UAlg
UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

REGULATION OF NEUROGENESIS BY CALPAINS: RELEVANCE FOR POST-INJURY BRAIN REPAIR

Vanessa Mendes Machado

Tese para obtenção do Grau de Doutor em Ciências Biomédicas

Trabalho efectuado sob a orientação da
Professora Doutora Inês Maria Pombinho de Araújo

2016

REGULATION OF NEUROGENESIS BY CALPAINS: RELEVANCE FOR POST-INJURY BRAIN REPAIR

Declaração de autoria de trabalho

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.

Vanessa Mendes Machado

Aos meus pais

Agradecimentos

Gostaria de agradecer a todos os que de alguma forma contribuíram para a realização desta tese.

À minha orientadora, Professora Doutora Inês Araújo, e à Professora Doutora Caetana Carvalho, pelos ensinamentos e aconselhamentos transmitidos ao longo da minha carreira em investigação.

A toda a gente do CNC, em especial ao Bruno e à Inês, que sempre se disponibilizaram para ajudar nos dilemas da investigação, à Manuela, que conseguiu transformar um início tremido numa boa amizade, e ainda às técnicas e funcionárias que tanto apoio me deram no CNC e que tanta falta me fizeram quando vim para Faro!

De Faro há muito mais gente para agradecer, pois foi onde estive mais tempo e onde realizei a maior parte deste trabalho. A lista é longa, e agradeço a todos, mas em especial a todos os técnicos que me ensinaram e ajudaram sempre que necessário, e ao pessoal do laboratório, em especial à Joana, Daniela, Ana e Adélia, e um agradecimento ainda maior à Sofia, que já me atura há quase 20 anos!! A tua presença, e das tuas crianças, e ajuda tornaram a realização deste trabalho bastante mais suportável.

Ao pessoal da bola, por proporcionarem momentos de descontracção que me permitiram abstrair de todos os problemas e tensões durante a parte mais crítica da realização deste trabalho.

Aos senhores jardineiros, sem os quais a realização dos testes comportamentais com a piscina teria sido bem mais morosa, e ao Sr. Paulo Jorge, por toda a ajuda com o material para os testes de comportamento e com a impressão desta tese.

Ao meu irmão, por ter disponibilizado parte do seu tempo para me dar uma ajuda preciosa com as bases dos esquemas da introdução desta tese.

Ao Doutor Mala Rao e ao Doutor Takaomi Saido, pela ajuda e disponibilização dos murganhos utilizados nesta tese.

Ao próprio CNC e ao CBMR, por me terem acolhido nas suas instalações, e um especial agradecimento à FCT, por ter financiado este trabalho.

Por fim, agradeço de coração a todos os que aturam os meus constantes devaneios (vocês sabem quem são!) e claro, à minha famíla, que sempre me apoiou em tudo o que precisei, e que eu sei que sempre estará presente, venha o que vier.

Abstract

New neurons are continuously added in the adult mammalian brain, in two main neurogenic regions: the subventricular zone, in the walls of the lateral ventricles, and the subgranular zone, in the dentate gyrus of the hippocampus. Neural stem cells present in these niches are able to proliferate and originate migrating neuroblasts, which ultimately differentiate into new neurons. Interestingly, in the event of a brain lesion, neural stem cell proliferation increases, possibly in an attempt to repair the damage. However, this repair is limited by impaired cell migration or decreased survival of new neurons. Discovering how to increase the efficiency of post-injury neurogenesis may therefore be beneficial for brain repair. On the other hand, brain damage can also activate several proteases, including calpains. Calpains are ubiquitous proteases known to be involved in cell proliferation, migration and differentiation in several systems. In the brain, calpain inhibition has been shown to be neuroprotective, but their effects on neurogenesis remain elusive. Deletion of the endogenous inhibitor of calpains, calpastatin, has previously been shown to impair early stages of neurogenesis *in vivo*. In the present work, we show that calpain inhibition is able to reverse this impairment, further implicating them in the process. Since calpain inhibition offers a promising target for the enhancement of post-injury neurogenesis, we evaluated different stages of the process and cognitive behavior recovery after seizure-induced hippocampal lesion in mice overexpressing calpastatin, and also the effects of the absence of conventional calpains on the early stages of hippocampal neurogenesis. We observed that the pattern of enhancement of post-injury neurogenesis was mostly maintained in mice overexpressing calpastatin, though some cognitive recovery indicates some degree of neuroprotection, which by itself may have masked the influence of calpain inhibition on neurogenesis. Mice lacking calpains, however, presented unaltered levels of the early stages of hippocampal neurogenesis, suggesting that calpains only affect this process when their activity is over a certain threshold, under which neurogenesis is not affected. This means that calpain inhibition could potentially be useful to stimulate the formation of new neurons after injury, given that the levels of calpain activity could be reduced under a certain threshold.

Keywords: Adult neurogenesis, brain injury, calpains, hippocampus, memory.

Resumo

No cérebro adulto dos mamíferos, novos neurónios são continuamente adicionados em duas regiões neurogénicas principais: a zona subventricular, nas paredes dos ventrículos laterais, e a zona subgranular, no giro dentado do hipocampo. As células estaminais neurais presentes nestes nichos têm a capacidade de proliferar e dar origem a neuroblastos em migração, que acabam por se diferenciar em novos neurónios. Curiosamente, no evento de uma lesão cerebral, a proliferação das células estaminais neurais aumenta, possivelmente numa tentativa de reparação do dano cerebral. No entanto, esta reparação é limitada pela redução da migração celular ou pela diminuição da sobrevivência dos novos neurónios. Descobrir como aumentar a eficiência da neurogénese após lesão poderá ser portanto benéfico para a reparação cerebral. Por outro lado, o dano cerebral também pode activar diversas protéases, incluindo as calpaínas. As calpaínas são proteases ubíquas, sabendo-se que estão envolvidas na proliferação, migração e diferenciação celular em diversos sistemas. No cérebro, a inibição das calpaínas tem efeito neuroprotector, mas os seus efeitos na neurogénese permanecem ainda pouco conhecidos. A delecção do inibidor endógeno das calpaínas, a calpastatina, já anteriormente mostrou ser capaz de reduzir as primeiras fases da neurogénese *in vivo*. No presente trabalho, mostramos que a inibição das calpaínas é capaz de reverter este efeito, implicando-as ainda mais no processo. Uma vez que a inibição das calpaínas parece ser um alvo promissor para o aumento da neurogénese após lesão, avaliámos várias fases do processo neurogénico, bem como a recuperação das alterações do comportamento cognitivo após lesão no hipocampo induzida por crises epilépticas, em murganhos com sobrexpressão de calpastatina. Avaliámos ainda os efeitos da delecção das calpaínas 1 e 2 nas primeiras fases da neurogénese no hipocampo. Observámos que o padrão de aumento da neurogénese após lesão foi mantido em murganhos com sobrexpressão de calpastatina, apesar de alguma recuperação cognitiva indicar algum grau de neuroprotecção, que por si só poderá ter mascarado a influência da inibição das calpaínas na neurogénese. Murganhos com falta de calpaínas, no entanto, não apresentaram alterações das primeiras fases da neurogénese no hipocampo, sugerindo que as calpaínas apenas afectam este processo quando a sua actividade está acima de um certo limite, abaixo do qual a neurogénese não é afectada. Isto significa que a inibição das calpaínas poderá potencialmente ser útil para prevenir a redução da formação de novos neurónios após lesão, caso os níveis de actividade das calpaínas possam ser reduzidos abaixo de um certo limite.

