, calpain 2, or both calpains.

In this work, 12 week old calpain knockout mice for calpain 1, calpain 2, both, or wild type (WT) littermates were used. The mice were tested in the open field, object recognition, Morris water maze, contextual and cued fear conditioning, passive avoidance, elevated plus maze and forced swimming tests, to evaluate neurological function. Results from the elevated plus maze show that calpain2 knockout mice and double knockout mice present an anxious phenotype comparing with WT mice, suggesting that calpain 2, but not calpain 1, is involved in anxiety. Memory, learning, locomotor activity and exploratory behaviour as well as helplessness were similar in calpain knockout and WT mice. Neurogenesis in calpain knockout mice was also similar to WT mice.

Overall, our work shows that calpain 2 is involved in anxiety and a clear phenotype was identified in calpain knockout mice regarding their involvement in memory and learning, which is agreement with previously published data using mice that lack the small regulatory subunit of calpains.

KEY WORDS: *Calpain1, Calpain 2, cognitive behaviour, neurogenesis, memory, anxiety, hippocampus*

RESUMO

A neurogénese corresponde ao processo pelo qual novos neurónios são formados e ocorre em regiões específicas do cérebro, chamadas de nichos neurogénicos, tais como no hipocampo e nas paredes dos ventrículos laterais. Actualmente, sabe-se que a neurogénese está envolvida em funções cognitivas dependentes dessas mesmas regiões. Para além disso, tem-se vindo a identificar a influência das calpaínas na neurogénese, mas ainda pouco se sabe sobre de que forma as calpaínas influenciam este processo. Neste trabalho, de modo a avaliar o envolvimento das calpaínas no comportamento cognitivo e emocional, foram utilizados murganhos geneticamente modificados (murganhos sem calpaína1, sem calpaína2 e sem as duas).

Neste trabalho, utilizámos murganhos com 12 semanas geneticamente modificados e os que apresentavam um genótipo WT, foram utilizados como controlo. De modo a avaliar as funções neurológicas, estes murganhos foram submetidos a vários testes de comportamento: campo aberto, reconhecimento de objectos, labirinto aquático, condicionamento por medo associado ao contexto e estímulo, esquiva aversiva, labirinto elevado em forma de cruz e natação forçada. No teste do labirinto elevado em forma de cruz, murganhos com knockout para a calpaína2 e para as duas calpaínas, apresentaram um fenótipo mais ansioso comparativamente com os murganhos WT. Estes resultados sugerem que a calpaína2, mas não a calpaína1, está envolvida na ansiedade. A memória, aprendizagem, actividade motora e exploratória, bem como a tendência para a depressão foram semelhantes nos murganhos knockout e nos murganhos WT.

No geral, o nosso trabalho mostra que a calpaína2 está envolvida na ansiedade. No entanto, o fenótipo identificado nos murganhos knockouts relativos à memória e aprendizagem é normal. Estes resultados estão de acordo com informação publicada anteriormente em murganhos sem a subunidade pequena das calpaínas.

PALAVRAS-CHAVE: *Calpaína1, Calpaína2, comportamento cognitivo, neurogénese, memória, ansiedade, hipocampo.*

1. INTRODUCTION

1.1 NEUROGENESIS

For a long time, the brain was considered an immutable organ (Diamond 2001). However, with the development of new techniques, this dogma was gradually disassembled.

In 1890, William James introduced the term plasticity to the neurosciences for the first time by observing that behaviour could modify the human brain (James 1890). But only in 1904, Ramon y Cajal associated plasticity with neurons, throughout his description of neuronal structure (Ramón y Cajal 1904). However, Ramon y Cajal determined that neurogenesis could only occur in a developing brain, and not in an adult brain. They created a dogma that was widely accepted by the scientific community for many years (Pascual-Leone et al. 2005).

In 1962, Altman, with an autoradiographic investigation using thymine, observed proliferation of neurons, suggesting the occurrence of neurogenesis in the brain of rats(Altman 1962). However, this discovery was widely disregarded at the time.

Raisman, in 1969, induced a selective lesion in the brain of an adult rat and showed the formation of new synapses after the injury (Raisman 1969).

The neurogenic mechanism was also intensively studied in other species: birds (Goldman and Nottebohm 1983), lizards (Lopez-Garcia et al. 1988), fish (Kaslin et al. 2008) and monkeys (Kornack and Rakic 1999). The field of cerebral plasticity research was influenced tremendously by the fact that neurogenesis can also occur in the adult brain.

Nowadays, there are still many aspects of neurogenesis that are not well understood and are being actively investigated.

1.1.1 ADULT NEUROGENESIS

Human adult neurogenesis comprises the formation of new neurons in specific regions of the human brain. Neurogenesis occurs throughout life mainly in the hippocampus and in the wall of lateral ventricles (Kuipers et al. 2014).

Neurogenesis has also been described to take place in other brain regions such as in the neocortex (Cameron and Dayer 2008), piriform cortex (Bernier et al. 2002), striatum (Ernst et al. 2014) and hypothalamus (Kokoeva et al. 2005).

1.1.1.1 NEUROGENIC NICHES

In the adult mice brain, neurogenesis occurs in the dentate gyrus, similarly as in humans. It also occurs in the ventricular-subventricular zone (V-SVZ) of lateral ventricle walls. In V-SVZ, newborn neurons leave the neurogenic niche and migrate towards the olfactory bulb, through the rostral migratory stream (RMS). In the olfactory bulb, neuroblasts (newborn neurons) differentiate into interneurons (granule and periglomerular cells) and integrate the neuronal olfactory circuit. (Fuchs and Flugge 2014, Ming and Song 2011).

Neurogenesis in humans occurs in two distinct zones of the human brain: hippocampus (in the dentate gyrus) and in ventricles (lining the ventricle walls, in the ventricular-subventricular zone (V-SVZ). In those regions, neuronal stem cells (NSC) proliferate continuously, maintaining the neurogenic niche and leading to the formation of new neurons.

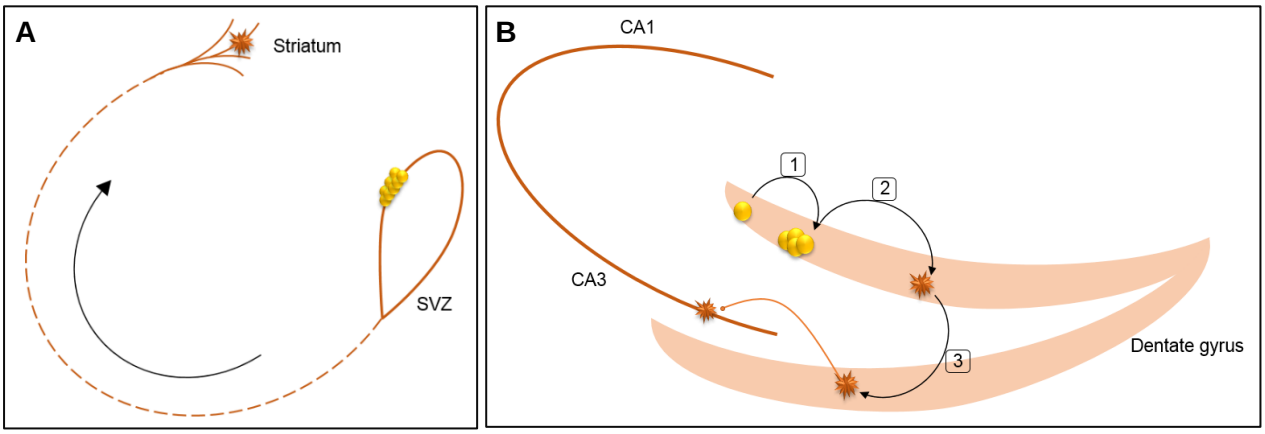

Figure 1.1 Representative images of the two neurogenic niches in humans. A, in the ventricular-subventricular zone (V-SVZ), neuroblasts formed in the V-SVZ migrate to the striatum where they differentiate into mature neurons; **B,** immature neurons of the subgranular zone, migrate to the granular zone where they differentiate into mature neurons.

In the V-SVZ, neuronal stem cells give rise to neuroblasts (newborn neurons), leave the neurogenic niche and migrate toward the striatum where they differentiate into mature neurons (Figure 1.1) (Ernst et al. 2014).

Initially, it was thought that neuroblasts migrated to the olfactory bulb, as explained previously for mice. However, in humans, it has recently been demonstrated that neuroblasts migrate to the striatum and not to the olfactory bulb (Bergmann et al. 2012, Ernst et al. 2014). These findings are also supported by the study of neuroblasts marked for doublecortin, where it was found that the number of stained neuroblasts in the striatum was similar to those in the hippocampus (Kang et al. 2011). Additionally, Ernst and Frisé also analysed ^{14}C incorporated in the human body after the nuclear bomb test and after the nuclear explosion (Spalding et al. 2013). Ernst found high incorporations of this radioisotope in striatum and hippocampus (Ernst et al. 2014).

The dentate gyrus is composed of two main layers: granular zone (GZ) and subgranular zone (SGZ). Newborn neurons (immature neurons) are formed in this neuronal niche in the SGZ and then proliferate and migrate to the GZ (Figure 1.2). In the granular zone, immature neurons differentiate into mature neuronal cells (granular cells) that project their axons to the CA3 (Taupin 2006), which in turn extend their axons to the CA1.

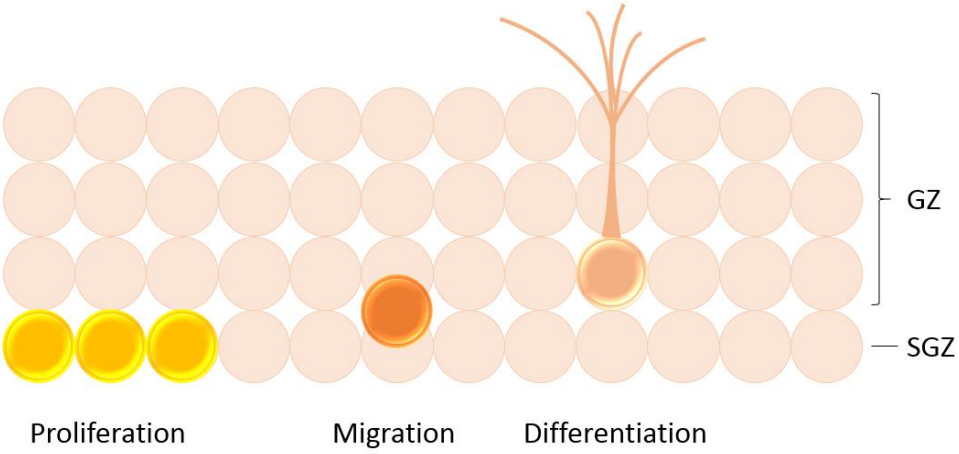

Figure 1.2 The maturation of newborn neurons in the dentate gyrus. Neuronal stem cells, in the subgranular zone (SGZ), go through proliferation, followed by migration and differentiation to the granular zone (GZ) or granular zone. (Adapted from (Lucassen et al. 2010))

1.1.1.2 INTEGRATION OF NEUROBLASTS IN NEURONAL CIRCUITS

Mature neurons, in the GZ, expand their axons (mossy fibres) to the CA3 zone (Zhao et al. 2006). In that zone, these neurons play a role in spatial learning, long-term spatial memory retention, trace conditioning, and contextual fear conditioning (Ming and Song 2011).

Briefly, the dentate gyrus and CA3 are involved in the rapid spatial processing (essential for episodic memory formation) (Hunsaker et al. 2008). The dentate gyrus receives the information from the various areas of the brain and encodes it, to make it useful for CA3. In turn, CA3 processes spatial information and sends the information to the CA1 that binds both the spatial and temporal contexts and sends the information to the entorhinal cortex for long-term storage (Hunsaker et al. 2008, Kempermann et al. 2004).

Mature neurons originated from the V-SVZ, migrate to the striatum. In the striatum, they play a major role in cognitive flexibility. Until recently, the striatum was thought to be essentially associated with motor functions. Nowadays, the involvement of striatum in cognitive functions is widely accepted (Ernst and Frisen 2015).

Mature neurons, in the olfactory bulb, are involved in the olfactory memory and olfactory circuit plasticity (Ernst and Frisen 2015). Moreover, neurons in the olfactory bulb send axons to the hippocampus, which in turn is involved in some olfactory memory tasks (Dusek and Eichenbaum 1997).

1.1.2 REGULATION OF NEUROGENESIS

Neurogenesis can be regulated by a wide range of factors, both intrinsic and extrinsic. In this section, some of these factors will be discussed.

1.1.2.1 INTRINSIC FACTORS

Ageing is one of the intrinsic factors that regulate neurogenesis. It is widely known that neurogenesis decreases with age in a diversity of mammals. This phenomenon also happens in humans and it was first described by Manganas (Manganas et al. 2007).

Hormones can also regulate neurogenesis. Estrogen, an adult sex steroid, can transiently stimulate proliferation, one of the phases of neurogenesis in the dentate gyrus (Tanapat et al. 1999). These stimuli depend on the estrous cycle, reproduction, stress and ageing (Pawluski et al. 2009).

BDNF, the brain-derived neurotrophic factor, enhances brain plasticity and modulates brain energy metabolism (Rothman et al. 2012). Moreover, BDNF increases the survival of newborn neurons and increases neurogenesis in the dentate gyrus (Rossi et al. 2006).

Vascular endothelial growth factor (VEGF) enhances cell proliferation in V-SVZ and SGZ in mice exposed to an enriched environment or having performed voluntary exercise (for example in a running wheel) (Cao et al. 2004). VEGF also influences learning and memory processes and modulates the synaptic transmission (see (Warner-Schmidt and Duman 2007) for review). Interestingly, VEGF also mimics the action of antidepressants in the behavioural tests such as the forced swimming test (Zhao et al. 2008).

1.1.2.2 EXTRINSIC FACTORS

Stress is a factor that can modulate negatively or positively neurogenesis. When people are exposed to a small amount of stress (stressful challenges), it can be beneficial for brain plasticity and can even, in some cases, increase neurogenesis (Kuipers et al. 2014). However, when exposed to high levels of stress, or chronic stress, it may lead to physical and psychiatric diseases such as major depressive disorder, anxiety disorders, posttraumatic stress disorder and schizophrenia (Fuchs and Flugge 2014). Chronic stress also decreases, in some cases, the proliferation and survival of new neurons in the dentate gyrus (Ferragud et al. 2010).

Recently, the diet has been described as a positive modulator of neurogenesis when having an appropriate dietary intake. Diet is also a cost-effective and non-invasive way to prevent and improve the quality of life of some neuropsychiatric disorders such as Alzheimer's disease. An appropriate diet can also benefit cognition and mood (Murphy et al. 2014).

Studies in mice have demonstrated that exercise also modulates neurogenesis. Voluntary exercise, such as running, can increase cell proliferation, survival and neuronal differentiation in the dentate gyrus of mice. Van Praag saw that the number of surviving neurons, when mice performed voluntary exercise, was similar to the ones related to the enrichment of the environment (Kempermann et al. 1997, van Praag et al. 1999).

Acute sleep deprivation, in adult rats, increases the proliferation of granule cell precursor and even lead to symptoms of depression (Grassi Zucconi et al. 2006). However, if the sleep deprivation is prolonged, it can act as a trigger to depression and anxiety disorders (Kahn-Greene et al. 2007).

Social interaction studies in mice found that this type of interactions enhances proliferation and integration of new neurons in the olfactory bulb. In the case of mice, this social interaction is important to prevent inbreeding, learn the odour of the offspring and even to avoid pregnancy block (Bruce effect) (Peretto and Paredes 2014).

1.1.3 NEUROGENESIS AND DISEASES

Alterations in neurogenesis may be related to both acute neurological conditions and neurodegenerative disorders, as discussed below.

Parkinson's disease is characterised by several symptoms such as bradykinesia, rigidity, resting tremor and postural instability. This disease is caused by a degeneration of dopaminergic neurons in the *substantia nigra* and by the presence of intracytoplasmic protein aggregates (Lewy body inclusions) (Esteves et al. 2010). In this disease, adult neurogenesis

is severely affected and in transgenic animal models an impairment of proliferative activity was observed (Winner et al. 2011).

Patients with Alzheimer's disease develop progressively olfactory deficits, memory impairment and severe neurodegeneration, with cognitive and functional deterioration. This disease is caused by the accumulation of amyloid-β peptide (Aβ) and is characterized by a neuronal and synaptic loss. In an early stage, stimulation of the endogenous neurogenesis may promote the formation of newborn neurons that could somehow prevent the neuronal and synaptic loss. In contrast, those newborn neurons are synthesized in a lower rate than the degeneration of the mature neurons, leading to a progressive loss of neuronal functions (Mu and Gage 2011).

Huntington's disease is caused by a mutation by repetition of the CAG sequence in the huntingtin gene (MacDonald et al. 1993). Patients with this illness develop symptoms such as choreatic movements and cognitive decline (Walker 2007). In patients with Huntington's disease, high levels of proliferation and neurogenesis were observed in V-SVZ. This findings may indicate that immature neurons are migrating to the caudate nucleus in response to the severe neuronal cell loss (Curtis et al. 2003). Neuroblasts in the striatum were also present, but could not mature, indicating the presence of a toxic microenvironment or even an incapacity of neuroblats to differentiate (Winner and Winkler 2015).

Brain damage originated by stroke, epileptic episodes or traumatic brain injury create an environment that stimulates the proliferation of neural stem cells. Newly formed neuroblasts migrate to the damaged region, but not all survive because of the toxic environment that was created. The surviving neuroblats are not displayed in an organized manner. This way, when stimulated, those neurons create an excitotoxic environment that is susceptible for new epileptic crises to occur, for example. It is important to understand, that after a traumatic brain injury or stroke, the probability to develop epilepsy or have epileptic seizures increases significantly (reviewed by Sawada and Sawamoto 2013).