Palavras-chave: Neurogénese, lesão cerebral, calpaínas, hipocampo, memória.

Table of contents

Abbreviations

BrdU (5-bromo-2'-deoxyuridine)
BSA (Bovine serum albumin)
***Capn1&2* cDKO** (Calpains 1 and 2 conditional double knockout mice)
***Capn1* KO** (Calpain 1 knockout mice)
***Capn2* cKO** (Calpain 2 conditional knockout mice)
CAST (Calpastatin)
***Cast* Ht** (Calpastatin heterozygous mice)
***Cast* KO** (Calpastatin knockout mice)
CNS (Central nervous system)
Cre Ctrl (Cre control)
D-MEM/F-12 (Dulbecco's modified eagle medium: nutrient mixture)
DCX (Doublecortin)
DG (Dentate gyrus)
EDTA (Ethylenediaminetetraacetic acid)
EdU (5-ethynyl-2'-deoxyuridine)
EGF (Epidermal growth factor)
FGF (Basic fibroblast growth factor)
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)
GZ (Granular zone)
hCAST (Calpastatin overexpressed mice)
HBSS (Hank's balanced salt solution)
i.p. (intraperitoneal)
KA (Kainic acid)
NeuN (Neuronal nuclei)
NSC (Neural stem cell)
OB (Olfactory bulb)
PBS (Phosphate buffer saline)
RMS (Rostral migratory stream)
s.c. (subcutaneous)
SAL (Saline)
SDS (Sodium dodecyl sulfate)
SGZ (Subgranular zone)
SVZ (Subventricular zone)

Ta (Annealing tempearature)

TBS-T (Tris buffer saline with tween 20)

WT (Wild-type)

Chapter 1

General Introduction

Chapter 1

1.1. Neurogenesis in the adult mammalian brain

The brain is the most complex organ in our body. Everything we perceive of the world, every feeling, emotion, behavior, from every movement we make to everything we learn, all is processed through the brain. However, it was not until 1965, with the discovery of mitotic cells in the adult rat brain (Altman & Das 1965), that this organ started to be regarded as potentially plastic in terms of introduction of new neurons during adulthood, and not as immutable as previously thought. The existence of new neurons being formed in the adult brain has shed new light for the possibility of brain repair, boosting investigation in the field. 50 years later, aided by technological advances, the progress so far has been remarkable, allowing for a better understanding of the characterization, regulation and functionality of these cells, as well as their implications for therapeutic applications. However, given the complexity of the brain, there is always something more to be discovered, and thus something else that can be improved, in order to help increasing the efficiency of brain recovery.

1.1.1. Neurogenic regions in the adult brain

Neural stem cells (NSCs) are self-renewing, multipotent cells that can give origin to new neurons in the adult brain, in a process designated by neurogenesis. Neurogenesis occurs mainly in two regions: the subventricular zone (SVZ), in the wall of the lateral ventricles, and the dentate gyrus (DG) of the hippocampus (reviewed in Bond *et al.* 2015) (Fig. 1.1A). There are also reports of neurogenesis occurring in other regions of the brain, such as the neocortex (Bernier *et al.* 2002, Gould *et al.* 1999), striatum (Bedard *et al.* 2002, Ernst *et al.* 2014) and hypothalamus (Kokoeva *et al.* 2005), though to a much inferior degree. NSCs constitute a pool of relatively quiescent astrocyte-like cells that, when activated, can originate transient amplifying progenitor cells, which in turn give rise to neuroblasts, commiting to the neuronal lineage. During the first week of life, neuroblasts from the SVZ migrate in chains, through the rostral migratory stream (RMS), into the olfactory bulb (OB), where they start to migrate radially (Fig. 1.1B). By the end of the second week, migration is complete, and the immature neurons in the OB start growing apical and basal dendrites. After one month, the newborn interneurons complete synapse development and integration into the local networks, and comprehend mostly inhibitory granule cells, but also some periglomerular cells (Jin 2016). Neuroblasts from the DG, in turn, are

generated in the subgranular zone (SGZ), a monolayer of cells between the granular zone (GZ) and the hilus, in the hippocampus. These neuroblasts migrate shorter distances, into the GZ, and start to extend limited cellular processes during the first week after birth. During the following week, they start to extend apical dendrites through the GZ and axons to the CA3 region of the hippocampus (Jin 2016). In the third week, the immature neurons finally start to connect with the local neuronal network, but only complete maturation after 8 weeks, becoming new granule cells in the DG (Aimone *et al.* 2014) (Fig. 1.1B, amplified panel). Overall, neurogenesis can thus be divided into 3 main stages: NSC proliferation, neuroblast migration and neuronal differentiation and integration. However, survival of the newborn cells is very limited, with the majority of SVZ-derived cells being eliminated after reaching the OB and, in the DG, before leaving the SGZ (Biebl *et al.* 2000).