However, there are still a lot of questions without a solution that needs a deeper investigation, and advances in technology are also required (reviewed by Marxreiter et al. 2013).

1.2 CALPAINS

1.2.1 BIOLOGY OF CALPAINS

Calpains are a family of calcium-dependent, neutral, soluble and cysteine proteases that have been identified in a variety of organisms: vertebrates, nematodes, insects (ex.: *Drosophila melanogaster)*, yeast (ex.: budding yeast), bacteria and even in plants and fungi (Sorimachi and Suzuki 2001).

Calpains were first described by Gordon Guroff in 1964 and nowadays, there are 15 isoforms described in the human genome (Table 1.1) (Guroff 1964). However only two of them are ubiquitously expressed in the human brain (more specifically in neurons and glia): μ-calpain (calpain1) and m-calpain (calpain2), commonly named as the conventional calpains (Sorimachi and Suzuki 2001). The nomenclature of *μ* and *m* refers to the optimal amount of calcium needed to activate these proteins *in vitro*: 10–50 μM and 0.3–0.35 mM, respectively (Baudry et al. 2013, Dutt et al. 2006).

Calpains perform their function by modifying the 3D structure of its substrates by cleavage and, therefore, hampering the function of the target proteins (Baudry and Bi 2016, Baudry et al. 2013, Zadran et al. 2013).

Table 1.1 Calpain isoforms and tissue-specific expression in humans.

Calpain	Calpain Gene	Tissue-specific
Calpain1	*CAPN1*	Ubiquitous
Calpain2	*CAPN2*	Ubiquitous
Calpain3	*CAPN3*	Skeletal muscle, lens and retina
Calpain5	*CAPN5*	Ubiquitous (high in colon, small intestine and testis)
Calpain6	*CAPN6*	Embryonic muscle, placenta
Calpain7	*CAPN7*	Ubiquitous
Calpain8	*CAPN8*	Gastrointestinal tract (stomach mucosa)
Calpain9	*CAPN9*	Gastrointestinal tract
Calpain10	*CAPN10*	Ubiquitous
Calpain11	*CAPN11*	Testis
Calpain12	*CAPN12*	Hair follicle
Calpain13	*CAPN13*	Ubiquitous
Calpain14	*CAPN14*	Oesophagus
Calpain15	*CAPN15*	Ubiquitous
Calpain16	*CAPN16*	Testis
CalpainS1	*CAPNS1*	Ubiquitous
CalpainS2	*CAPNS2*	Ubiquitous

1.2.1.1 STRUCTURE

Calpains are constituted by two subunits, known as the small and the large subunits (see Figure 1.3).

The small subunit (CAPNS1 or calpain4) is the regulatory subunit and is present in both calpain1 and 2. This subunit has a molecular mass of 30kDa.

The large subunit (calpain1 or calpain2) is responsible for the catalytic activity and, for this reason, it is also called the catalytic subunit. This subunit has a 55–65% of homology between calpain1 and calpain2 and has a molecular mass of approximately 80kDa (Branca 2004).

The large and the small subunits bind to form a heterodimer structure, which is activated by calcium binding (Baudry et al. 2013, Sorimachi and Suzuki 2001).

1.2.1.1.1 DOMAINS

Each calpain subunit is composed of specific domains (Figure 1.3).

Regarding the small subunit (CAPNS1), it comprises two major domains: V and VI. On the other hand, the large catalytic subunit consists of four major domains (from I to IV). Each of them plays a specific role in calpain activity.

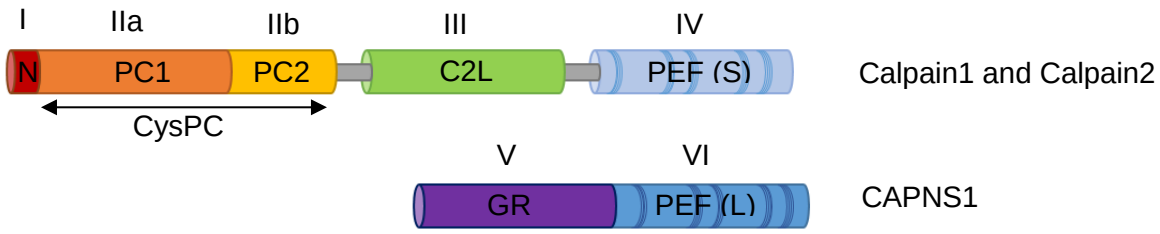

Figure 1.3 Schematic structure of calpain 1 and 2 domains. Calpain1 and calpain2 (large subunit) is composed by domains I to IV and has a molecular mass of 80kDa. CAPNS1 (small subunit) comprises domains V and VI with a molecular mass of 30kDa. The 5 EF-hand motifs are represented by blue bars in domains IV and VI.

The N domain (I) is the N-terminal region and is the most conserved domain. This domain is responsible for the autolysis and even for the separation of both subunits (large and small) upon calcium activation (Nakagawa et al. 2001). The catalytic domain (II, CysPC) is composed of Cys, His and Asn residues and has two core domains (IIa and IIb) which, after binding calcium on each core domain, fuse forming a cysteine catalytic region. This catalytic domain is responsible for interacting with substrates and calpastatin (endogenous calpain inhibitor). Domain III (C2L) is involved in calcium and phospholipid binding, interacts with cell membranes and is responsible for calpain regulation (Gil-Parrado et al. 2003, Hosfield et al. 1999).

The IV (S – small subunit) and VI (L – large subunit and the C-terminal of the small subunit) domains have 5 EF-hand calcium-binding motifs that interact between them forming a heterodimer. The IV domain has a partial homologous region to calmodulin. The V domain is the N-terminal of the small subunit and is composed of Gly residues, which play a role in the hydrophobic behaviour and may be involved in membrane anchor mechanisms (Ferreira 2012, Moldoveanu et al. 2004, Wu and Lynch 2006).

1.2.1.3 REGULATORY MECHANISMS

Calpains are regulated by a variety of mechanisms. As previously mentioned, calcium is one of the molecules responsible for regulatory mechanisms of calpains. Each calpain (1 and 2) has a determined quantity of calcium needed for its activation. However, the amount of calcium available in the cytoplasm is substantially lower than the milimolar requirements of calcium for calpain2, for instance. According to the literature, some mechanisms can decrease the quantity of calcium needed for activation of conventional calpains, such as phosphorylation, binding of phospholipids and even cleavage of N-terminal (Kovacs 2014). Other authors associate the increase in calcium levels with the activation of ionotropic receptors and voltage-gated calcium channels present in the membrane or by the release of intracellular stores of calcium (e.g.: endoplasmic reticulum and mitochondria) (Baudry et al. 2013, Campbell and Davies 2012, Wu and Lynch 2006).

In vertebrates, calpains can also be regulated by calpastatin, its specific endogenous inhibitor (Goll et al. 2003). When the concentration of intracellular calcium increases, calpastatin migrates to the cytosol, is phosphorylated and consequently the affinity to bind to calpain1 and 2 increases. At that point, calpastatin is able to modify calpain activity, by inhibiting calpain2 and lowering the inhibition of calpain1 (Salamino et al. 1994).

Phosphorylation is also a mechanism of inhibiting the activity of calpain2. This modification occurs in the domain III, specifically in serine 369 and tyrosine 370 by kinase A (Kovacs 2014).

There are other described ways of regulating calpain activity, such as the activation by the formation of heterodimers and by autolysis of N-terminal, or the activation by association with membrane phospholipids and by interaction with regulatory proteins (e.g.: Gas2) (Branca 2004).

1.2.1.2 BIOLOGICAL FUNCTIONS

Calpains play a major role in a wide range of cell functions such as proliferation, migration, division, apoptosis and cytoskeletal remodelling (Ferreira 2012).

In the nervous system, calpains are involved in axonal guidance, growth cone motility, learning, memory and neurodegenerative processes (Baudry et al. 2013, Wu and Lynch 2006). In addition, it has been proposed a hypothesis that calpain1 and calpain2 are also involved in synaptic plasticity (Baudry et al. 2011).

1.2.1.4 DYSFUNCTION OF THE CALPAIN SYSTEM

Over the years, it has been discovered that calpains are involved in a wide range of mechanisms underlying numerous diseases. An imbalance in regulatory mechanisms of calpains (overactivation by an increase of calcium concentration or mutations in calpain genes, for example) may cause diseases (Table 1.2). Among those diseases are type 2 diabetes, cataracts, Duchenne's muscular dystrophy, rheumatoid arthritis, various platelet syndromes, hypertension, liver dysfunction, and certain kinds of cancer (Khorchid and Ikura 2002).

Table 1.2 Examples of calpain-related diseases.

Calpain	Related diseases	References
Calpain3	Limb-girdle muscular dystrophy, lens fibre differentiation.	(Hauerslev et al. 2012), (De Maria et al. 2009)
Calpain5	Huntington's disease, regulation of blood pressure and cholesterol levels.	(Gafni et al. 2004), (Saez et al. 2007)
Calpain6	Bone reabsorption and cytoskeletal organisation.	(Hong et al. 2011)
Calpain7	Huntington's disease, impairement in endosomal pathway, cataract.	(Gafni et al. 2004), (Osako et al. 2010), (De Maria et al. 2009)
Calpain8	Gastric and mucosal defence.	(Hata et al. 2010)
Calpain9	Gastric cancer.	(Yoshikawa et al. 2000)
Calpain10	Huntington's disease, type-2 diabetes, polycystic ovary syndrome, metabolic syndrome.	(Gafni et al. 2004), (Bodhini et al. 2011), (Dasgupta et al. 2012), (Perez-Martinez et al. 2011)
Calpain11	Sperm functional processes.	(Ben-Aharon et al. 2006)
Calpain12	Impaired acrosome reaction or motility in spermatozoa.	(Ashizawa et al. 2006)
Calpain15	Hereditary cataract with microphthalmia.	(Kamei et al. 1998)

In the central nervous system, calpains 1 and 2 are involved in Parkinson's disease, Alzheimer's disease, ischemia, stroke, traumatic brain injury, hypoxia and spinal cord injury (Huang and Wang 2001).

Parkinson's disease, as previously described, is defined by a progressive loss of dopaminergic neurons as well as by the presence of intracytoplasmic protein aggregates (Lewy body inclusions).

Lewy body inclusions aggregate and lead to an increase of calcium inside the neurons. That rise in calcium levels disrupts the mitochondria, increasing even more the levels of calcium. This destabilization of calcium overactivates calpains (Esteves et al. 2010). Calpains, when activated, possibly lead to cytoskeleton disturbance, proteasomal failure, and endoplasmic reticulum stress and consequently leads to apoptosis (Domingues et al. 2008).

Alzheimer's disease, as described above, is characterised by the deposition of beta-amyloid. This deposition starts with proteolytic cleavage of amyloid precursor protein, creating peptides that aggregate forming deposits of beta-amyloid. That deposition of beta-amyloid enhances extracellular calcium influx in the hippocampal neurons. Consequently, this increase in calcium concentrations leads to the activation of calpains. (reviewed by Ferreira 2012).

Calpains, in this context, when overactivated lead also to the cleavage of Tau proteins (a microtubule-associated protein essential to stabilise the neuron structure) by beta-amyloid. Tau dissociates from microtubules and aggregates forming neurofibrillary tangles that inhibit the fast axonal transport (reviewed by Iqbal et al. 2009).

Huntington's disease is characterised by repetitions of CAG in the huntingtin gene. Huntingtin is a substrate of calpains and therefore is cleaved by activated calpains following calcium excitotoxicity (Gafni and Ellerby 2002, Goffredo et al. 2002). This excitotoxicity is created by mitochondrial defects induced by mutant huntingtin (Gu et al. 1996). In pathologic conditions, when huntingtin is cleaved by caspases creates a toxic peptide. Calpains create an even smaller peptide with an even higher level of toxicity (Hackam et al. 1998).

1.2.2 ANIMAL MODELS FOR STUDYING CALPAINS

Animal models have long been used in research (since ancient Greece) and are a fundamental key to understanding the mechanisms, and pathophysiology, underlying countless human diseases (Ericsson et al. 2013).

Throughout the years, animal models allowed to understand, initially, the physiology of human body (by comparative biology) and progressively allowed understanding of the mechanism of numerous diseases. The development of treatments was gradual and even the medical devices had a significant advance in these last years (Ericsson et al. 2013, van Meer et al. 2015).

In the end of the 20th century, animal models started to be used as a research tool for measuring the dosing parameters of drug candidates and even its toxicity levels (McGonigle 2014).

Regarding the animal species used in biomedical research, there are a lot of them used as models for a variety of illnesses: fruit fly, guinea pig, mouse, yeast, etc. Zebrafish is an example of a model used aiming to understand regenerative processes and diseases linked with bone degeneration/regeneration (Vijayakumar et al. 2013).

Mice are widely used as models of human behaviour and disease. There are several reasons for choosing mice as biomedical models: small life cycle (2-3 years of life expectancy), 1-10 pups per litter, easy to maintain (small space occupied and up to 5 per cage) and sexual maturity reached at 5 to 7 weeks of life. Additionally, they share 99% of their genome with humans, the mice genome can be easily manipulated and analysed, and they mimic adequately several human diseases, making them an appropriate model for biomedical research (Lieschke and Currie 2007, Richard et al. 2000).

The development of genetically modified mice (*Mus musculus*, C57Bl/6 strain) for the calpain system has allowed the investigation of the involvement of calpains in physiology and disease. Such mice are very useful for studying changes in brain physiology and behaviour.

Nowadays, there are two main ways to study calpains. One of them is by inducing modifications in the mice genome creating knockouts for calpains and knockouts for its endogenous inhibitor, calpastatin. Another way is by using of calpain pharmacological inhibitors synthesised in vitro.

1.2.3 CALPAIN KNOCKOUT MICE

In the last years, several knockout approaches for calpains were established in mice (Table 1.3).

In the beginning, the knockout for the small regulatory subunit (present in both calpain1 and calpain2) was established by removing the small subunit gene (*Capn4*) from mice embryonic stem cells. However, *Capn4*$^{-/-}$ mice embryos died at midgestation due to cardiovascular system defects, which reveals the importance of the calpain small subunit during gestation. In contrast, *Capn4*$^{+/-}$ mice were viable, fertile and also present a normal phenotype (Arthur et al. 2000).

Tan created a conditional knockout for the small subunit flanking the exons 9, 10 and 11 by loxP and Cre recombinase was expressed under the under the control of the cytomegalovirus promoter (Tan et al. 2006). Also, Amini created a conditional knockout for the small subunit, but using the nestin promoter, which allows the inhibition of this subunit only in the nervous system (Amini et al. 2013).

A knockout for the catalytic subunit of the calpain1 was also developed. A sequence in exon4 (encodes a critical part of the catalytic domain) of calpain gene was excised and replaced by a nonsense cassette creating a normal or conventional knockout form of the calpain1 gene (Azam et al., 2001).

For calpain2, the conventional knockout of the catalytic subunit could not be implemented, also due to embryonic lethality (Dutt et al. 2006, Tan et al. 2006). The only way to resolve this issue was to implement a conditional knockout for calpain2. In this knockout, a Cre construction was introduced in the mice genome as well as the LoxP construction, surrounding exon3 from the calpain2 gene (flox). Cre construction is activated in the presence of nestin in neuronal cells (nestin binds to its specific promoter which is associated with Cre construction). When Cre is present, it recombines the LoxP regions, which in turn deletes (flox) the gene of interest, forming a conditional knockout.

For the double knockout, a simple way to silence both calpain1 and 2, was to excise the small subunit since it is present in both calpains, but, as explained before, it leaded to embryonic lethality. This way, the only way to make it functional was by creating a conditional double knockout, using also the Cre construction as described before (Takano et al. 2011).

There are other calpain knockouts, such as calpain3 knockout that conducts to increased muscular dystrophy (Richard et al. 2000).

Table 1.3 Calpains and types of knockouts

Calpains	Knockout	Consequences	References
Small subunit	Knockout of the small subunit (CAPNS1) (conventional knockout)	Embryonic lethal	(Arthur et al. 2000)
	Conditional Knockout of the small subunit (CAPNS1)	Normal development	(Amini et al. 2013)
Calpain1	Knockout of the catalytic subunit of calpain 1/μ-calpain (conventional knockout)	Normal development	(Azam et al. 2001)
Calpain2	Knockout of the catalytic subunit of calpain (conventional knockout)	Embryonic lethal	(Dutt et al. 2006)
	Conditional calpain2 knockout	Normal development	(Takano et al. 2011)
Calpain1 and 2	Conditional double knockout	Normal development	(Takano et al. 2011)
Calpain3	Conventional knockout	Muscular dystrophy	(Richard et al. 2000)

1.2.4 CALPAINS AND NEURONAL PLASTICITY

1.2.4.1CALPAINS AND SYNAPTIC PLASTICITY

Synaptic plasticity is the process by which synapses can be modulated according to one or several stimuli that is induced and was first described by Ramon y Cajal (Cajal 1906). Synapses may be strengthened by long-term potentiation (LTP). LTP is closely related with learning a memory processes (Briz and Baudry 2016).

In 1984, Lynch and Baundry, described for the first time the involvement of calpains in the synaptic plasticity (Lynch and Baudry 1984). Calpains modulate synaptic plasticity by regulating neurotransmitter release and synaptic structural stabilization (by spectrin modulation) (Wu and Lynch 2006). Briz and Baudry, proposed the cleavage of spectrin (a skeletal protein) by calpains, leading to the insertion of glutamate receptors in the postsynaptic membrane of postsynaptic neurons. This phenomenon increases the susceptibility for LTP and leads to alterations in the dendritic spines (Briz and Baudry 2016).

Baudry and Bi proposed a model in which calpain1, after activated, starts a signalling cascade leading to modulations in the synaptic structure and potentiating LTP. On the other hand, calpain2 stops the signalling cascade (Baudry and Bi 2016).

This field is continuously being investigated, because there are still numerous mechanisms that must be clarified.

1.2.4.2 CALPAINS AND NEUROGENESIS

As explained before, when a brain damaged occurs, there is the formation of an excitotoxic environment. This environment leads to an increase of calcium levels at an intracellular level. As a result of the increasing of calcium levels, calpains are overactivated, leading to post-ischemic neuronal death (Neumar et al. 2001). Santos found that calpain1 represses differentiation of the neural stem cells, and calpain2 increases glial differentiation (Santos et al. 2012).

Previews work of our group, showed that by inhibiting calpains with calpeptin proliferation and migration of neural stem cells increased. Also, in calpastatin knockout mice, proliferation and migration were decreased (Machado et al. 2015).

Amini created a conditional knockout for the small subunit and observed a decrease in LTP and spatial memory as well as deficits in spine density. Neuronal death was also linked to calpain functions. Consequently, Calpain1 and calpain2 are involved in plasticity and neuronal death (Amini et al. 2013).

1.3 OBJECTIVES

Previous work in our group showed a link between calpains and neurogenesis. Knowing that neurogenesis can alter the behaviour, the aim was to characterise neurogenesis and the behavioural phenotype of calpain1 and calpain2 knockout and conditional double knockout for these calpains.

2. METHODS

2.1IN VIVO

2.1.1 ANIMAL MODEL

2.1.1.1CALPAIN KNOCKOUT MICE

Calpain1 knockout mice was created by excising the exon4 of the catalytic subunit (large subunit), and replacing it with a nonsense sequence (a phosphoglycerate kinase-cassette).

Calpain2 conditional knockout was created using a frameshift mutation in exons 3 to 7. Moreover, the exon3 was flanked using the Cre loxP construction with nestin promotor. Cre construct is activated only in present of nestin, which means that is only activated in the central nervous system.

Calpain1&2 conditional double knockout has a similar construct to calpain2 conditional knockout and have also the calpain1 knockout.

Cre control mice were created inserting Cre construct in the genome, without any other modification and were used as comparison groups. Cre control mice were used to certify that it does not have any interference in the behavioural phenotype outcome.

Calpain1 Knockout (Azam et al., 2001), Cre control, Calpain2 conditional knockout and cDKO mice were kindly provided by Dr. Takaomi Saido (Takano et al. 2011). Wild Type (WT) mice littermates were used as controls.

2.1.1.2 MICE HUSBANDRY

Mice (*Mus musculus*) were kept in the animal facilities (Universidade do Algarve) until 2 days before the beginning of the tests with the following conditions: room temperature at 23ºC±1ºC, bedding, food, water *ad libitum* and enrichment (paper rolls).

While in the animal facilities, mice with specific genotypes: Wild type (WT), *Capn1* KO, *Capn2* cKO, *Capn1&2* cDKO (refered in this work as cDKO) and Cre Ctrl, under the C57Bl/6J background, were used for breeding to obtain the animals used in this study. Embryonic development of mice, usually, lasts 18 to 22 days; 6 to 8 days after birth, littermates were tagged (toe fingers cut, corresponding to a specific number). Four weeks after birth, mice reached maturity and were weaned, i.e. the litter was separated from its progenitors. At that time, females and males were also separated per cages (Figure 2.1).

Figure 2.1 Mice Timeline. 6 to 8 days after birth, littermates were tagged with toe marks and at 1 month age were weaned and separated by sex. At 3 months (12 weeks) age littermates were ready to perform behavioural tests.

When mice reached 12 weeks, they were able to start the behavioural tests.

Two days before the tests begin, the animals were submitted to a period of habituation, where was simulated the handling by the experimenter inside the testing room. The main purpose is to eliminate the stress caused by handling, avoiding potential bias while performing the tests.

In some cases, when the animal was too stressed out, the habituation was conducted more than twice a day.

All experiments were performed in accordance with institutional and European guidelines (2010/63/EU) for the care and use of laboratory animals.

2.1.2 GENOTYPING

2.1.2.1 DNA EXTRACTION

For DNA extraction, tail tips were cut and stored at -20ºC for later extraction using the NZY Tissue gDNA Isolation kit.

DNA samples were quantified using NanoDrop, and stored at 4ºC (if utilized in the next day) or at -20ºC (if used later than 24h after extraction).

2.1.2.2 PCR

For amplification of DNA samples previously collected, the following primers were used: Cre Primer (forward and reverse), GAPDH Primer (forward and reverse), Capn1 Primer (forward, normal and reverse), Capn2 Primer (forward and reverse) (see Table 2.1). NYZTaq 2x Green Master Mix (DNA Taq purified from *Escherichia coli* as well as dNTPs) was used to amplify specific DNA sequences.

The Master Mix had two different dyes, which allowed the tracking of electrophoresis progress.

Using Thermal Cycler, primers and Master Mix were used to amplify the DNA samples (denaturation: 5min at 95ºC; 30 cycles of denaturation: 30s at 95ºC, annealing: 45s at 64ºC for Capn2 and 60ºC for Cre and Capn2 and elongation: 45s at 72ºC; final extension step: 5min at 72ºC).

Table 2.1 Table of primers* used throughout the genotyping protocol.

Primer	Reference/Sequence
Capn1 Primer (Forward)	5'-CTC ACT TAG CAT AGG CTT TCT CCA GCA GTG-3'
Capn1 Primer (N)	5'-CCT GAA CGA GAT CAG CAG CCT CT-3'
Capn1 Primer (Reverse)	5'-CAG AAC CAC TGA CAC GGT CCA GAT CTG-3'
Capn2 Primer (Forward)	5'-ATA GCT CCT GTG TAT CAG GCA CAG AGC TGG-3'
Capn2 Primer (Reverse)	5'- GGA GCT CAT CTG TGT CTC CAA AGC C-3'
Cre Primer (Forward)	5'-CGC AGA ACC TGA AGA TGT TC-3'
Cre Primer (Reverse)	5'-CGA AAT CAG TGC GTT CGA AC-3'
GAPDH Primer (Forward)	5'-ACC ACA GTC CAT GCC ATC AC-3'
GAPDH Primer (Reverse)	5'-TCC ACC ACC CTG TTG CTG-3'

*All of the primers were acquired from NZYTech, with head office in Lisbon, Portugal.

2.1.2.3 ELECTROPHORESIS

After PCR, DNA samples were separated by size using a 2% agarose gel in TAE buffer. Green Safe Premium (a nucleic acid stain) was added during the agarose gel preparation aiming to detect DNA by fluorescence as it binds to DNA during gel casting.

2.1.2.4 ANALYSIS

Aiming to observe the presence or absence of Cre (GAPDH is used as a control), Capn1 and Capn2, electrophoresis gel was placed gently inside the ChemiDoc and an image was acquired and analysed, see examples (Figure 2.2).

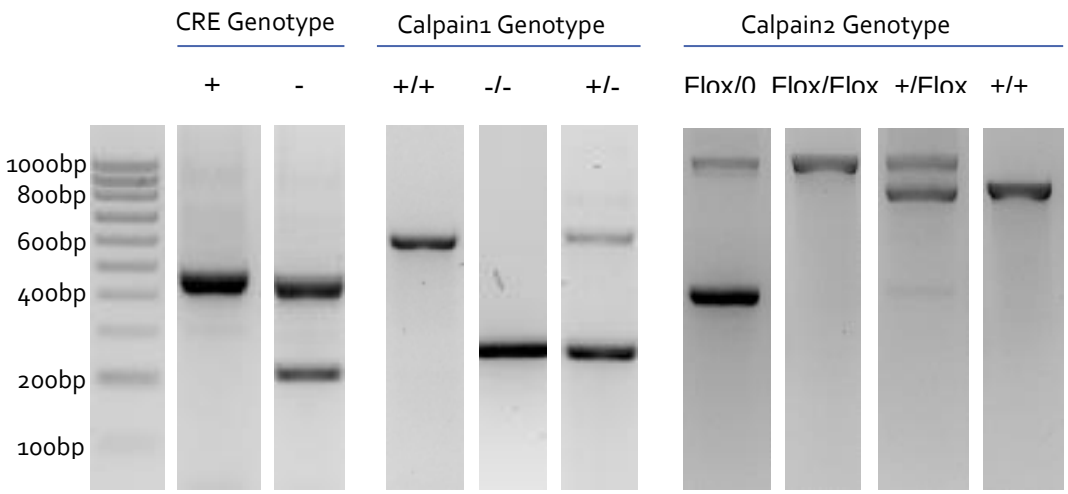

Figure 2.2 Examples of possible genotypes for each gene/construction. **Cre Construction** may be present and in that case, will be presented by two bars (GAPDH – 480bp and Cre – 220bp). If Cre is not present, only one bar will appear (GAPDH – 480pb, control). **Calpain1** gene may be represented by +/+ (630bp which represent the wild type form – gene not excise in both alleles), -/- (270bp which means Calpain1 gene excise in both alleles) and +/- (Calpain1 Gene excise in one allele). **Calpain2** gene may be represented by 4 forms: flox/0 (1045bp and 421bp – one allele excised and one not existent), flox/flox (1045bp – both alleles excised), +/flox (838bp and 1045bp – one allele present and one excised) and +/+ (838bp – both alleles present, WT form).

2.1.3 BEHAVIOURAL TESTS

Two days before the tests start, animals were moved to the test room and placed inside a ventilated cabinet (Ultragene, Canada) which maintained the temperature at 22ºC±1 and the day/night cycle (day- from 8am to 8pm; night – from 8pm to 8am). Tests were performed as represented in Figure 2.1.

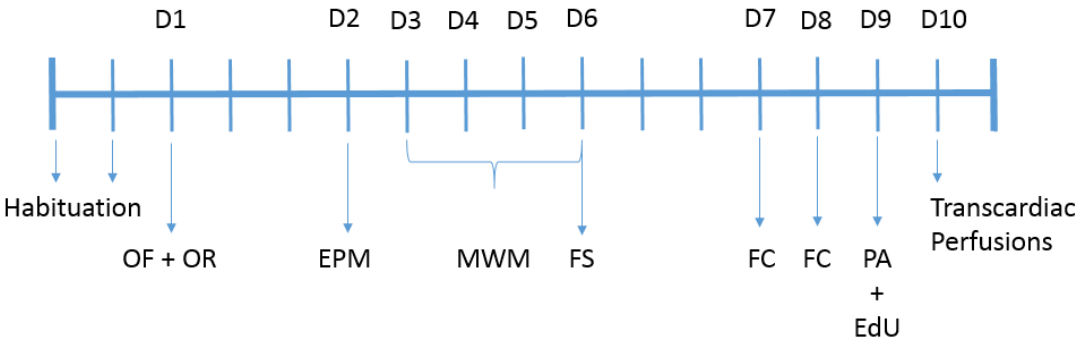

Figure 2.3 Behavioral tests schedule. Before tests begin, two days of habituation are required to allow mice to accustom to handling. On day 1, mice perform the Open Field Test and the Novel Object Recognition Test. On day 2, Elevated Plus Maze test is implemented. From day 3 to day 6, Morris Water Maze test is performed and in the day 6, 4h after the MWM, the Forced Swimming Test is performed. On days 7 and 8, the Fear Conditioning Test is performed and in the day 9 Passive Avoidance Test is performed. 12h before transcardic perfusions, mice are submitted to two infections of EdU 2h apart from one another.

General considerations: Before and after starting a test, all the test apparatus and material was cleaned with ethanol 10%. As well as between trials and animals. The objective was not to influence the performance of the mice by having other smells inside the labyrinth/box.

Every test was recorded in a video file which was analysed in the ANY-maze software (Stoelting, Wood Dale, USA), using a GoPro Hero Camera (GoPro, San Mateo, California, USA).

2.1.3.1 OPEN FIELD TEST

In order to access mice exploratory, locomotor and anxious behaviour, the Open Field Test was performed. In this test (Walsh and Cummins 1976), mice were placed inside an empty acrylic box with 40x40x40cm^3, divided into 9 squares (Figure 2.5). The mice were recorded inside the box for 5 minutes, after which they were removed and the box was cleaned with alcohol 10%.

After recording, the video file was opened in the ANY-Maze software that allows the analysis of line crossings and rearing episodes (the exploratory and locomotor activity), as well as the time in centre (in seconds) and number of central entries (anxiety).

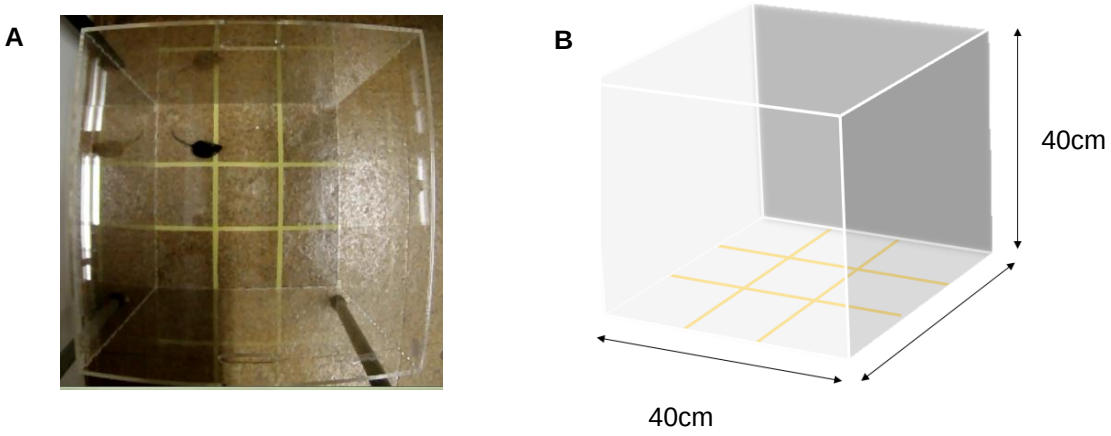

Figure 2.4 Open Field Test. A, Real Time test in the Open Field Box; **B,** Open Field Box dimensions.

2.1.3.2 NOVEL OBJECT RECOGNITION TEST

Aiming to understand the recognition memory of knockout mice, Novel Object Recognition Test was performed. This test was composed of two parts. In the first one, mice were introduced to two objects (A and B) and in the second part, mice were presented to object A and C, the novel object (Bevins and Besheer 2006).

Two hours after the Open Field test, mice were placed gently inside the acrylic box used before, with two distinct objects (A – a small paper cup cut in half and B – a small and normal paper cup) for 5 minutes (Figure 2.5-A). Two hours later, the object B was removed and replaced by a new one, the object C (a box with 6.5x6.5x2cm dimensions). Again, mice were placed inside the acrylic box with object A and C for 5 minutes (Figure 2.5-B).

After that time, mice were removed, the apparatus and the objects were cleaned with alcohol 10% and data collected was analysed (percentage of time spent exploring each object).

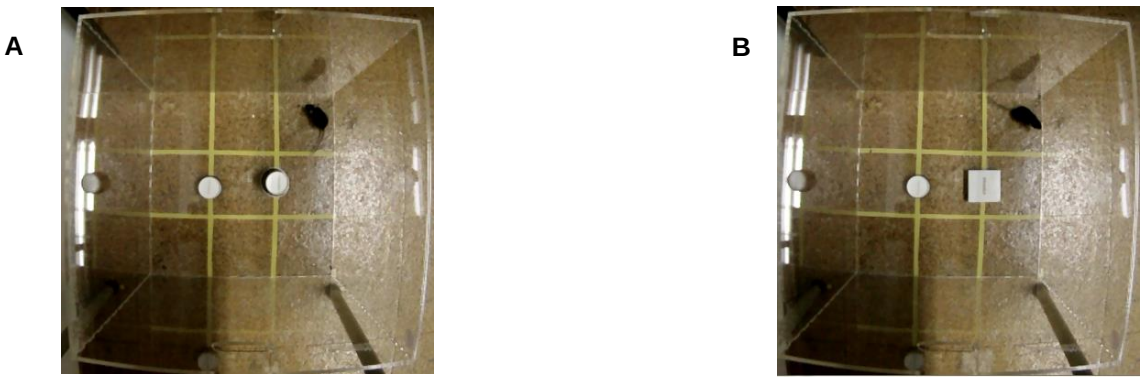

Figure 2.5 Object Recognition Test. A, Part One (object A and B); **B,** Part 2 (object A and C).

2.1.3.3 ELEVATED PLUS MAZE TEST

The Elevated Plus Maze Test (Walf and Frye 2007) evaluates the anxiety behaviour of mice. When mice are too anxious, they spent more time in the closed arms. This test consists of a cross-like labyrinth (with 5cm width) with two open arms and two closed arms (35cm length) and elevated 70cm from the ground (Figure 2.6). The closed arms have walls with 15cm height, in which mice cannot see beyond them. The open arms do not have walls so the mice were aware of the height of the apparatus.

In this test, mice were placed gently in the middle of the labyrinth, facing the closed left arm, and were able to explore it freely for 5min. After that time, the animal was removed, the apparatus was cleaned with alcohol 10% and the percentage of time exploring the open and the closed arms were analysed.

As the labyrinth is black and mice also have a black coat, they were painted gently on the back with non-allergic facial paint (Water Make-up 001 White, from Grimas, Holland), using a brush.