Of interest, neurogenesis can also be observed in humans. The first clear evidence of newborn neurons in the adult human brain was observed in *post-mortem* samples of the hippocampus of cancer patients that had been administered a thymidine analogue for diagnostic purposes (Eriksson *et al.* 1998). The evaluation of the presence of mitotic cells requires incorporation of a distinct tag into the DNA of these cells which can later be analysed. The work of Eriksson *et al.* was possible because the patients had been administered a thymidine analogue for other purposes (cancer followup). However, administration of anything that can be integrated in the DNA can be mutagenic and the samples would only become available after the subject had died, which difficult these kind of studies in humans. Other approaches were therefore necessary for further studies on adult human neurogenesis. The fact that NSCs originate neuroblasts, which can be detected by the presence of doublecortin, led to the discovery that humans present a migratory extent of the lateral ventricles, similarly to the RMS (Curtis *et al.* 2007). Despite this curious finding, doublecortin-labeling does not directly indicate the presence of newborn neurons. More notable advances have only started to take place after the development of a distinctive method for neuronal birth dating developed by the group of Jonas Frisén (Spalding *et al.* 2005). This method takes advantage of the extreme increase in ^{14}C levels in the atmosphere during the above-ground nuclear bomb testing between 1955 and 1963, their steady decrease after that period and, more importantly, the fact that the DNA of dividing cells incorporates ^{14}C in levels similar to the ones present in the atmosphere at the time of division, being stable in non-dividing cells. So far, three major findings already derived from the use of the ^{14}C methodology. First, there is no significant neuronal turnover in the human OB (Bergmann *et al.* 2012), unlike what is seen in other mammals, where newborn cells

derived from the SVZ end up becoming new interneurons in the OB. However, the apparent existence of a RMS in humans led to further investigation on the destination of these newborn cells. Curiously, newborn neurons are present in the human striatum, suggesting that, in humans, SVZ-derived cells end up in the striatum, instead of the OB (Ernst *et al.* 2014). Additionally, the human hippocampus was found to harbor substantial neurogenesis, with a relative increase in neuronal turnover compared to other regions (Spalding *et al.* 2013). Thus, neurogenic regions are also present in the adult human brain, and even though SVZ-derived newborn cells are not directed to the OB, it may still prove itself useful for possible applications on neuronal replacement after brain injury.

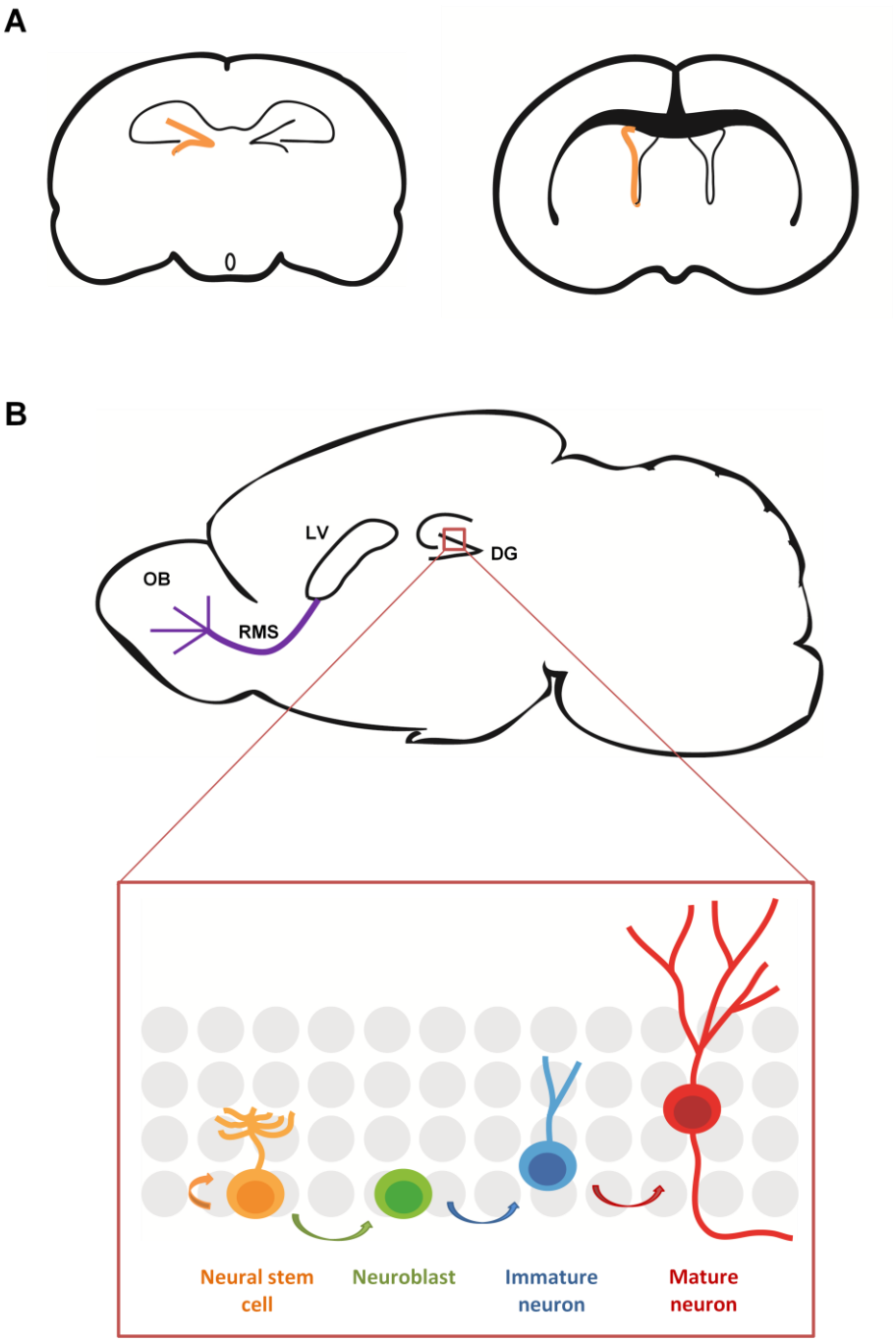

Figure 1.1 - Neurogenic niches in the adult mammalian brain. (**A**) Coronal sections highlighting in orange the dentate gyrus (left) and the subventricular zone (right). (**B**) Sagittal illustration showing the migrating route (rostral migratory stream, RMS) of neural stem cells from the subventricular zone onto the olfactory bulb (OB), where they migrate radially and differentiate. Amplified panel shows a representation of neurogenesis in the hippocampus, with self-proliferating neural stem cells originating neuroblasts, which then become immature neurons in the granular zone of the dentate gyrus (DG), where they become fully mature neurons. LV, lateral ventricle.