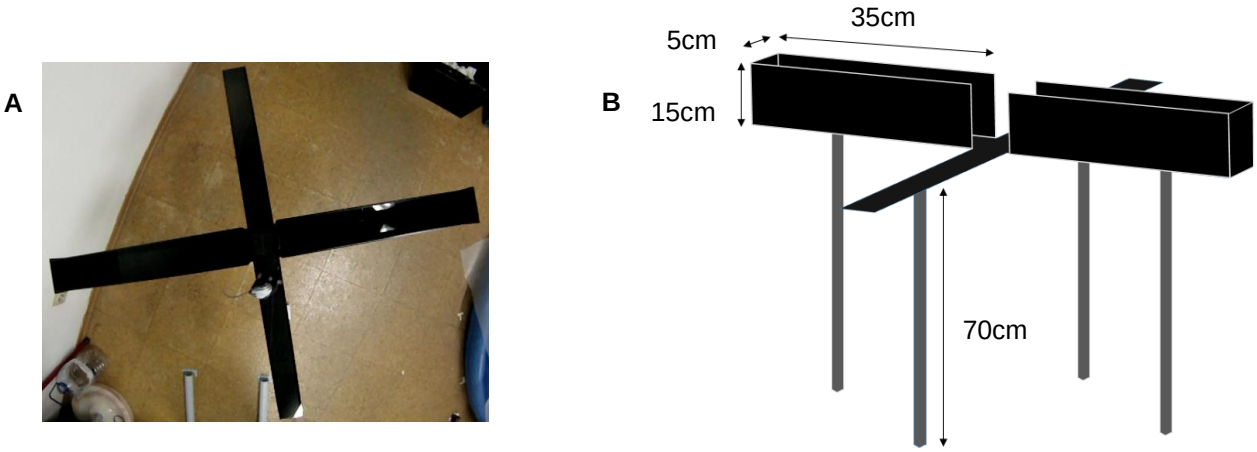

Figure 2.6 Elevated Plus Maze Test. **A,** Photography of a real time test; **B,** EPM apparatus dimensions.

2.1.3.4 MORRIS WATER MAZE TEST

In order to access spatial and learning memory, a Morris Water Maze Test was used (Morris 1984).

The pool utilized in this test had 1.50m of diameter and was filled with water that was maintained at 22ºC± 1ºC, having a translucent acrylic platform (with 10cm of diameter and carved to increase grip) inside and covered with 0.5cm of water (Figure 2.7). The objective is to test if mice can find the platform guiding only by spatial wall cues. These cues are placed on the surrounding walls and have patterns to help mice locate the platform, and consequently the way out of the pool.

The first 3 days have the aim to allow the mice to learn the platform location. In those three days, mice were placed successively in the four quadrants (one different quadrant at each time and with a 30min time between quadrants) and allowed to swim and find the platform for only 1min. If mice did not find the platform after 1min, they were placed on the platform for about 10s to allow them to perceive the platform location by viewing the surrounding cues.

In the final day (4th day), the platform was removed and mice were placed in the 4th quadrant (the farther one from the platform quadrant). For 1min, they were allowed to swim and find the location of the platform.

The latency to find the platform is measured for the 3 first days and in the 4th day, the time spent in the platform quadrant is measured, as well as the number of platform zone entries.

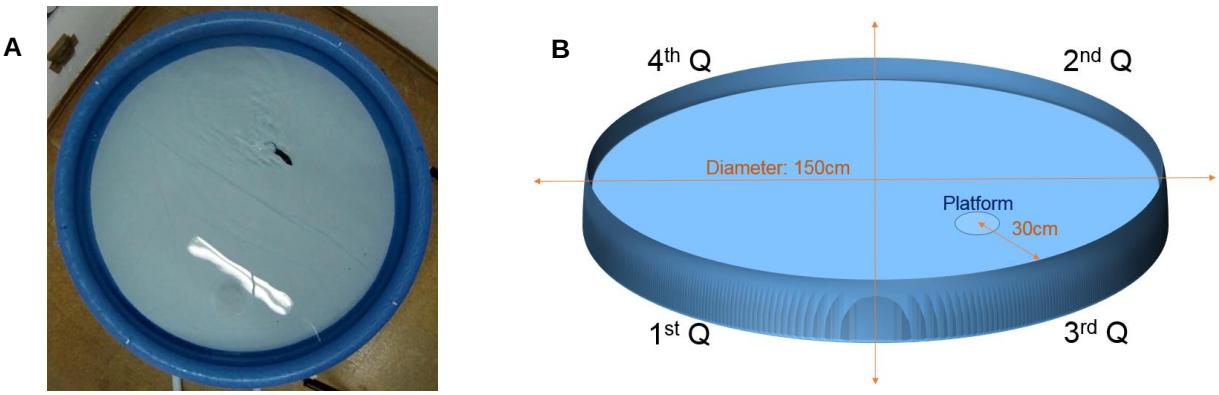

Figure 2.7 Morris Water Maze Test. A, Real time image; **B,** Water Maze apparatus dimensions.

2.1.3.5 FORCED SWIMMING TEST

The objective of this test was to define the time mice spent immobile during those 6min and, consequently, the tendency to have a helpless behaviour (the more depressive, the more immobile).

This test consists of placing mice inside a cylinder recipient (with a 10cm diameter and a 48cm height) filled with water (5cm below the surface at 23ºC±1) for 6min (Porsolt et al. 1977) (Figure 2.8).. In this test, mice usually start trying to escape, then stop swimming, and start trying to escape again. For this reason, a threshold line time of 6min total was settled.

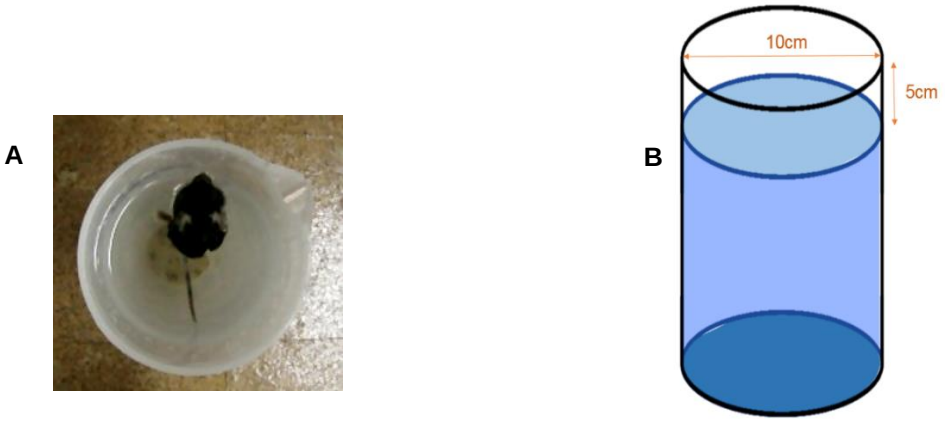

Figure 2.8 Forced Swimming Test. **A** – Photography of mice during the forced swimming test; **B** – Forced Swimming apparatus dimensions.

2.1.3.6 FEAR CONDITIONING TEST

The Fear Conditioning Test (Wehner and Radcliffe 2004) was performed to access contextual memory that is associated with hippocampus and amygdala-dependent memory (reviewed in Curzon et al. 2009).

This test is composed of 3 parts. In the first part, mice were placed gently inside a chamber with a grid floor, inside a soundproof box, with background sound (white noise), 70% ethanol scent and 100% lighting, for 6min and 30s. In the first 2min, mice were allowed to explore the chamber freely (habituation or acquisition). Following the habituation period, mice heard 30s of tone (80dB), and in the last 2s of it, mice were submitted to a 0.7mA footshock. After this cue period, mice were able to rest for 1min (Figure 2.9). This sequence of events is repeated 2times.

On the next day, mice were placed inside the box, under the same conditions as the previous part (70% alcohol scent, 100% lighting and background white noise) for 5 min. During this time, mice were not submitted to tone or footshock. The main purpose was to identify if mice associated the same context with footshock, i.e. to understand the associative memory.

For the third part of the test, three hours later, the context was completely changed (listed walls, white floor, vanilla scent, background fan noise, and 10% lighting). Mice had a period of habituation of 3min and then were submitted to the tone for 3min (total of 6min test). This last part had the aim of understanding if mice associated the tone with the footshock, in an entirely different context.

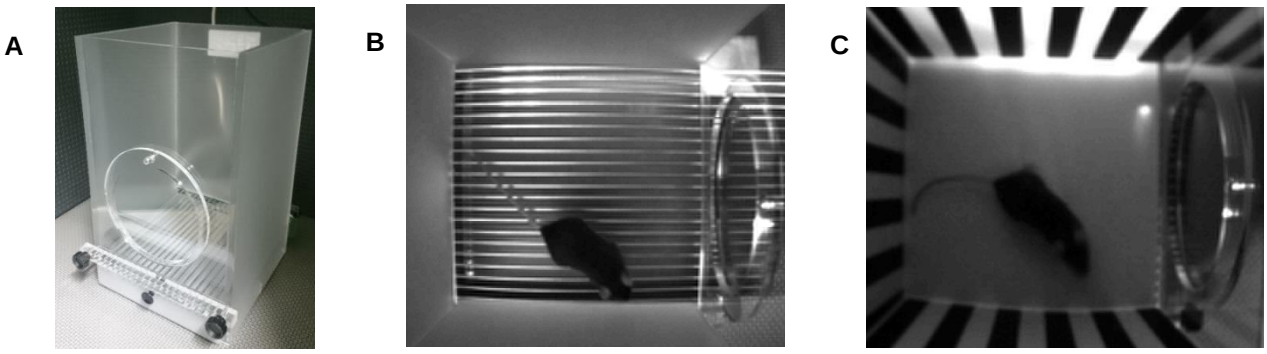

Figure 2.9 Fear Conditioning Test. **A -** FC Apparatus; **B -** Cued Test**; C -** New Context Test

2.1.3.7 PASSIVE AVOIDANCE

The fear-based conditioned avoidance learning was accessed using the Passive Avoidance Test.

The purpose of this test was to understand if the mice could learn how to avoid foot shock by staying on the cardboard platform (Kazlauckas et al. 2005). In order to do that, the device used in the previous test was also utilized in this test (soundproof box with a chamber with a metallic grid). To avoid this foot shock a cardboard box platform (6.5x6.5x2), which cannot carry electricity, was placed over the metallic grid, near the back wall, allowing mice to be on top of the platform and consequently will protect them from getting an electrical shock (Figure 2.12).

Ninety minutes after the first part of the test, mice were placed again inside the box on top pf the cardboard box, but not submitted to any footshock. The latency to put four paws down on the metallic grid was measured.

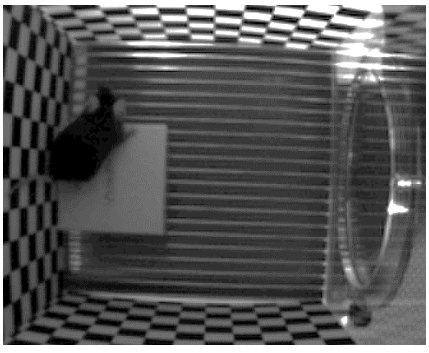

Figure 2.10 Passive Avoidance Test

2.1.3.8 ANALYSIS

Statistical analysis was performed in GraphPad 5 Prism (version 5.00), using One-way ANOVA and Two-way ANOVA statistical tests, and Bonferroni's Multiple Comparison Test was used as a parametric test. Statistical significant differences were considered when $P<0.05$.

2.2 HISTOLOGY AND IMAGING – DENTATE GYRUS PROLIFERATION

2.2.1 EdU INJECTIONS

After the last behavioural test, mice were submitted to two intraperitoneal injections of EdU (5-ethynyl-2'-deoxyuridine, 50 mg/kg), in 0.01M PBS, two hours apart from one another, twelve hours before sacrifice.

2.2.2 TRANSCARDIAC PERFUSIONS

Mice were transcardially perfused, using 50ml of 0.9% sodium chloride (NaCl) and then 50ml of 4% paraformaldehyde (PFA) in 0.1M of phosphate buffer (PBS) as a fixative.

The brain was removed from mouse cranial cavity and stored overnight with 4ml of PFA, at 4ºC.

In the next day, the PFA was removed and replaced by a solution composed by 20% sucrose in PBS and sodium azide (to prevent possible fungi contaminations) and stored at 4ºC, with the aim to dehydrate the brains and cryoprotect them.

2.2.3 BRAIN CORONAL SECTIONS

Brains were stored at least 48h after perfusion, at 4ºC, and afterwards sectioned in a cryostat (Thermo Scientific Cryostar NX50) in coronal sections with a thickness of 30µm. The sections were stored in series of 6, with 4ml of Walter Antifreeze solution (0.5 M Phosphate Buffer (1.7 g NaH2PO4.H2O, 5.45 g $Na2HPO_4.2H_2O$, 400 ml dest. H_2O), Etylenoglycol, Glycerol), at 4ºC.

2.2.4 STAINING FOR EdU AND DOUBLECORTIN (IMMUNOHISTOCHEMISTRY)

Brain tissues were submitted to a process of staining using antibodies.

This process started with the rinsing of free-floating brain sections with 3% BSA-PBS and then being incubated for 45min with 0.5% Triton-PBS. Following, the brain sections were rinsed twice with 3% BSA-PBS and Click-iT reaction cocktail (Click-iT EdU Alexa Fluor 488 HCS Assay kit) was prepared and brain sections were incubated in this solution for 1h, protected from light henceforward.

After the incubation period, brain sections were rinsed again in 3%BSA-PBS and blocked for 1h with 5% of blocking solution (NHS in 0.25% Triton-PBS), at room temperature. The next 36h to 48h brain sections were incubated with primary antibody (goat anti-DCX, 1:400), at 4ºC.

After rising once with 0.25% Triton-PBS and twice with 2% of blocking solution, brain sections were incubated with secondary antibody solution (donkey anti-goat, diluted 1:200) for 2h, at room temperature.

Nuclei were stained with Hoechst 33342 for 10min and the free-floating brain sections were washed with PBS 3 times and mounted, in 1% gelatine-coated slides. The coverslip was placed gently with the help of Dako fluorescence mounting medium.

The samples were stored at 4ºC, in an appropriate box, protected from light.

2.2.5 FLUORESCENCE MICROSCOPY

Mounted brain sections in 1% gelatine-coated slides were analysed under Axio Imager Z2 (Zeiss, Oberkochen, Germany) microscope, always using the same settings to avoid bias, and allowing the analysis of two processes: proliferation and migration.

Proliferation process was detected by the presence of positively stained EdU cells that incorporate EdU (thymine analogue) during the S-phase of the dividing cell cycle. Those dividing cells could be observed in the dentate gyrus granular zone and the subgranular zone in both hemispheres.

Migration process could be observed through DCX staining which allowed to quantify the percentage of immunoreactive area and perceive which cells were migrating as the doublecortin is a neuronal migration protein.

2.2.4 ANALYSIS

The percentage of immunoreactive area was analysed using ImageJ 1.50i (National Institute Health, USA).

3. MATERIALS

120S/R Pump (010.6131.R00) was purchased from Watson-Marlow Pumps (UK).

Thin Wall Needles 25G x 5/8" (NN – 2516R) were purchased from Terumo Company (Leuven, Belgium) and Sterile Luer Slip 1ml Syringes (300013) were purchased from BD Plastipak (Spain).

ANY-maze software (version 4.99) and Soundproof box were purchased from Stoelting Co. (Wood Dale, IL, USA).

C1000 Touch™ Thermal Cycler and Chemidoc –XRS+ were purchased from Bio-Rad Laboratories, Lda (Hercules, California, USA).

NanoDrop 2000 and CryoStar NX50 were purchased from Thermo Scientific (Wilmington, USA).

Antibody Donkey anti-goat (A11058) was purchased from Invitrogen (Carlsbad, California, USA) and antibody Doublecortin (C-18) anti-goat (SC-8066) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

Green Safe Premium (MB13201), NZYDNA Ladder V (MB06101, 200 lanes) and NZYTaq 2x Green Master Mix (MB03902) were purchased from NZYTech (Lisbon, Portugal).

EdU (E10187) and Click-iT® EdU Alexa Fluor® 488 HCS Assay (C10350) were purchased from Molecular Probes (OR, USA).

Axio Imager Z2 was purchased from Zeiss, Oberkochen, Germany.

Other material was purchased from NZYTech (Lisbon, Portugal).

4. RESULTS

4.1 BEHAVIOURAL PHENOTYPE OF CALPAIN KNOCKOUT MICE IS SIMILAR TO WILD TYPE MICE

4.1.1CALPAIN KNOCKOUT MICE DO NOT EXPERIENCE ALTERATIONS IN EXPLORATION/LOCOMOTOR ACTIVITY

Exploratory/locomotor activity of mice was analysed by the Open Field Test. When mice have a more exploratory behaviour, they spend more time moving and exploring the box. When they move across the box, as the box was divided into symmetric squares, they cross those dividing lines. The more they explore the box, the more lines are crossed.

The number of line crossings was significantly different between the different genotypes studied (Figure 4.1-A) (One-way ANOVA (Bonferroni's post-test) [F= 1.520; dF=4; P>0.05]). All of them crossed similarly the same amount of dividing lines compared with the control WT=144.2 ±16.8. The number of rearing episodes was also unaffected by genotype (Figure 4.1-B), comparing with WT mice = 38.8±5.8 (One-way ANOVA (Bonferroni's post-test) [F= 1.971; dF=4; P>0.05]).

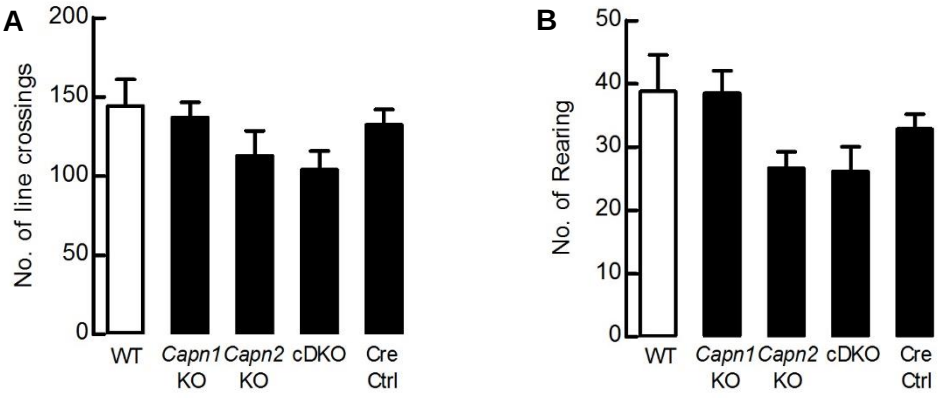

Figure 4.1 Absence of calpain1, 2 or both does not lead to alteration in exploratory and locomotor activity of calpain knockout mice. During the Open Field Test analysis by ANY-Maze software, the number of line crossed (**A**) and mice rearing behaviour (**B**) were counted. Data represents means ± SEM, One-way ANOVA, n=6-14.

4.1.2 LEARNING AND MEMORY ARE NOT AFFECTED BY CALPAIN DELECTION

The learning and memory acquisition processes were tested through the Novel Object Recognition Test, Morris Water Maze Test, Fear Conditioning Test and Passive Avoidance Test.

In the first part of the Novel Object Recognition test, mice were expected to explore the two objects equally, i.e., spend the same percentage of time exploring each object. However, according to the test results, mice with calpain1 knockout (Capn1 KO) explored significantly more the object A (62.80±4.43) than the object B compared with control (43.89±4.04) (Two-way ANOVA (Bonferroni's post-test); [t=5.250; P<0.001]) (Figure 4.2-A). All of the other genotypes explored the object A and B equally (Two-Way ANOVA (Bonferroni's post-test) [*F*=0.0; *dF*=4; P>0.05]).

In the second part of Novel Object Recognition Test, object B was replaced by object C. As mice were previously introduced to object A and B, they were expected to remember those two objects and spend more time exploring the object C, the novel object. However, according to the results, all genotypes tested spent similar time exploring the object C, compared with the WT (51.87±6.01)(Figure 4.2-B) (Two-way ANOVA (Bonferroni's post-test) [*F*=0.0; d*F*=4; P>0.05]) and clearly in Figure 4.2-C (One-way ANOVA (Bonferroni's post-test) [*F*=0.893; d*F*=4 , P>0.05]).

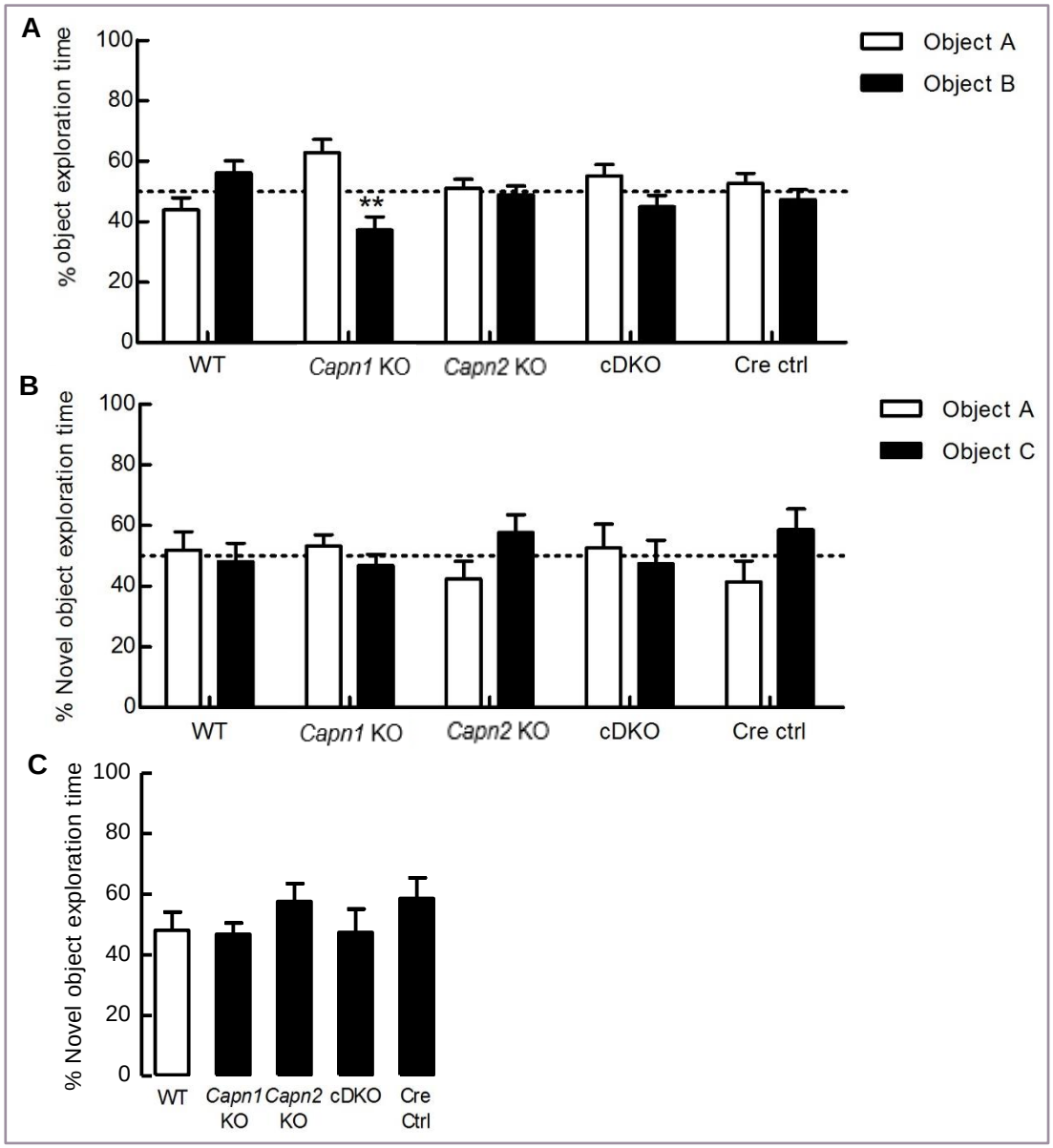

Figure 4.2 Recognition memory is not influenced by calpain knockout mice. Mice explored similarly the presented objects without significant differences. **A,** Presentation of object A and B (Two-way ANOVA); **B,** Presentation of object A and C (Two-way ANOVA); **C,** Presentation of object C (One-way ANOVA). Data are expressed in Means ± SEM, N=6-13.

Morris Water Maze Test tests the learning process as mice should find a platform location hidden under water through spatial cues on the walls of the testing room.

According to the test results (Figure 4.3-A), in the first 3 days of water maze test, mice were able to find the platform location through spatial cues faster each day, independently of their genotype (Two-way ANOVA; F= 1.417; dF=4; P>0.05). However, on the second day, calpain1 knockout mice had a significantly better performance (25.93±3.06)as they found the platform faster than on the other days compared with the control (37.01±2.21)(Two-way ANOVA (Bonferroni's Post-test); [t=2.696; P<0.001]).

In the last day of Morris Water Maze Test (4^{th} day), the platform was removed and the percentage of time spent in each quadrant was calculated.

During the first testing days, mice had the opportunity to find the platform location through spatial cues. If the mice learned the platform location, they would spend more time in the third quadrant (where the platform was located).

Regarding the results (Figure 4.3-B and C), mice spent more time in the third quadrant, independently of the genotype (WT= 51.36±36) (Figure 4.3-B, Two-way ANOVA (Bonferroni's Post-test) [F=0.00002176; dF=4; P<0.001]). However, there were no significant differences between genotypes (Figure 4.3-C, Two-way ANOVA (Bonferroni's Post-test) [F=0.00002176; dF=4; P>0.05]).

The number of entries in the platform area (the area where was the platform on the first three days of the test) was also quantified (Figure 4.3-D).

Although there was no statistical difference between genotypes, compared with control (3.43±0.75) (One-way ANOVA (Bonferroni's Post-test) [F=0.976; dF=4; P>0.05]), a slight tendency for an increase is observed in cDKO mice and a trend to decrease is seen in mice with calpain2 Knockout.

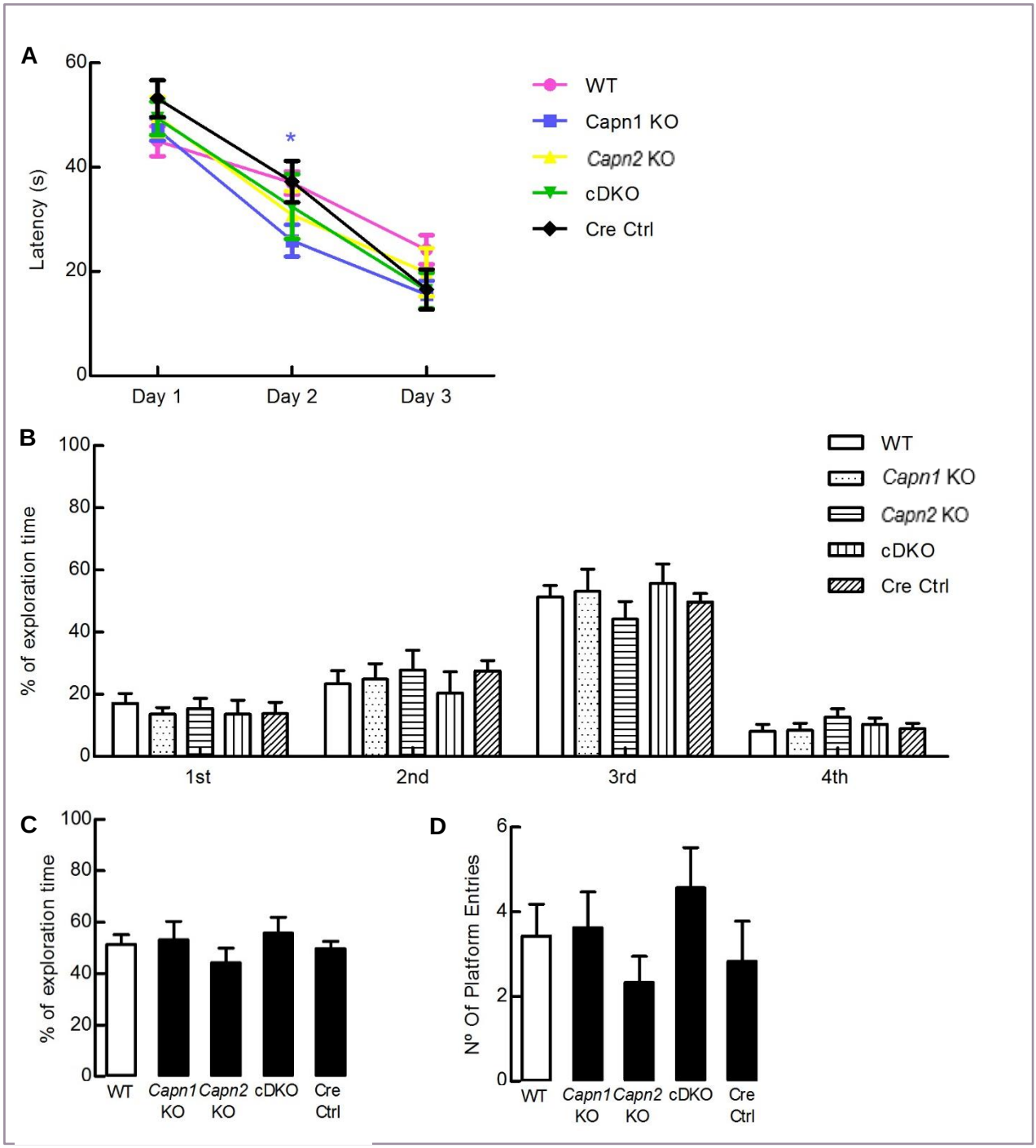

Figure 4.3 Spatial memory is not affected by calpain knockout mice. Throughout the Water Maze test, in the first 3 days, latency (s) to find the platform location was measured (**A,** Two-way ANOVA, N=6-14) and in the last day (4th day), the percentage of time spent on each quadrant was measured (**B,** Two-way, N=6-8). **C,** Corresponds to the percentage of time exploring the quadrant 3 in the 4th day (One-way ANOVA, N=6-8).Data are presented in means ± SEM. Adding, also in 4th day, the number of platform area entries was also taking in count (**D,** One-way ANOVA, N=6-8).

The Fear Conditioning Test was also used to complement the information obtained before about learning and memory processes and was perform on day 7 and 8. This test evaluates the contextual memory that is associated with the hippocampus and the amygdala-dependent memory.

In the first day, mice were submitted to a footshock after a tone (repeated 2 more times) and the freezing time was measured. Observing the test results (Figure 4.4-A), it has a clear increase in the percentage of freezing time throughout the cues. However, between genotypes, there are no significant differences compared with control: Two-way ANOVA (Bonferroni's Post-test) [F=1.748; dF=4; P>0.05]).

In the second day (Figure 4.4-B), the association between the context A and tone and footshock was analysed by placing the mice inside the FC box, but without the presentation to tone nor footshock (association with context A). The freezing time was also measured and it showed no differences between genotypes, compared with the WT mice (51.82±4.07) (One-way ANOVA (Bonferroni's Post-test) [F=0.777; dF=4; P<0.05]).

Three hours later, mice were placed for the third time inside the FC box, but this time with a new context (context B). The association between tone - footshock and the new context was analysed one more time through freezing time (%) in the habituation (Figure 4.4-C) phase and during the exposition to tone (Figure 4.4-D). However, no statistically significant difference between genotypes was observed neither in the habituation phase (WT= 22.26±4.25) (One-way ANOVA (Bonferroni's Post-test) [F= 2.977; dF=4; P<0.05]) nor the cued test (WT=44.49±9.24) (One-way ANOVA (Bonferroni's Post-test) [F=1.743; dF=4; P<0.05]).

Also, the freezing time in context A is higher than the freezing time in context B. This means that mice can associate that they are placed in a new context (Figure 4.4-A).

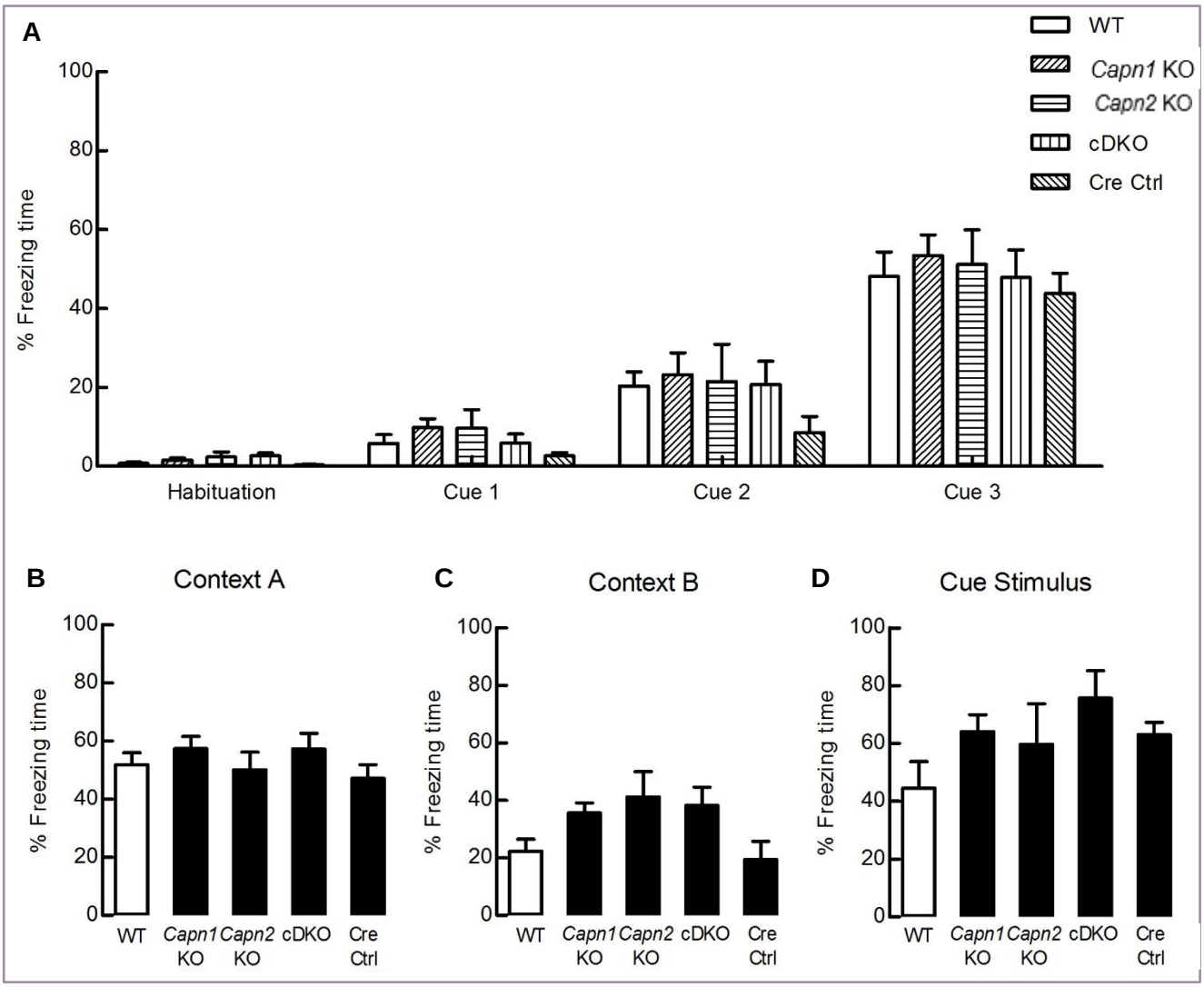

Figure 4.4 Associative memory is not influenced by calpain knockout mice. Mice were submitted to Fear Conditioning Test and the freezing time was measured. **A,** Test trials (Two-way ANOVA); **B,** Association with context A (One-way ANOVA); **C,** Habituation to the Context B (One-way ANOVA); **D**, Cue Stimulus with context B (One-way ANOVA).Data are presented in means ± SEM, N=6-14.