1.1.2. Functional significance of adult neurogenesis

Adult neurogenesis from the SVZ and the DG has been consistently implicated with learning and memory. Newborn neurons from both regions have a period of higher hyperexcitability and enhanced synaptic plasticity, which may contribute for distinct functions in the integrated circuits (Bond *et al.* 2015, Ge *et al.* 2008). Investigation of the roles of SVZ neurogenesis under physiological conditions has been relatively less extensive than in the hippocampus. Nonetheless, given that newborn cells from the SVZ end up mostly differentiating in the OB, their association with olfaction is not surprising. More specifically, newborn neurons in the OB have been shown to contribute to the maintenance of existing circuits and to odor learning and memory (Lazarini & Lledo 2011). Additionally to the generation of interneurons in the OB, NSCs from the SVZ can also originate a small portion of oligodendrocytes, which integrate the *corpus callosum* to myelinate axons (Xing *et al.* 2014). The fact that the OB in humans is significantly less developed, since humans do not need to rely on olfaction as much as other mammals, may justify the lack of newborn cells in that region. The significance of newborn neurons in the striatum instead still remains somewhat elusive, though this region can also have an involvement in cognitive functions, particularly when challenges in the environment require behavioral adaptation (Cools *et al.* 2006). Moreover, since the striatum is mainly related with motor control, there is a possibility that newborn cells in this region may be involved in learning of new motor skills.

Adult hippocampal neurogenesis has been more extensively studied than OB neurogenesis in the rodent brain, possible because it simulates better what happens in humans. The involvement of hippocampal neurogenesis on cognition can be assessed by modulating neurogenesis and performing cognitive behavioral tests that evaluate specific kinds of memory. Several methods have been used to impair neurogenesis, including the use of antimitotic drugs (Shors *et al.* 2001), irradiation (Madsen *et al.* 2003), viruses (Zhang *et al.* 2008, Jessberger *et al.* 2009) or transgenic animals (Dupret *et al.* 2008, Saxe *et al.* 2006, Imayoshi *et al.* 2008, Garcia *et al.* 2004). Experimental data from these studies was sometimes inconsistent, which may be explained by different responses between species or strains, efficiency and specificity of the methods used to impair neurogenesis, as well as differences in the performance of a specific test or the parameters evaluated (Deng *et al.* 2010). Nevertheless, the fact that several studies where adult hippocampal neurogenesis has been impaired reveal alterations in learning and memory indicate that DG neurogenesis is important for the maintenance of these processes (Deng et al. 2010). Interestingly, the hippocampus is

involved in the processes of pattern separation, the ability to discriminate between two similar episodes, and pattern completion, which consists of the reconstruction of a previous memory based only in partial elements of an episode. Since young granule cells seem to mediate pattern separation and old granule cells pattern completion (Nakashiba *et al.* 2012), neurogenesis can affect these processes, by altering the balance in the proportions of these cells. More specifically, increased neurogenesis may enhance pattern separation, increasing discrimination, and, conversely, impaired neurogenesis may enhance pattern completion, increasing generalization (Sahay *et al.* 2011). If the imbalance is too pronounced, it may lead to disorders such as autism or anxiety, respectively, which highlights the importance of maintaining neurogenesis tightly regulated.

1.1.3. Regulation of adult neurogenesis

Adult neurogenesis can be regulated by a plethora of factors, starting with the own neurogenic niches, the microenvironments where neurogenesis occurs. Both the SVZ and the DG usually present optimal conditions for the normal maintenance of neurogenesis. This is accomplished by several players in the niche, including astrocytes, microglia, endothelial cells and also, in the case of the SVZ, ependymal cells. All of these cells can secrete signaling factors that will help in maintaining a pool of self-renewing NSCs, as well as directing migration, fate commitment, maturation and survival of the newborn cells (Aimone *et al.* 2014, Ma *et al.* 2009, Ming & Song 2011). Curiously, before integrating the existing networks, newborn cells can already be sensitive to neurotransmitters, such as γ-aminobutyric acid and glutamate, the primary neurotransmitters in the brain (Berg *et al.* 2013). The close proximity of both niches with blood vessels and of the SVZ with the lateral ventricles may also allow for other factors from the blood and the cerebrospinal fluid to enter the niches and interfere with neurogenesis (Mirzadeh *et al.* 2008, Tavazoie *et al.* 2008, Palmer *et al.* 2000, Shen *et al.* 2008).

Despite the fine-tuning of adult neurogenesis occurring in the niches, several extrinsic factors are known to interfere with the process. Some examples include age, stress, enrichment, voluntary exercise and learning. With increased incidence of neurodegenerative disorders in the older population, it is important to understand the influence that age may have on the generation of new neurons. In fact, it has been demonstrated that aging impairs progenitor cell proliferation, as well as neuronal

differentiation and survival (Bondolfi *et al.* 2004, Kuhn *et al.* 1996, Heine *et al.* 2004). Moreover, direct proof of a decline in adult hippocampal neurogenesis with age has been recently reported to occur in humans, although to a less extent (Spalding *et al.* 2013). Furthermore, both acute and chronic stressful events can also cause impairment of hippocampal cell proliferation and survival (Aimone *et al.* 2014). Even though age and stress negatively regulate adult neurogenesis, some extrinsic factors are actually able to enhance it. That is the case with enrichment of the environment and voluntary exercise, such as running. Enrichment is able to enhance survival of new granule neurons (Kempermann *et al.* 1997), while exercise is able to boost cell proliferation and survival (Snyder *et al.* 2009, van Praag *et al.* 1999). Interestingly, both enrichment and exercise have been shown to improve the impairment in neurogenesis that occurs with age (van Praag *et al.* 2005, Kempermann *et al.* 2002). The effect of learning on adult neurogenesis is not as straightforward. Spatial learning of hippocampal-dependent tasks can either promote or hinder survival of newborn cells, depending on their age at the time of learning (Dobrossy *et al.* 2003), which is just another indication of how complex neuronal systems can be.

Additionally, and perhaps the regulatory event that most impact has in a potential clinical setting, adult neurogenesis can be deregulated after a pathologic event. Alterations in neurogenesis have been reported both in neurodegenerative diseases, such as Huntington's, Parkinson's and Alzheimer's disease, and after acute brain insults, such as traumatic brain injury, ischemic stroke and epileptic seizures, addressed in the next section.

1.1.4. Post-injury neurogenesis

Neurodegenerative diseases are characterized by slow and progressive neuronal death. In Huntington's disease, this is caused by a genetic mutation that causes neuronal loss mostly in the striatum and cortex, but can also affect the hippocampus. Hippocampal neurogenesis in animal models of this disease is impaired (Gil *et al.* 2005, Lazic *et al.* 2004, Simpson *et al.* 2011, Kandasamy *et al.* 2010), while reports both in rodents and in humans indicate an enhancement of cell proliferation in the SVZ (Tattersfield *et al.* 2004, Curtis *et al.* 2003). Parkinson's disease, in turn, is characterized by a selective loss in dopaminergic neurons in the substantia nigra. Cell proliferation both in the SVZ and in the hippocampus of patients with Parkinson's disease is impaired, which seems to be caused by loss of dopaminergic input

(Hoglinger *et al.* 2004). Recently, accumulation of α-synuclein in the hippocampus was also shown to be involved in the decreased survival of newly generated neurons in a mouse model of this disease (Schlachetzki *et al.* 2016). In Alzheimer's disease, accumulation of β-amyloid peptide induces severe neuronal loss, starting in the hippocampus. Impairment of cell proliferation and survival of newborn neurons have been reported in several animal models of the disease, but alterations of these processes in patients with Alzheimer's disease have not been very clear (Mu & Gage 2011).