In the last day of behavioural tests, Passive Avoidance Test was performed. On training time, mice were placed over a small box and 60s after test start and every time mice placed four paws down on the grid floor they received 3 footshocks of 2s, with 1s of interval and with 0,7mA. Analysing the latency (s) between genotypes (Figure 4.5-A), there is a small increasing tendency of cDKO mice (compared with WT mice, 108,60±42,10) to take more time to place four paws down after the habituation period. However, this tendency is not significant from the statistical point of view (One-way ANOVA and Bonferroni's Multiple Comparison Test [*F*= 0.984; *dF*=4; P>0.05]).

90min after the training session (Figure 4.5-B), the test was performed and started immediately after placing mice on the platform and the test had a performance time of 300s. This time, mice did not receive any footshock. Analysing the graphic, a tendency to Calpain2 KO mice spend all the test time on the platform can be observed. However, once more, no statistically significant difference was found between genotypes comparing with WT (187.91±46.12) (One-way ANOVA and Bonferroni's Multiple Comparison Test [*F*=1.849; *dF*=4; P>0.05]).

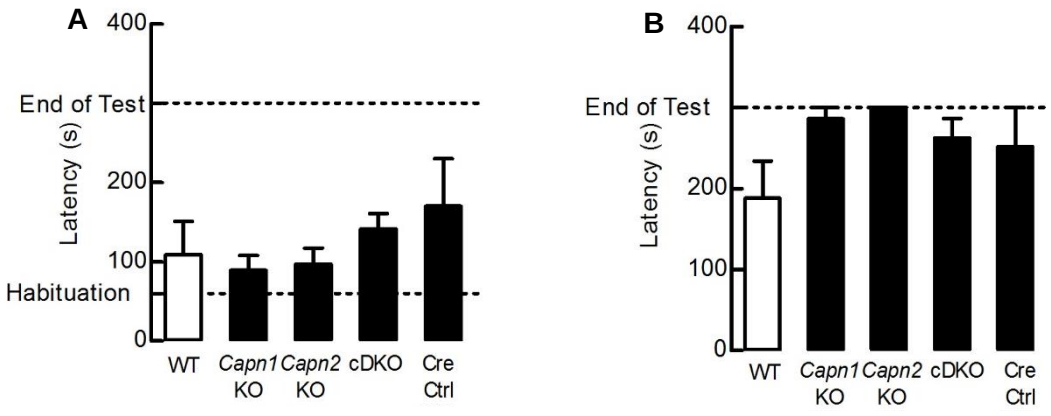

Figure 4.5 Short-term memory is not affected in calpain knockout mice. In passive avoidance test mice performed similarly in training (A, One-way ANOVA) and in test (B, One-way ANOVA). Data are presented in means ± SEM, N=4-11.

4.1.3 ANXIETY AND DEPRESSION LEVELS REMAIN UNALTERED

Anxiety and depression were tested using the following tests: Open Field Test, Elevated Plus Maze Test and Forced Swimming Test.

When mice are anxious, they spend more time in the corners avoiding the more exposed areas. Taking this into count, the Open Field Test were performed to test the levels of anxiety in mice with specific genotypes. The following graphics show no significant difference between genotypes (WT= 16.90±2.48): One-way ANOVA and Bonferroni's Post-Test [*F*= 0.260; *dF*=4; P>0.05] (Figure 4.6-A). In Figure 4.6-B, although it can be seen a tendency in mice with conditional double knockout (cDKO) to spend more time in the centre it is not statistically a significant difference (WT= 18,880±3,90) (One-way ANOVA and Bonferroni's Post-Test [*F*= 2.353; *dF*=4; P>0.05]).

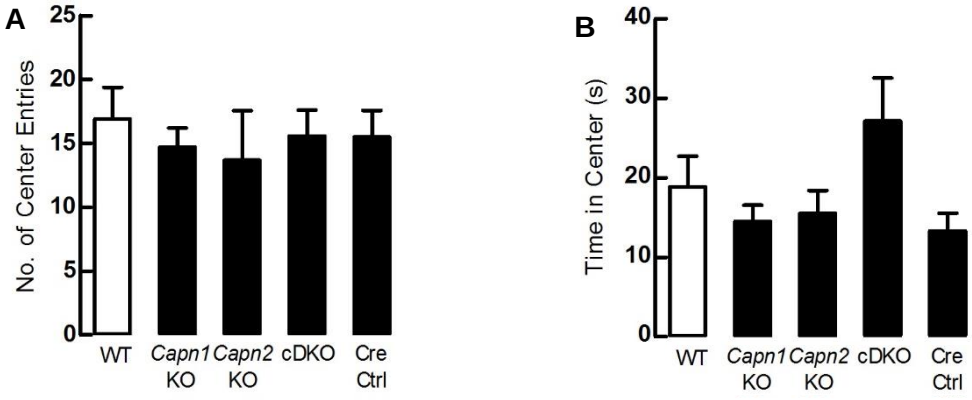

Figure 4.6 Anxiety levels remained unaltered as evaluated by Open Field Test. Knockout mice performed similarly, both in the number of centre entries (**A,** One-way ANOVA) and in the time spent in centre (**B,** One-way ANOVA). Data are presented in means ± SEM, N=6-14.

In the Elevated Plus Maze Test, mice spent more than 80% of the time inside the closed arms comparatively with the percentage of time spent in the open arms and even in the central zone.

Moreover, mice with Capn2 knockout (93.40±1.45) and mice with conditional double knockout (92.79±1.27) spent significantly more time in the closed arms compared with control (83.60±2.91)(Figure 4.7) (Two-way ANOVA and Bonferroni's Post-Test [*F*= 0.004; *dF*=4; P<0.05]).

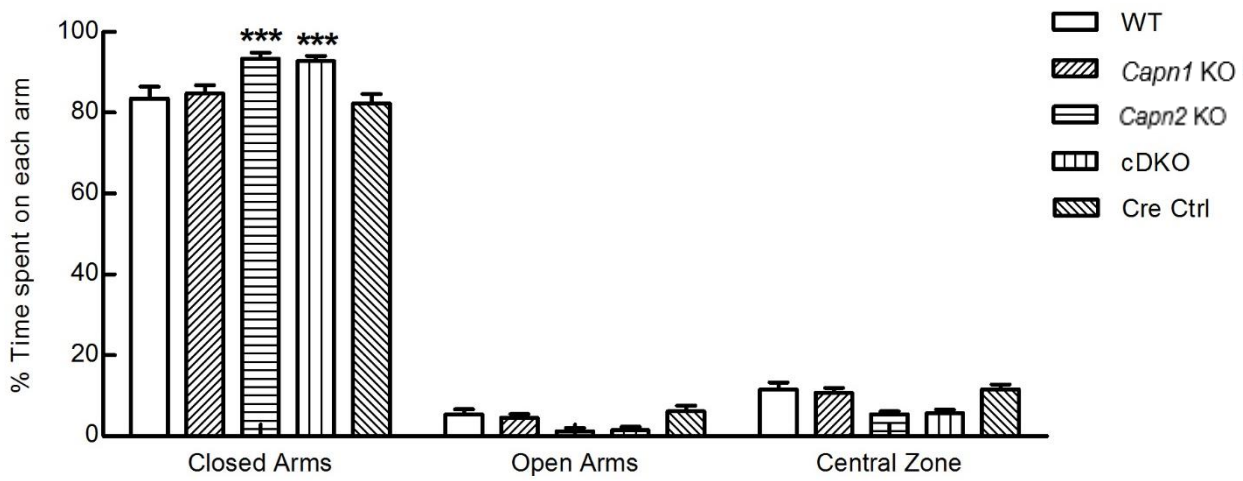

Figure 4.7 Calpain2 and cDKO mice are more anxious than the other genotypes compared with WT mice. Anxiety levels were testes throughout the elevated Plus Maze Test and Calpain2 and conditional double knockout mice spent preferentially more time inside closed arms (Two-way ANOVA) comparatively with the open arms and central zone. Other genotypes did not present any significant difference between them. Data are showed in means ± SEM, N=6-14.

Forced Swimming Test is also used as a pro-depressive behavioural measure method and throughout this test time spent immobile by mice was measured. Analysing the following graphic, it can be seen a tendency of Capn2 knockout to spend more time immobile comparing with the other genotypes. However, this tendency is not supported by One-way ANOVA [F=3.692; dF=4; P>0.05]. All genotypes performed similarly compared with WT (237.49±11.05).

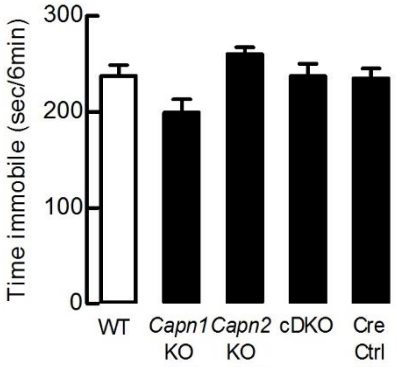

Figure 4.8 Depression levels are unaltered by calpain knockout mice. Mice performance was analysed with ANY-Maze software and time mice spent immobile was measured. No differences were observed (One-way ANOVA). Data are presented in means ± SEM, N=6-8.

4.2 NEUROGENESIS IS UNCHANGED

4.2.1 PROLIFERATION AND MIGRATION IN DENTATE GYRUS ARE PRESERVED

Proliferation and migration processes were analysed and the number of EdU positive stained cell were counted (Figure 4.9). Cells present in the sub granular zone were counted to analyse the proliferation (Figure 4.9-A). Cells found in the granular zone of the dentate gyrus of hippocampus were counted to analyse migration (Figure 4.9-B).

No significant difference was observed neither in the proliferation process (One-way ANOVA [F= 0.7061; dF=4; P>0.05]), nor in the migration process (One-way ANOVA [F= 0.837; dF=4; P>0.05]), compared with control (F, WT= 12.15±2.15 and G, WT= 3.20±0.59)

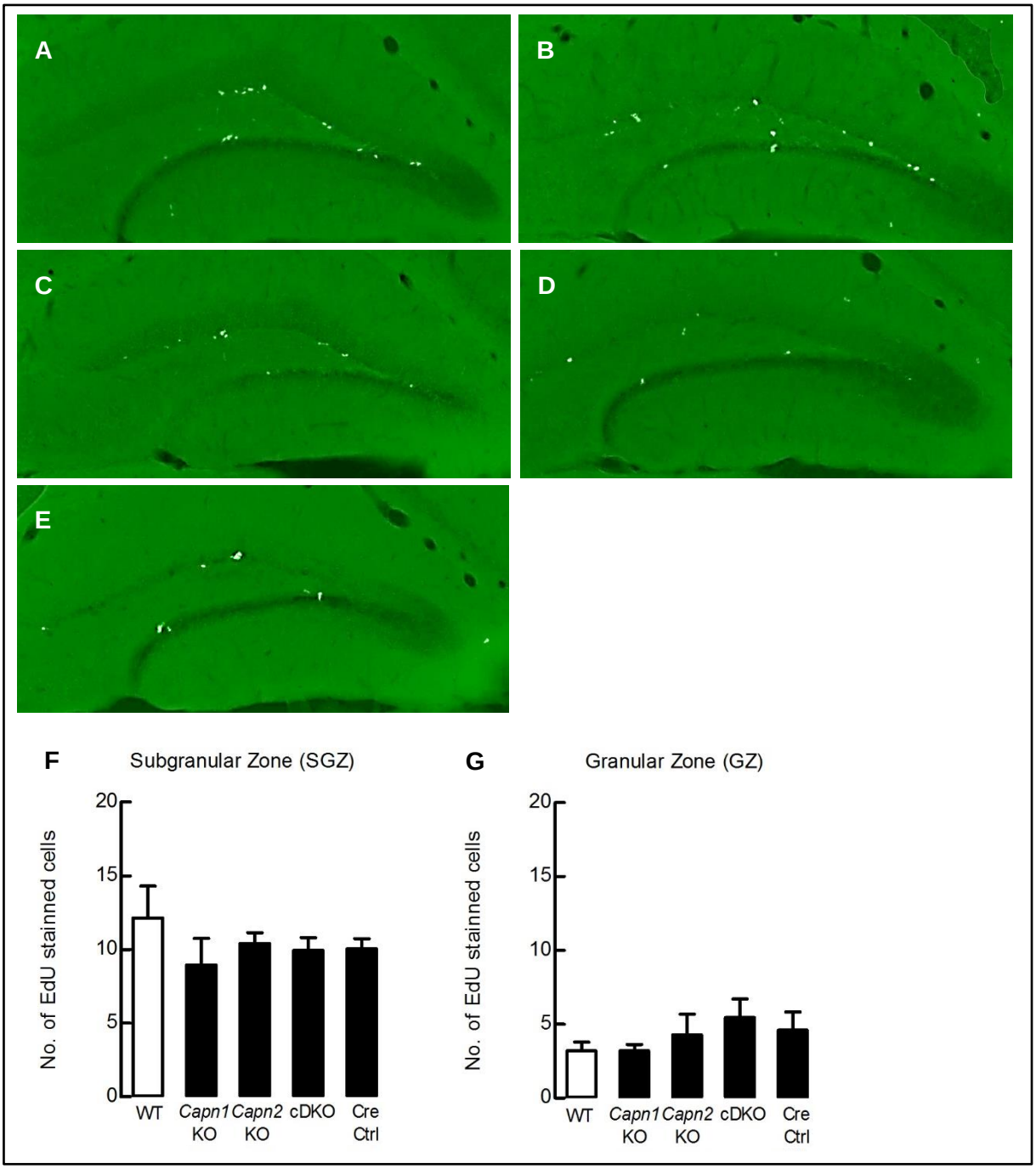

Figure 4.9 Proliferation and migration in the dentate gyrus of calpain knockout mice is similar to wild type mice. Representative Imagens of EdU staining of left dentate gyrus of mice hippocampus. A, Wild Type mice; **B**, Cre control**; C**, Calpain1 knockout; **D**, Calpain2 Knockout**; E**, CDKO; **F,** Number of EdU stained cells the subgranular zone (One-way ANOVA); **G,** Number of EdU stained cells in the granular zone (One-way ANOVA). Data are presented in means ± SEM.

4.2.1 NEUROGENESIS IS MAINTAINED BY CALPAIN KNOCKOUT

The neurogenesis was also analysed with staining with DCX that label immature (Figure 4.10). From the various genotypes, there were no statistical differences between them, comparing with the control: WT= 2.03±0.44 (One-way ANOVA [*F*=0.705; *dF*=4; P>0.05]) (Figure 4.12).

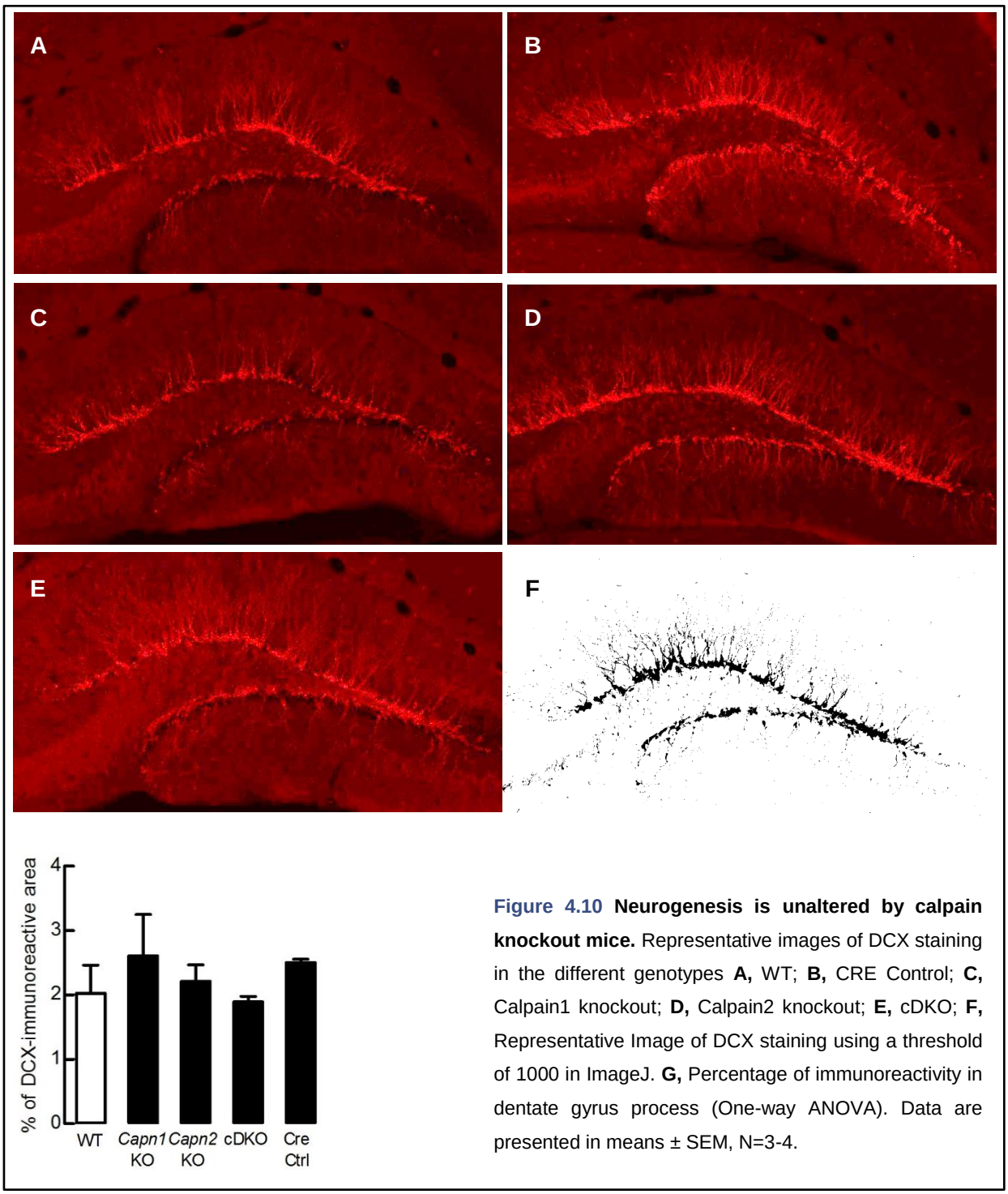

Figure 4.10 Neurogenesis is unaltered by calpain knockout mice. Representative images of DCX staining in the different genotypes **A,** WT; **B,** CRE Control; **C,** Calpain1 knockout; **D,** Calpain2 knockout; **E,** cDKO; **F,** Representative Image of DCX staining using a threshold of 1000 in ImageJ. **G,** Percentage of immunoreactivity in dentate gyrus process (One-way ANOVA). Data are presented in means ± SEM, N=3-4.