Regarding acute brain lesions, the alterations observed in neurogenesis are mostly different. In the event of a traumatic brain injury, the hippocampus can be particularly affected, leading to deficits in learning and memory (Hilton 1994). Cognitive improvement may occur, but it is very limited and takes a considerable amount of time (Schmidt *et al.* 1999). Interestingly, however, cell proliferation in the SVZ and DG was shown to be increased in animal models of traumatic brain injury (Chirumamilla *et al.* 2002, Dash *et al.* 2001, Rice *et al.* 2003, Gao *et al.* 2009). Moreover, newborn granule cell integration in the hippocampal network was seen to be associated with cognitive recovery (Emery *et al.* 2005, Sun *et al.* 2007). This enhancement of cell proliferation has also been observed following ischemic brain stroke, even in humans (Jin *et al.* 2001, Jin *et al.* 2006, Macas *et al.* 2006, Marti-Fabregas *et al.* 2010, Tonchev *et al.* 2003). Moreover, newborn cells are able to migrate into the damaged regions, where they differentiate and integrate the existing circuitry (Arvidsson *et al.* 2002, Nakatomi *et al.* 2002, Yamashita *et al.* 2006, Zhang *et al.* 2001). However, most of the newborn cells die in the process, with only around 0.2% of lost neurons ending up being replaced in the damaged striatum (Arvidsson *et al.* 2002). Furthermore, alterations of neurogenesis are also observed after seizures. The hippocampus is especially affected in temporal lobe epilepsy, the most common seizure-related disorder in humans. Several animal models of this condition show a significant increase in the early stages of neurogenesis following seizures (Carreira *et al.* 2015, Gray & Sundstrom 1998, Jiang *et al.* 2003, Nakagawa *et al.* 2000, Parent *et al.* 2002, Parent *et al.* 1997, Scott *et al.* 2000). However, some of the newborn cells seem to integrate abnormally in the networks, with ectopic granule cells being observed particularly in the hilus, possibly aggravating the outcome of the disease (Cameron *et al.* 2011, Dashtipour *et al.* 2001, Jessberger *et al.* 2007b, Scharfman *et al.* 2000). In patients with temporal lobe epilepsy, evidence of increased cell proliferation (Blumcke *et al.* 2001, Crespel *et al.* 2005) and ectopic granule cells (Parent *et al.* 2006, Parent *et al.* 1997) have also been observed. Moreover, most of the newborn cells seemingly become astrocytes, reducing

differentiation into new neurons (Carreira *et al.* 2015, Hattiangady & Shetty 2010, Sierra *et al.* 2015).

Overall, adult neurogenesis in neurodegenerative diseases appears to be negatively affected, while in acute disorders there seems to be an attempt to replace neurons lost after an insult to the brain, albeit not very efficient. If we discover how to boost or make the process more efficient, this endogenous potential of the brain to repair itself could be very useful for treatment of neurodegenerative diseases or to improve the outcome of acute brain disorders. Apart from the enhancement of endogenous neurogenesis, other strategies have also been considered for brain repair, such as transplantation of stem cells from different origins (Lindvall & Kokaia 2010), or even by direct conversion of astrocytes into neurogenic cells (Magnusson *et al.* 2014, Niu *et al.* 2013). However, changes in the microenvironment after injury that may affect cell survival must not be excluded, and understanding how they can affect neurogenesis may contribute to a successful outcome for brain repair.

1.2. Calpains

Calpains are a group of intracellular Ca^{2+}-dependent cysteine proteases, first described over 50 years ago in the rat brain (Guroff 1964). Since then, calpains have been characterized in almost all eukaryotes and some bacteria. In humans, 18 genes coding for calpains and calpain-related proteins have already been reported (Sorimachi *et al.* 2011). Calpain isoforms are either ubiquitous or tissue-specific, and their importance is highlighted by the fact that their deregulation can be related to several diseases, including cancer, diabetes, muscular dystrophy and brain disorders (Chakraborti *et al.* 2012). In this work, we will focus on the most abundant and well characterized ubiquitous calpain isoforms, calpain 1 and calpain 2, also known as conventional calpains.

1.2.1. Calpain structure and modulation

Calpain 1 and calpain 2 function as heterodimers, presenting a large catalytic subunit (80 kDa; CAPN1 and CAPN2, respectively) in association with an identical small regulatory subunit (CAPNS1) (Goll *et al.* 2003). The catalytic subunit can be divided in four domains (I to IV), while the regulatory subunit is divided in two (V and VI) (Fig. 1.2).

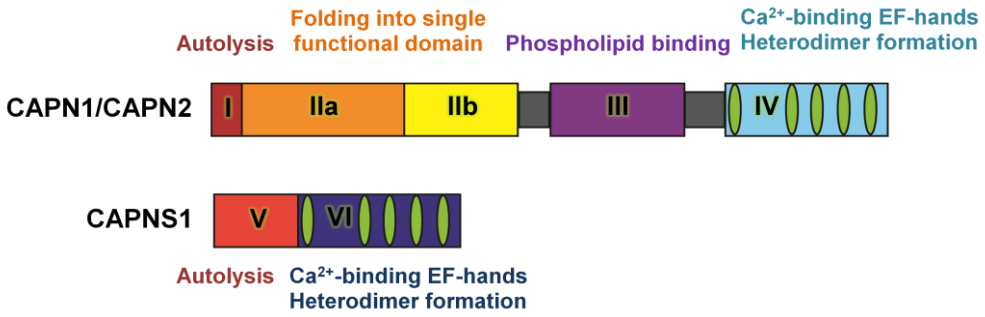

Figure 1.2 - Calpain structure. Structure of the large catalytic subunit of conventional calpains, CAPN1 and CAPN2 (domains I to IV), and of the small regulatory subunit CAPNS1 (domains V and VI). Upon activation, domains I and V can be autolyzed, domains IIa and IIb fold into a single functional domain, domain III is responsible for phospholipid binding and domains IV and VI present five CA^{2+}-binding EF-hands each (in green), of which one is responsible for heterodimer formation between the large and the small subunits.

Domain I is located at the N-terminal of the catalytic subunit, and can be autolyzed in the presence of Ca^{2+}, lowering the Ca^{2+} concentration required for calpain activation (Cong *et al.* 1989). Domain II presents two core domains (subdomains IIa and IIb), which fold into a single functional domain after binding with Ca^{2+} (Moldoveanu *et al.* 2002). Ca^{2+} also binds to domain III, regulating phospholipid binding (Tompa *et al.* 2001), and to the five EF-hands present in domain IV of the catalytic subunit and domains VI of the regulatory subunit (Minami *et al.* 1987). The fifth EF-hand motif of each of these domains, located in COOH-terminal, contribute to the formation of heterodimers (Imajoh *et al.* 1987). Lastly, domain V, in the N-terminal of the regulatory subunit, has glycine-rich clusters, which can be autolyzed upon activation (Sorimachi *et al.* 2011).

Calpain 1 and calpain 2 require elevated levels of Ca^{2+} to be activated maximally *in vitro* (2-80 μM and 0.2-0.8 mM, respectively), which do not normally exist in the cell. Some other regulatory mechanisms for calpains have thus been proposed, which may help decrease the Ca^{2+} concentration requirement of calpains (Franco & Huttenlocher 2005). One of them has already been mentioned, the autolysis of the domain I of the catalytic subunit of calpains. This truncated form of the protein has lower Ca^{2+} requirements and allows folding of domain II into a functional domain (Baki *et al.* 1996, Elce *et al.* 1997, Imajoh *et al.* 1986). Binding of phospholipids can also reduce the Ca^{2+} requirements of calpains (Melloni *et al.* 1996, Arthur & Crawford 1996), as well as several proteins identified as calpain activators (Salamino *et al.* 1993, Melloni *et al.* 1998, Melloni *et al.* 2000). Moreover, phosphorylation has also been shown to modulate calpains, either by activating (phosphorylation by mitogen-activated protein kinase or protein kinase C) or inhibiting (phosphorylation by protein kinase A) them (Glading *et al.* 2000, Shiraha *et al.* 2002, Smith *et al.* 2003, Xu & Deng 2006). Lastly, but importantly, calpains are also regulated by their endogenous inhibitor, calpastatin (CAST), the only known endogenous calpain inhibitor (Murachi 1984). CAST is ubiquitously expressed and selective for the conventional calpains (calpain 1 and calpain 2), although calpain 8 and calpain 9 have been shown to be inhibited by CAST *in vitro* (Hata *et al.* 2007, Lee *et al.* 1999). CAST has four inhibitory domains, being thus able to bind and inhibit four calpain molecules (Emori *et al.* 1987, Hanna *et al.* 2007). A plethora of pharmacologic inhibitors of calpains have been developed, in an effort to mimic CAST efficiency pharmacologically, or to selectively inhibit calpain 1 or calpain 2 (Donkor 2015). Although a lot of progress has been made regarding, e.g., stability, selectivity of calpains over other proteases and reduced toxicity, selective inhibition of calpain 1 or calpain 2 has been more difficult. Nonetheless, for

experimental studies to evaluate the roles of calpains, animal models lacking calpains or CAST, or overexpressing CAST, for example, can be used. To be noted, however, that knockout mice for calpain 2 or for the small regulatory subunit are embryonically lethal (Arthur *et al.* 2000, Dutt *et al.* 2006, Takano *et al.* 2011). In this case, conditional knockout animals must be used, in order to deplete the desired protein in a specific adult tissue, without compromising embryonic development.

1.2.2. Calpain functions and dysfunctions

One curious feature that distinguishes calpains from other proteolytic systems is the fact that products of calpain cleavage can maintain activity, indicating that they may modulate, rather than degrade, their substracts. Along with the fact that calpains can cleave over 100 substracts, including cytoskeletal and structural proteins, membrane-bound-receptors, signaling enzymes and transcription factors, it is no wonder that they appear to have a role in several different biological processes (Goll et al. 2003). Studies that evaluate the effects of calpain inhibition have particularly brought some insights into the functions of calpains in several systems. However, the observed effects may vary, depending on the cell type examined. Decreased calpain activity has been associated with reduced proliferation in several cell types, including vascular smoothmuscle cells, Chinese hamster ovary cell colonies, osteoblasts, chondrocytes and lung endothelial cells (Ariyoshi *et al.* 1998, Kashiwagi *et al.* 2010, Qiu *et al.* 2006, Shimada *et al.* 2008, Xu & Mellgren 2002). However, impaired cell proliferation was also shown to occur both by calpain inhibition in mouse NSC lines (Santos *et al.* 2012), but also in hippocampal NSC, by removing CAST inhibition (Machado *et al.* 2015), suggesting a participation of calpains in neurogenesis. These seemingly contradictory effects can also be seen regarding cell migration, where calpain inhibition was shown to reduce migration of pancreatic beta-cells, vascular smooth muscle cells, T-cell and lung endothelial cells (Parnaud *et al.* 2005, Paulhe *et al.* 2001, Qiu *et al.* 2006, Rock *et al.* 2000), while also increasing neutrophil spreading ability (Lokuta *et al.* 2003). Furthermore, contradictory effects can be seen even in the same cell type, such as an increase or decrease in platelet spreading with calpain inhibition (Croce *et al.* 1999, Kuchay *et al.* 2012). Therefore, not only how calpains function in different cells but also what triggers their activity must be investigated with further detail, to account for these differences.

In addition to the physiologic roles of calpains, it is also interesting to evaluate their effects under pathologic conditions. After a brain lesion, there is overactivation of glutamate receptors, leading to an increase of calcium intake by the cells. Since calpains are activated by increased levels of Ca^{2+}, it is also not surprising that they are involved in neuronal damage (Araujo *et al.* 2010). Deregulation of calpain activity has thus been implicated in several brain disorders, such as ischemic stroke, traumatic brain injury and Alzheimer's, Parkinson's and Huntington's diseases, and calpain inhibition has shown potential in conferring neuroprotection (reviewed in Saez *et al.* 2006). Our group has also demonstrated that calpain inhibition is neuroprotective in a seizure-induced model of temporal lobe epilepsy (Araujo *et al.* 2008). Moreover, since calpains are implicated not only in cell proliferation and migration, but have also been reported to interfere with cell differentiation (Kashiwagi *et al.* 2010, Yajima & Kawashima 2002) and synaptic plasticity (Baudry & Bi 2016), it would be interesting to evaluate whether calpain inhibition could also enhance neurogenesis, being capable of helping regenerating the brain, in addition to being neuroprotective.