5. DISCUSSION

Our aim was to characterize neurogenesis and the behavioural phenotype produced by calpain deletion. Results showed that calpain2 knockout mice and cDKO are more anxious than calpain1 knockout mice and WT mice, suggesting a role for calpain 2 in anxiety. Memory, learning, locomotor activity and helplessness were normal among genotypes. The neurogenesis was also normal in all genotypes.

In our work, calpain1 knockout mice present a similar learning and memory phenotype to WT mice. It was established by Oliver et al. that calpain inhibition with leupeptin decreases the magnitude of LTP (Oliver et al. 1989). More recently, Grammer demonstrated that calpain1 knockout does not affect behaviour or long-term potentiation (LTP), which means calpain1 knockout mice have the same ability as WT mice to create new memories (Grammer et al. 2005). Although calpains do not seem to be involved in LTP, calpain1 knockout leads to changes in the erythrocyte structure. This finding demonstrates an important function of calpain1 to suppress erythrocyte deformability, with implications for development of treatment for disorders such as sickle cell disease (Wieschhaus et al. 2012).

Our data shows higher levels of anxiety in cDKO and calpain2 knockout mice in the elevated plus maze test. This suggests that calpain 2, but not calpain 1, is involved in anxiety behaviour. Compared with the open field test results, those of the elevated plus maze test, are more accurate due to the fact that the open field is more focused on the locomotor activity of mice. Any subtle differences might only be observed in the elevated plus maze test. Adding, emotionality can vary in different environments, leading to different behavioural responses (Ramos 2008). Previous work by Nakajima et al (2008) also showed that calpastatin, the endogenous calpain inhibitor, is also associated with affective and emotional behaviour in mice (Nakajima et al. 2008). The aim was to characterize neurogenesis and the behavioural phenotype produced by calpain knockout. Results showed that calpain2 knockout mice and cDKO are more anxious than calpain1 knockout mice and WT mice. Memory, learning, locomotor activity and pro-depressive profile were normal among genotypes. The neurogenesis was also normal in all genotypes.

Our work now shows that calpain 2 is also associated with emotional behaviour. Helplessness was similar in all genotypes, which suggests that calpains are not associated with depressive behaviour.

Learning and memory remains normal as well as the exploratory and the helpless behaviour. In contrast, Liu using low doses of a specific inhibitor (C2I), saw that normal learning was restored in calpain1 knockout mice in the novel object recognition test and that

the calpain2 limits the extent of learning (Liu et al. 2016). In another study, calpain inhibition with calpeptin leaded to an impaired spatial and working memory (Olson et al. 2015).

The possibility that mice carrying Cre construction could have an impact on the parameters being evaluated was also addressed. Cre control mice did not show any significant difference in any of the performed experiments compared with control (WT mice). Giusti also tested mice with Nestin-Cre construct through a battery of behavioural tests and also found no influence of this construct in the phenotype of mice. However, he also observed that this construct may slightly influence the results of the fear conditioning test and highlight the need to have appropriate controls to validate the results, which was not observed in our study (Giusti et al. 2014).

Studies using knockout mice and pharmacological inhibition of calpains may present different results when investigating the function of calpains. Since knockout mice survived all their life without calpains, they could have developed a compensatory mechanism to overcome the absence of calpains. Another hypothesis is that the calpain system which could be a redundant mechanism (Hepple et al. 2005). Those compensatory mechanisms could be somehow linked to more than 200 described substrates of calpains (such as growth factor receptors, cytoskeletal proteins, microtubule-associated proteins, and mitochondria), which, consequently, are involved in a variety of pathways (Franco and Huttenlocher 2005, Piatkov et al. 2014, Yildiz-Unal et al. 2015).

In this work, mice were used independently of the sex. That may be a reason for not having noticed any alteration in the phenotype. The small difference between genders cannot be observed if the tests that were performed independently of the mice gender. Moreover, female mice have estrogens that have a neuroprotective ability and may mask the effect of calpain absence (Dubal and Wise 2001). Another point to have in mind is the fact that, in the human genome, there may be more calpains to be discovered. Moreover, these calpains may be involved in the redundant mechanism (Huang and Wang 2001).

Adult neurogenesis in calpain knockout mice was surprisingly similar to WT mice. The study of neurogenesis, in this context, was made having in account the previous knowledge of alterations in behaviour that could be a result of the new neuron formation. Previous work of our group showed that calpains affect neurogenesis, consequently it would be expected to see a decrease in migration and proliferation (Machado et al. 2015). However, according with the results, migration and proliferation are not affected by calpain deletion. A study from another group, also demonstrated a decrease in proliferation of neural stem cells when calpains were inhibited (Santos et al. 2012). Again, this lack of differences could be explained

by the fact of having performed the tests with gender mix. Mirescu proved that cellular proliferation depends on the animal gender (Mirescu and Gould 2006).

Since neurogenesis is a process that leads to adaptations in central nervous system, those adaptations may be significant to a point that expresses alteration in the behavioural phenotype of mice. However, it also can result in slight alteration, small enough to not be verified when analysing the behaviour of mice. In the last case, those little adaptations must be observed at a neuron level, i.e., for instance, through the observation of the increase/decrease in dendritic spine formation or neuronal activity and synaptic transmission. Dendritic spines are involved in learning and memory acquisition. Every time mice learn a new task, there is a change, i.e. an increase or decrease in the formation of dendritic spines in specific neurons. Another way of analysing the formation of new synapses and dendritic spines is through the analysis of electrophysiology (using Long-term potentiation) that is closely related to dendritic spines formation. LTP can modulate the formation of dendritic spines and could also complement the presented results (Baudry et al. 2013). As calpains are involved in cytoskeletal modulation, they may also participate in dendritic spine alteration and LTP. In a near future, we aim to explore the LTP in calpain knockout mice and understand if there is any alteration at a neuronal level, even though that there was no behavioural phenotype.

The need for selective inhibitors for a given calpain, which only inhibit one calpain isoform, is a major focus to understand calpain role in cellular physiology. Indeed, specific inhibitors for only one calpain are currently under research. BDA-410 is one example of calpain1 specific inhibitor that is being studied to prevent or even treat sickle cell disease (De Franceschi et al. 2013). For calpain2, Z-Leu-Abu-CONH-CH2-C6H3 (3, 5-(OMe)2 (C2I) is a specific inhibitor of calpain2. Liu showed that using this inhibitor, learning was increased in normal conditions and even in genetic models with impaired learning (Liu et al. 2016).

6. CONCLUSIONS

Finally, this work can be summarised in three key conclusions:

i) Calpain2 is associated with anxiety behaviour;

ii) Memory and learning are not affected by calpain deletion in mice;

iii) Adult neurogenesis in calpain knockout mice is normal.

7. BIBLIOGRAPHIC REFERENCES

Altman J (1962). "Are new neurons formed in the brains of adult mammals?" Science **135**(3509): 1127-1128.

Amini M, CL Ma, R Farazifard, G Zhu, Y Zhang, J Vanderluit, JS Zoltewicz, F Hage, JM Savitt, DC Lagace, RS Slack, JC Beique, M Baudry, PA Greer, R Bergeron and DS Park (2013). "Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury." J Neurosci **33**(13): 5773-5784.

Arthur JS, JS Elce, C Hegadorn, K Williams and PA Greer (2000). "Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division." Mol Cell Biol **20**(12): 4474-4481.

Ashizawa K, GJ Wishart, S Katayama, D Takano, M Maeda, E Arakawa and Y Tsuzuki (2006). "Effects of calpain and Rho-kinase inhibitors on the acrosome reaction and motility of fowl spermatozoa in vitro." Reproduction **131**(1): 71-79.

Azam M, SS Andrabi, KE Sahr, L Kamath, A Kuliopulos and AH Chishti (2001). "Disruption of the mouse mu-calpain gene reveals an essential role in platelet function." Mol Cell Biol **21**(6): 2213-2220.

Baudry M and X Bi (2016). "Calpain-1 and Calpain-2: The Yin and Yang of Synaptic Plasticity and Neurodegeneration." Trends Neurosci **39**(4): 235-245.

Baudry M and X Bi (2016). "Calpain-1 and Calpain-2: The Yin and Yang of Synaptic Plasticity and Neurodegeneration." Trends Neurosci.

Baudry M, X Bi, C Gall and G Lynch (2011). "The biochemistry of memory: The 26year journey of a 'new and specific hypothesis'." Neurobiol Learn Mem **95**(2): 125-133.

Baudry M, MM Chou and X Bi (2013). "Targeting calpain in synaptic plasticity." Expert Opin Ther Targets **17**(5): 579-592.

Ben-Aharon I, PR Brown, R Shalgi and EM Eddy (2006). "Calpain 11 is unique to mouse spermatogenic cells." Mol Reprod Dev **73**(6): 767-773.

Bergmann O, J Liebl, S Bernard, K Alkass, MS Yeung, P Steier, W Kutschera, L Johnson, M Landen, H Druid, KL Spalding and J Frisen (2012). "The age of olfactory bulb neurons in humans." Neuron **74**(4): 634-639.

Bernier PJ, A Bedard, J Vinet, M Levesque and A Parent (2002). "Newly generated neurons in the amygdala and adjoining cortex of adult primates." Proc Natl Acad Sci U S A **99**(17): 11464-11469.

Bevins RA and J Besheer (2006). "Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'." Nat Protoc **1**(3): 1306-1311.

Bodhini D, V Radha, S Ghosh, KR Sanapala, PP Majumder, MR Rao and V Mohan (2011). "Association of calpain 10 gene polymorphisms with type 2 diabetes mellitus in Southern Indians." Metabolism **60**(5): 681-688.

Borsini A, PA Zunszain, S Thuret and CM Pariante (2015). "The role of inflammatory cytokines as key modulators of neurogenesis." Trends Neurosci **38**(3): 145-157.

Branca D (2004). "Calpain-related diseases." Biochem Biophys Res Commun **322**(4): 1098-1104.

Briz V and M Baudry (2016). "Calpains: Master Regulators of Synaptic Plasticity." Neuroscientist.

Cajal Ry (1906). The structure and connexions of neurons**:** 220-253.

Cameron HA and AG Dayer (2008). "New interneurons in the adult neocortex: small, sparse, but significant?" Biol Psychiatry **63**(7): 650-655.

Campbell RL and PL Davies (2012). "Structure-function relationships in calpains." Biochem J **447**(3): 335-351.

Cao L, X Jiao, DS Zuzga, Y Liu, DM Fong, D Young and MJ During (2004). "VEGF links hippocampal activity with neurogenesis, learning and memory." Nat Genet **36**(8): 827-835.

Curtis MA, EB Penney, AG Pearson, WM van Roon-Mom, NJ Butterworth, M Dragunow, B Connor and RL Faull (2003). "Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain." Proc Natl Acad Sci U S A **100**(15): 9023-9027.

Curzon P, NR Rustay and KE Browman (2009). Cued and Contextual Fear Conditioning for Rodents. Methods of Behavior Analysis in Neuroscience. J. J. Buccafusco. Boca Raton (FL).

Dasgupta S, PV Sirisha, K Neelaveni, K Anuradha and BM Reddy (2012). "Association of CAPN10 SNPs and haplotypes with polycystic ovary syndrome among South Indian Women." PLoS One **7**(2): e32192.

De Franceschi L, RS Franco, M Bertoldi, C Brugnara, A Matte, A Siciliano, AJ Wieschhaus, AH Chishti and CH Joiner (2013). "Pharmacological inhibition of calpain-1 prevents red cell dehydration and reduces Gardos channel activity in a mouse model of sickle cell disease." FASEB J **27**(2): 750-759.

De Maria A, Y Shi, NM Kumar and S Bassnett (2009). "Calpain expression and activity during lens fiber cell differentiation." J Biol Chem **284**(20): 13542-13550.

Diamond MC (2001). "Response of the brain to enrichment." An Acad Bras Cienc **73**(2): 211-220.

Domingues AF, AR Esteves, RH Swerdlow, CR Oliveira and SM Cardoso (2008). "Calpain-mediated MPP+ toxicity in mitochondrial DNA depleted cells." Neurotox Res **13**(1): 31-38.

Dubal DB and PM Wise (2001). "Neuroprotective effects of estradiol in middle-aged female rats." Endocrinology **142**(1): 43-48.

Dusek JA and H Eichenbaum (1997). "The hippocampus and memory for orderly stimulus relations." Proc Natl Acad Sci U S A **94**(13): 7109-7114.

Dutt P, DE Croall, JS Arthur, TD Veyra, K Williams, JS Elce and PA Greer (2006). "m-Calpain is required for preimplantation embryonic development in mice." BMC Dev Biol **6**: 3.

Ericsson AC, MJ Crim and CL Franklin (2013). "A brief history of animal modeling." Mo Med **110**(3): 201-205.

Ernst A, K Alkass, S Bernard, M Salehpour, S Perl, J Tisdale, G Possnert, H Druid and J Frisen (2014). "Neurogenesis in the striatum of the adult human brain." Cell **156**(5): 1072-1083.

Ernst A and J Frisen (2015). "Adult neurogenesis in humans- common and unique traits in mammals." PLoS Biol **13**(1): e1002045.

Esteves AR, DM Arduino, RH Swerdlow, CR Oliveira and SM Cardoso (2010). "Dysfunctional mitochondria uphold calpain activation: contribution to Parkinson's disease pathology." Neurobiol Dis **37**(3): 723-730.

Ferragud A, A Haro, A Sylvain, C Velazquez-Sanchez, V Hernandez-Rabaza and JJ Canales (2010). "Enhanced habit-based learning and decreased neurogenesis in the adult hippocampus in a murine model of chronic social stress." Behav Brain Res **210**(1): 134-139.

Ferreira A (2012). "Calpain dysregulation in Alzheimer's disease." ISRN Biochem **2012**: 728571.

Franco SJ and A Huttenlocher (2005). "Regulating cell migration: calpains make the cut." Journal of Cell Science **118**(17): 3829-3838.

Fuchs E and G Flugge (2014). "Adult neuroplasticity: more than 40 years of research." Neural Plast **2014**: 541870.

Gafni J and LM Ellerby (2002). "Calpain activation in Huntington's disease." J Neurosci **22**(12): 4842-4849.

Gafni J, E Hermel, JE Young, CL Wellington, MR Hayden and LM Ellerby (2004). "Inhibition of calpain cleavage of huntingtin reduces toxicity: accumulation of calpain/caspase fragments in the nucleus." J Biol Chem **279**(19): 20211-20220.

Gil-Parrado S, O Popp, TA Knoch, S Zahler, F Bestvater, M Felgentrager, A Holloschi, A Fernandez-Montalvan, EA Auerswald, H Fritz, P Fuentes-Prior, W Machleidt and E Spiess (2003). "Subcellular localization and in vivo subunit interactions of ubiquitous mu-calpain." J Biol Chem **278**(18): 16336-16346.

Giusti SA, CA Vercelli, AM Vogl, AW Kolarz, NS Pino, JM Deussing and D Refojo (2014). "Behavioral phenotyping of Nestin-Cre mice: implications for genetic mouse models of psychiatric disorders." J Psychiatr Res **55**: 87-95.

Goffredo D, D Rigamonti, M Tartari, A De Micheli, C Verderio, M Matteoli, C Zuccato and E Cattaneo (2002). "Calcium-dependent cleavage of endogenous wild-type huntingtin in primary cortical neurons." J Biol Chem **277**(42): 39594-39598.

Goldman SA and F Nottebohm (1983). "Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain." Proc Natl Acad Sci U S A **80**(8): 2390-2394.

Goll DE, VF Thompson, H Li, W Wei and J Cong (2003). "The calpain system." Physiol Rev **83**(3): 731-801.

Grammer M, S Kuchay, A Chishti and M Baudry (2005). "Lack of phenotype for LTP and fear conditioning learning in calpain 1 knock-out mice." Neurobiol Learn Mem **84**(3): 222-227.

Grassi Zucconi G, S Cipriani, I Balgkouranidou and R Scattoni (2006). "'One night' sleep deprivation stimulates hippocampal neurogenesis." Brain Res Bull **69**(4): 375-381.

Gu M, MT Gash, VM Mann, F Javoy-Agid, JM Cooper and AH Schapira (1996). "Mitochondrial defect in Huntington's disease caudate nucleus." Ann Neurol **39**(3): 385-389.