1.3. Objectives

Calpains are known players in brain damage, and calpain inhibition is known to be neuroprotective in several neuronal disorders. These proteases are involved in the processes of cell proliferation, migration and differentiation in several systems, but not much is known of how they affect these processes during adult neurogenesis. Previous work by our group has shown that increased calpain activity impairs NSC proliferation in the DG and neuroblast migration both in the DG and in the RMS of adult mice. Moreover, calpain inhibition is able to enhance cell migration in SVZ explants. With this work, we proposed to further investigate the effects of calpains on neurogenesis, in order to determine how calpain modulation can contribute to enhance post-injury brain repair. We first wanted to evaluate whether the lack of CAST, the endogenous calpain inhibitor, also impaired SVZ-derived cell proliferation and migration *in vitro*, and, if that would be the case, whether calpain inhibition could reverse this impairment. Next, we used a model of seizure-induced neuronal lesion in mice overexpressing CAST, to assess the pattern of post-injury neurogenesis and cognitive recovery in these mice. Lastly, we used mice lacking one or both conventional calpains and evaluated whether absence of these calpains would affect early stages of hippocampal neurogenesis.

Chapter 2

Methods and Materials

2.1. Animals

In this study, we used several mouse lines that have different calpain or calpastatin (CAST) expression. Mice lacking CAST (*Cast* KO) (Takano *et al.* 2005) have polyadenylation signals in exon 6 to inhibit transcription of the calpain-inhibitory domains. Mice overexpressing CAST (hCAST) (Rao *et al.* 2008) have a human CAST construct (Hitomi *et al.* 1998) cloned into a Thymocyte differentiation antigen 1.1 expression cassette. For calpain knockout animals, mice lacking calpain 1 (*Capn1* KO), calpain 2 (*Capn2* cKO) or both (*Capn1&2* cDKO) were used. For *Capn1* KO mice (Azam *et al.* 2001), a phosphoglycerate kinase-cassette replaces a portion of exon 4 that belongs to a catalytic domain. Since conventional knockouts for calpain 2 are embryonically lethal (Dutt *et al.* 2006), a conditional knockout was used. *Capn2* cKO mice (Takano *et al.* 2011) have therefore exon 3 flanked by LoxP sites, which can be recombined by Cre. Cre recombinase, in this case, is in turn under the control of the promoter of Nestin, in order to achieve knockout animals for calpain 2 in the CNS. *Capn1&2* cDKO present both genotypes at the same time. All animals have been backcrossed in a C57BL/6J background, so C57BL/6J wild-type (WT) mice and WT littermates were used for comparison. To exclude the influence of the presence of Cre alone, animals that express Cre and express calpains 1 and 2 were also used as a comparison group (Cre Ctrl). The animals were kept in our animal facilities, at the Center for Neuroscience and Cell Biology (Coimbra) and at the University of Algarve (Faro), with food and water *ad libitum* in a 12-hour dark:light cycle. 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

DNA from each animal was obtained from the limbs (newborn mice) or tail clips (adult mice) and purified using the QIAamp DNA mini kit or the NZY Tissue gDNA Isolation kit. The genotypes were confirmed by PCR, using Platinum® Taq DNA Polymerase and dNTP Mix (for *Cast* KO) or NZYTaq 2x Green Master Mix, with the primers and annealing temperatures (Ta) listed on Table 2.1. The PCR products were run in a 2% agarose gel with ethidium bromide or GreenSafe Premium and visualized in a Gel Doc™ XR+ or a ChemiDoc XRS imaging system (BioRad Laboratories Inc., Hercules, CA, USA).

Table 2.1 - Primers and annealing temperatures (Ta) used for genotyping.

	Primers (5' - 3')	Ta
Cast KO	TCCGAAGCACACATCAAGTACAGG	54ºC
	CATTGCTCAGCGGTGCTGTCC	
	TTCTGTGTAAAGAAGCAAAAGGCAGC	
hCAST	AAGGCCATTCCAGTCAGCCA	60ºC
	AGCAACACTCTCTCCAC	
GAPDH (housekeeping)	ACCACAGTCCATGCCATCAC	
	TCCACCACCCTGTTGCTG	
Cre	CGCAGAACCTGAAGATGTTC	
	CGAAATCAGTGCGTTCGAAC	
Capn1 KO	CTCACTTAGCATAGGCTTTCTCCAGCAGTG	
	CCTGAAGAACGAGATCAGCAGCCTCT	
	CAGAACCACTGACACGGTCCAGATCTG	
Capn2 cKO	ATAGCTCCTGTGTATCAGGCACAGAGCTGG	64ºC
	GGAGCTCATCTGTGTCTCCAAAGCC	

2.2. Cell culture experiments

2.2.1. SVZ cultures

For the *in vitro* experiments, neural stem cells (NSCs) isolated from the subventricular zone (SVZ) of post-natal WT or *Cast* KO mice (0-3 days old) were used, prepared as previously described (Morte *et al.* 2013). Mice were decapitated and the brains removed and placed in a solution of 0.24% gentamicin in Hank's balanced salt solution (HBSS), where the meninges were removed. The brains were cut in 1 mm thick slices in a matrix and the SVZ was isolated under a stereomicroscope (Stemi DV4, Zeiss, Oberkochen, Germany), placed in clean 0.24% gentamicin/HBSS and digested with

0.025% trypsin/0.265 mM ethylenediaminetetraacetic acid (EDTA), for 15 min at 37ºC. After washing three times with 0.24% gentamicin/HBSS, the tissue was resuspended and the cells mechanically dissociated in warm Dulbecco's modified eagle medium: nutrient mixture F-12 (D-MEM/F-12) with 2 mM GlutaMAX™-I (L-Ala-L-Gln), supplemented with 1% B27, 1% antibiotic (10,000 units/ml of penicillin, 10 mg/ml streptomycin), 10 ng/ml epidermal growth factor (EGF) and 5 ng/ml basic fibroblast growth factor (FGF), and plated on uncoated flasks with filter cap, 100,000 cells/ml, in supplemented D-MEM/F-12/GlutaMAX™-I medium. These cells were maintained in culture as floating aggregates, neurospheres (Fig. 2.1), in a 95% air/5% CO_2 humidified atmosphere at 37ºC, and passaged at least once a week, by letting the neurospheres sediment, mechanically dissociate them and re-plating in new supplemented medium. Whenever possible, NSCs with at least two and up to twelve passages were used for these experiments.