Guroff G (1964). "A Neutral, Calcium-Activated Proteinase from the Soluble Fraction of Rat Brain." J Biol Chem **239**: 149-155.

Hackam AS, R Singaraja, CL Wellington, M Metzler, K McCutcheon, T Zhang, M Kalchman and MR Hayden (1998). "The influence of huntingtin protein size on nuclear localization and cellular toxicity." J Cell Biol **141**(5): 1097-1105.

Hata S, M Abe, H Suzuki, F Kitamura, N Toyama-Sorimachi, K Abe, K Sakimura and H Sorimachi (2010). "Calpain 8/nCL-2 and calpain 9/nCL-4 constitute an active protease complex, G-calpain, involved in gastric mucosal defense." PLoS Genet **6**(7): e1001040.

Hauerslev S, ML Sveen, M Duno, C Angelini, J Vissing and TO Krag (2012). "Calpain 3 is important for muscle regeneration: evidence from patients with limb girdle muscular dystrophies." BMC Musculoskelet Disord **13**: 43.

Hepple B, C Peckham, T Baldwin, M Brazier, R Brownsword, K Calman, P Harper, R Harries, P Lipton, Po Southwark, LR Plant, M Raff, N Ross, H Sewell, P Smith, M Strathern and A Williamson (2005). Genetically modified animals in the study of human disease. The ethics of researchminvolving animals, Nuffield Council on Bioethics.

Hong JM, SL Teitelbaum, TH Kim, FP Ross, SY Kim and HJ Kim (2011). "Calpain-6, a target molecule of glucocorticoids, regulates osteoclastic bone resorption via cytoskeletal organization and microtubule acetylation." J Bone Miner Res **26**(3): 657-665.

Hosfield CM, JS Elce, PL Davies and Z Jia (1999). "Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation." EMBO J **18**(24): 6880-6889.

Huang Y and KK Wang (2001). "The calpain family and human disease." Trends Mol Med **7**(8): 355-362.

Hunsaker MR, B Lee and RP Kesner (2008). "Evaluating the temporal context of episodic memory: the role of CA3 and CA1." Behav Brain Res **188**(2): 310-315.

Iqbal K, F Liu, CX Gong, C Alonso Adel and I Grundke-Iqbal (2009). "Mechanisms of tau-induced neurodegeneration." Acta Neuropathol **118**(1): 53-69.

James W (1890). The principles of Psychology.

Kahn-Greene ET, DB Killgore, GH Kamimori, TJ Balkin and WD Killgore (2007). "The effects of sleep deprivation on symptoms of psychopathology in healthy adults." Sleep Med **8**(3): 215-221.

Kamei M, GC Webb, IG Young and HD Campbell (1998). "SOLH, a human homologue of the Drosophila melanogaster small optic lobes gene is a member of the calpain and zinc-finger gene families and maps to human chromosome 16p13.3 near CATM (cataract with microphthalmia)." Genomics **51**(2): 197-206.

Kang HJ, YI Kawasawa, F Cheng, Y Zhu, X Xu, M Li, AM Sousa, M Pletikos, KA Meyer, G Sedmak, T Guennel, Y Shin, MB Johnson, Z Krsnik, S Mayer, S Fertuzinhos, S Umlauf, SN Lisgo, A Vortmeyer, DR Weinberger, S Mane, TM Hyde, A Huttner, M Reimers, JE Kleinman and N Sestan (2011). "Spatio-temporal transcriptome of the human brain." Nature **478**(7370): 483-489.

Kaslin J, J Ganz and M Brand (2008). "Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain." Philos Trans R Soc Lond B Biol Sci **363**(1489): 101-122.

Kazlauckas V, J Schuh, OP Dall'Igna, GS Pereira, CD Bonan and DR Lara (2005). "Behavioral and cognitive profile of mice with high and low exploratory phenotypes." Behav Brain Res **162**(2): 272-278.

Kempermann G, HG Kuhn and FH Gage (1997). "More hippocampal neurons in adult mice living in an enriched environment." Nature **386**(6624): 493-495.

Kempermann G, L Wiskott and FH Gage (2004). "Functional significance of adult neurogenesis." Curr Opin Neurobiol **14**(2): 186-191.

Khorchid A and M Ikura (2002). "How calpain is activated by calcium." Nat Struct Biol **9**(4): 239-241.

Kokoeva MV, H Yin and JS Flier (2005). "Neurogenesis in the hypothalamus of adult mice: potential role in energy balance." Science **310**(5748): 679-683.

Kornack DR and P Rakic (1999). "Continuation of neurogenesis in the hippocampus of the adult macaque monkey." Proc Natl Acad Sci U S A **96**(10): 5768-5773.

Kovacs LS, Yunchao (2014). "The Critical Role of Calpain in Cell Proliferation." Biomolecular Research & Therapeutics **3**(3).

Kuipers SD, CR Bramham, HA Cameron, CP Fitzsimons, A Korosi and PJ Lucassen (2014). "Environmental control of adult neurogenesis: from hippocampal homeostasis to behavior and disease." Neural Plast **2014**: 808643.

Lieschke GJ and PD Currie (2007). "Animal models of human disease: zebrafish swim into view." Nat Rev Genet **8**(5): 353-367.

Liu Y, Y Wang, G Zhu, J Sun, X Bi and M Baudry (2016). "A calpain-2 selective inhibitor enhances learning & memory by prolonging ERK activation." Neuropharmacology **105**: 471-477.

Lopez-Garcia C, A Molowny, JM Garcia-Verdugo and I Ferrer (1988). "Delayed postnatal neurogenesis in the cerebral cortex of lizards." Brain Res **471**(2): 167-174.

Lucassen PJ, P Meerlo, AS Naylor, AM van Dam, AG Dayer, E Fuchs, CA Oomen and B Czeh (2010). "Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action." Eur Neuropsychopharmacol **20**(1): 1-17.

Lynch G and M Baudry (1984). "The biochemistry of memory: a new and specific hypothesis." Science **224**(4653): 1057-1063.

MacDonald ME, CM Ambrose, MP Duyao, RH Myers, C Lin, L Srinidhi, G Barnes, SA Taylor, M James, N Groot, H MacFarlane, B Jenkins, MA Anderson, NS Wexler, JF Gusella, GP Bates, S Baxendale, H Hummerich, S Kirby, M North, S Youngman, R Mott, G Zehetner, Z Sedlacek, A Poustka, A-M Frischauf, H Lehrach, AJ Buckler, D Church, L Doucette-Stamm, MC O'Donovan, L Riba-Ramirez, M Shah, VP Stanton, SA Strobel, KM Draths, JL Wales, P Dervan, DE Housman, M Altherr, R Shiang, L Thompson, T Fielder, JJ Wasmuth, D Tagle, J Valdes, L Elmer, M Allard, L Castilla, M Swaroop, K Blanchard, FS Collins, R Snell, T Holloway, K Gillespie, N Datson, D Shaw and PS Harper (1993). "A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes." Cell **72**(6): 971-983.

Machado VM, MI Morte, BP Carreira, MM Azevedo, J Takano, N Iwata, TC Saido, H Asmussen, AR Horwitz, CM Carvalho and IM Araujo (2015). "Involvement of calpains in adult neurogenesis: implications for stroke." Frontiers in Cellular Neuroscience **9**.

Manganas LN, X Zhang, Y Li, RD Hazel, SD Smith, ME Wagshul, F Henn, H Benveniste, PM Djuric, G Enikolopov and M Maletic-Savatic (2007). "Magnetic resonance spectroscopy identifies neural progenitor cells in the live human brain." Science **318**(5852): 980-985.

Marxreiter F, M Regensburger and J Winkler (2013). "Adult neurogenesis in Parkinson's disease." Cell Mol Life Sci **70**(3): 459-473.

McGonigle P (2014). "Animal models of CNS disorders." Biochem Pharmacol **87**(1): 140-149.

Ming GL and H Song (2011). "Adult neurogenesis in the mammalian brain: significant answers and significant questions." Neuron **70**(4): 687-702.

Mirescu C and E Gould (2006). "Stress and adult neurogenesis." Hippocampus **16**(3): 233-238.

Moldoveanu T, Z Jia and PL Davies (2004). "Calpain activation by cooperative Ca2+ binding at two non-EF-hand sites." J Biol Chem **279**(7): 6106-6114.

Morris R (1984). "Developments of a water-maze procedure for studying spatial learning in the rat." J Neurosci Methods **11**(1): 47-60.

Mu Y and FH Gage (2011). "Adult hippocampal neurogenesis and its role in Alzheimer's disease." Mol Neurodegener **6**: 85.

Murphy T, GP Dias and S Thuret (2014). "Effects of diet on brain plasticity in animal and human studies: mind the gap." Neural Plast **2014**: 563160.

Nakagawa K, H Masumoto, H Sorimachi and K Suzuki (2001). "Dissociation of m-calpain subunits occurs after autolysis of the N-terminus of the catalytic subunit, and is not required for activation." J Biochem **130**(5): 605-611.

Nakajima R, K Takao, SM Huang, J Takano, N Iwata, T Miyakawa and TC Saido (2008). "Comprehensive behavioral phenotyping of calpastatin-knockout mice." Mol Brain **1**: 7.

Neumar RW, FH Meng, AM Mills, YA Xu, C Zhang, FA Welsh and R Siman (2001). "Calpain activity in the rat brain after transient forebrain ischemia." Exp Neurol **170**(1): 27-35.

Oliver MW, M Baudry and G Lynch (1989). "The protease inhibitor leupeptin interferes with the development of LTP in hippocampal slices." Brain Res **505**(2): 233-238.

Olson ML, AE Ingebretson and KM Harmelink (2015). "Hippocampal Cortactin Levels are Reduced Following Spatial Working Memory Formation, an Effect Blocked by Chronic Calpain Inhibition." Brain Sci **5**(2): 241-257.

Osako Y, Y Maemoto, R Tanaka, H Suzuki, H Shibata and M Maki (2010). "Autolytic activity of human calpain 7 is enhanced by ESCRT-III-related protein IST1 through MIT-MIM interaction." FEBS J **277**(21): 4412-4426.

Pascual-Leone A, A Amedi, F Fregni and LB Merabet (2005). "The plastic human brain cortex." Annu Rev Neurosci **28**: 377-401.

Pawluski JL, S Brummelte, CK Barha, TM Crozier and LA Galea (2009). "Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging." Front Neuroendocrinol **30**(3): 343-357.

Peretto P and RG Paredes (2014). Social Cues, Adult Neurogenesis, and Reproductive Behavior. Neurobiology of Chemical Communication. C. Mucignat-Caretta. Boca Raton (FL).

Perez-Martinez P, J Delgado-Lista, A Garcia-Rios, JF Ferguson, HL Gulseth, CM Williams, B Karlstrom, B Kiec-Wilk, EE Blaak, O Helal, M Malczewska-Malec, C Defoort, U Riserus, WH Saris, JA Lovegrove, CA Drevon, HM Roche and J Lopez-Miranda (2011). "Calpain-10 interacts with plasma saturated fatty acid concentrations to influence insulin resistance in individuals with the metabolic syndrome." Am J Clin Nutr **93**(5): 1136-1141.

Piatkov KI, JH Oh, Y Liu and A Varshavsky (2014). "Calpain-generated natural protein fragments as short-lived substrates of the N-end rule pathway." Proc Natl Acad Sci U S A **111**(9): E817-826.

Porsolt RD, A Bertin and M Jalfre (1977). "Behavioral despair in mice: a primary screening test for antidepressants." Arch Int Pharmacodyn Ther **229**(2): 327-336.

Raisman G (1969). "Neuronal plasticity in the septal nuclei of the adult rat." Brain Res **14**(1): 25-48.

Ramón y Cajal S (1904). " La Textura del Sistema Nerviosa del Hombre y los Vertebrados."

Ramos A (2008). "Animal models of anxiety: do I need multiple tests?" Trends Pharmacol Sci **29**(10): 493-498.

Richard I, C Roudaut, S Marchand, S Baghdiguian, M Herasse, D Stockholm, Y Ono, L Suel, N Bourg, H Sorimachi, G Lefranc, M Fardeau, A Sebille and JS Beckmann (2000). "Loss of calpain 3 proteolytic activity leads to muscular dystrophy and to apoptosis-associated IkappaBalpha/nuclear factor kappaB pathway perturbation in mice." J Cell Biol **151**(7): 1583-1590.

Rossi C, A Angelucci, L Costantin, C Braschi, M Mazzantini, F Babbini, ME Fabbri, L Tessarollo, L Maffei, N Berardi and M Caleo (2006). "Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment." Eur J Neurosci **24**(7): 1850-1856.

Rothman SM, KJ Griffioen, R Wan and MP Mattson (2012). "Brain-derived neurotrophic factor as a regulator of systemic and brain energy metabolism and cardiovascular health." Ann N Y Acad Sci **1264**: 49-63.

Saez ME, MT Martinez-Larrad, R Ramirez-Lorca, JL Gonzalez-Sanchez, C Zabena, MJ Martinez-Calatrava, A Gonzalez, FJ Moron, A Ruiz and M Serrano-Rios (2007).

"Calpain-5 gene variants are associated with diastolic blood pressure and cholesterol levels." BMC Med Genet **8**: 1.

Salamino F, R De Tullio, M Michetti, P Mengotti, E Melloni and S Pontremoli (1994). "Modulation of calpastatin specificity in rat tissues by reversible phosphorylation and dephosphorylation." Biochem Biophys Res Commun **199**(3): 1326-1332.

Santos DM, JM Xavier, AL Morgado, S Sola and CM Rodrigues (2012). "Distinct regulatory functions of calpain 1 and 2 during neural stem cell self-renewal and differentiation." PLoS One **7**(3): e33468.

Sawada M and K Sawamoto (2013). "Mechanisms of neurogenesis in the normal and injured adult brain." Keio J Med **62**(1): 13-28.

Sorimachi H and K Suzuki (2001). "The structure of calpain." J Biochem **129**(5): 653-664.

Spalding KL, O Bergmann, K Alkass, S Bernard, M Salehpour, HB Huttner, E Bostrom, I Westerlund, C Vial, BA Buchholz, G Possnert, DC Mash, H Druid and J Frisen (2013). "Dynamics of hippocampal neurogenesis in adult humans." Cell **153**(6): 1219-1227.

Takano J, N Mihira, R Fujioka, E Hosoki, AH Chishti and TC Saido (2011). "Vital role of the calpain-calpastatin system for placental-integrity-dependent embryonic survival." Mol Cell Biol **31**(19): 4097-4106.

Tan Y, N Dourdin, C Wu, T De Veyra, JS Elce and PA Greer (2006). "Conditional disruption of ubiquitous calpains in the mouse." Genesis **44**(6): 297-303.

Tanapat P, NB Hastings, AJ Reeves and E Gould (1999). "Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat." J Neurosci **19**(14): 5792-5801.

Taupin P (2006). "Adult neural stem cells, neurogenic niches, and cellular therapy." Stem Cell Rev **2**(3): 213-219.

van Meer PJ, ML Graham and HJ Schuurman (2015). "The safety, efficacy and regulatory triangle in drug development: Impact for animal models and the use of animals." Eur J Pharmacol **759**: 3-13.

van Praag H, G Kempermann and FH Gage (1999). "Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus." Nat Neurosci **2**(3): 266-270.

Vijayakumar P, V Laize, J Cardeira, M Trindade and ML Cancela (2013). "Development of an in vitro cell system from zebrafish suitable to study bone cell differentiation and extracellular matrix mineralization." Zebrafish **10**(4): 500-509.

Walf AA and CA Frye (2007). "The use of the elevated plus maze as an assay of anxiety-related behavior in rodents." Nat Protoc **2**(2): 322-328.

Walker FO (2007). "Huntington's Disease." Semin Neurol **27**(2): 143-150.

Walsh RN and RA Cummins (1976). "The Open-Field Test: a critical review." Psychol Bull **83**(3): 482-504.

Warner-Schmidt JL and RS Duman (2007). "VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants." Proc Natl Acad Sci U S A **104**(11): 4647-4652.

Wehner JM and RA Radcliffe (2004). "Cued and contextual fear conditioning in mice." Curr Protoc Neurosci **Chapter 8**: Unit 8 5C.

Wieschhaus A, A Khan, A Zaidi, H Rogalin, T Hanada, F Liu, L De Franceschi, C Brugnara, A Rivera and AH Chishti (2012). "Calpain-1 knockout reveals broad effects on erythrocyte deformability and physiology." Biochem J **448**(1): 141-152.

Winner B, Z Kohl and FH Gage (2011). "Neurodegenerative disease and adult neurogenesis." Eur J Neurosci **33**(6): 1139-1151.

Winner B and J Winkler (2015). "Adult neurogenesis in neurodegenerative diseases." Cold Spring Harb Perspect Biol **7**(4): a021287.

Wu HY and DR Lynch (2006). "Calpain and synaptic function." Mol Neurobiol **33**(3): 215-236.

Yildiz-Unal A, S Korulu and A Karabay (2015). "Neuroprotective strategies against calpain-mediated neurodegeneration." Neuropsychiatr Dis Treat **11**: 297-310.

Yoshikawa Y, H Mukai, F Hino, K Asada and I Kato (2000). "Isolation of two novel genes, down-regulated in gastric cancer." Jpn J Cancer Res **91**(5): 459-463.

Zadran S, G Akopian, H Zadran, J Walsh and M Baudry (2013). "RVG-mediated calpain2 gene silencing in the brain impairs learning and memory." Neuromolecular Med **15**(1): 74-81.

Zhao C, W Deng and FH Gage (2008). "Mechanisms and functional implications of adult neurogenesis." Cell **132**(4): 645-660.

Zhao C, EM Teng, RG Summers, Jr., GL Ming and FH Gage (2006). "Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus." J Neurosci **26**(1): 3-11.