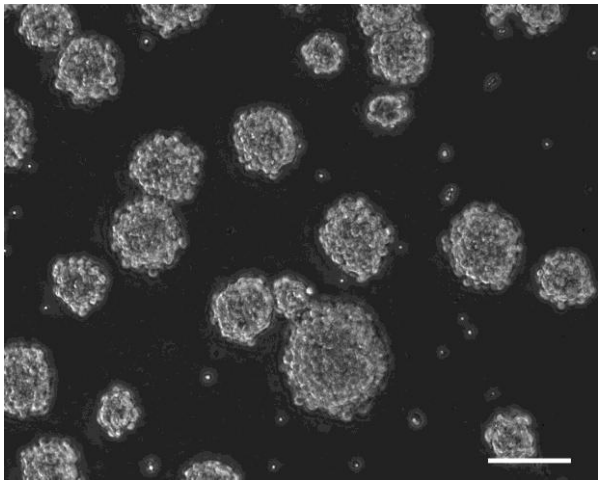

Figure 2.1 - Floating neurospheres. Cells isolated from the SVZ of post-natal mice in culture, as neurospheres. Scale bar: 100 µm.

2.2.2. αII-spectrin breakdown analysis

To analyze αII-spectrin breakdown products, neurospheres were collected, gently dissociated and plated on 0.1 mg/ml poly-L-lysine-coated plates, in supplemented medium, until at least 70% confluency was reached (Fig. 2.2). The NSCs were then treated with the calcium ionophore A23187 10 µM for 1h30 and the levels of full-length αII-spectrin and its breakdown products detected by Western blot (Sengoku *et al.*

2004). For that purpose, the medium was removed, and after washing once with 0.01 M phosphate buffer saline (PBS), cytosolic cell extracts were prepared by lysing the cells in 100 mM Tris-HCl, 10 mM ethylene glycol tetraacetic acid, 1% triton X-100 and 2 mM $MgCl_2$, pH 7.4, supplemented with 1x PhosSTOP phosphatase inhibitors, 1x cOmplete, Mini protease inhibitors and 1 µM dithiothreitol, at 4ºC. The lysates obtained were then sonicated 6 times with 2-second pulses, 4 seconds apart, and centrifuged for 10 min, 15,800 g, at 4ºC. The protein concentration in the supernatant was determined by the bicinchoninic acid method, using the BCA protein kit, according to the manufacturer's instructions. 6x concentrated sample buffer was added (0.5 M Tris-HCl/0.4% sodium dodecyl sulfate (SDS) pH 6.8, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% bromophenol blue) and the lysates were denatured at 95ºC for 5 min. Equal amounts of protein (15 µg) were separated by electrophoresis on SDS-polyacrylamide gels, using MiniPROTEAN® 3 systems (BioRad Laboratories Inc., Hercules, CA, USA). Electrophoresis gels composition was 6% bis-acrilamide in 1.5 M Tris-HCl pH 8.0 (for the resolving gel) and 4% bis-acrilamide in 0.5 M Tris-HCl pH 6.8 (for the stacking gel), plus 0.1% SDS, 0.05% tetramethylethylenediamine and 0.05% ammonium persulfate, in ultrapure water. Gels were submerged in running buffer (25 mM Tris, 25 mM bicine, 0.1% SDS, pH 8.3), the samples loaded onto the gel and the electrophoresis started at 60 V for 10 min, followed by 140 V until proper band separation was achieved. Polyvinylidene difluoride membranes were activated in 100% methanol for about 5 s and for 15-30 min in electrotransfer buffer (N-cyclohexyl-3-aminopropanesulfonic acid 10 mM, methanol 10%, pH 11.0) and the proteins were then transferred electrophoretically (350 mA for 1 h) to the membranes, submerged in electrotransfer buffer, using a MiniPROTEAN® Tetra Cell apparatus (BioRad Laboratories Inc., Hercules, CA, USA). Membranes were blocked for 1 h at room temperature, in Tris buffer saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% tween-20 (TBS-T) and 5% low-fat dry milk. Incubations with the primary antibodies (mouse anti-αII-spectrin 1:2,000 and mouse anti- glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1:5,000 for the loading control, in TBS-T with 1% low-fat dry milk) were performed overnight, at 4ºC. The membranes were then washed in TBS-T (three 5 min washes, with 2 quick washes before and after) and incubated for 1 h, at room temperature, with the correspondent horseradish peroxidase-conjugated secondary antibody (anti-mouse 1:2,000 in TBS-T with 1% low-fat dry milk). Membranes were washed again in TBS-T, incubated 5 min with the Clarity™ Western ECL substrate and bands were then visualized in a ChemiDoc XRS imaging system, using the ImageLab software (version 4.0), from BioRad Laboratories Inc. (Hercules, CA, USA).

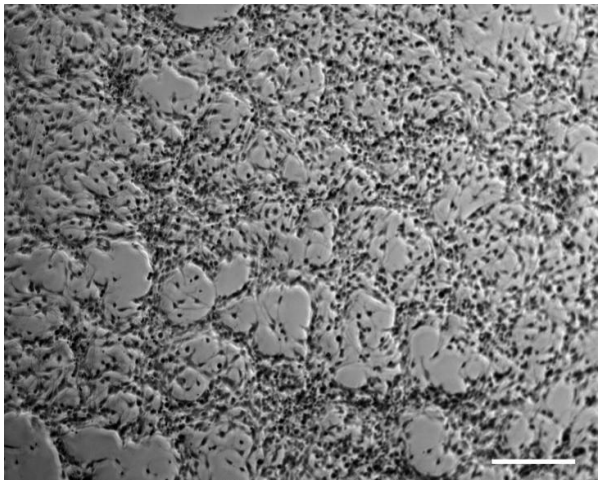

Figure 2.2 - Plated NSCs. Dissociated cells isolated from the SVZ of post-natal mice, plated on a poly-L-lysine-coated well. Scale bar: 200 µm.

2.2.3. Cell proliferation

For cell proliferation assays, dissociated NSCs were plated on 0.1 mg/ml poly-L-lysine-coated glass coverslips and kept in culture, in supplemented medium, until 40-60% confluency was reached. The cells were left untreated or were treated with calpeptin 25 µM for 6 h and fixed with 4% paraformaldehyde/4% sucrose in PBS. 5-ethynyl-2'-deoxyuridine (EdU) 10 µM was given to the cells 4 h before fixation and its detection was performed using a commercially available kit (Click-iT® EdU Alexa Fluor® 488 HCS Assay). The cells were rinsed with 3% bovine serum albumin (BSA) in PBS, permeabilized with 0.5% triton X-100 in PBS for 15 min at room temperature and a reaction cocktail (reaction buffer, $CuSO_4$, Alexa Fluor 488 and reaction buffer additive) was added for 30 min, at room temperature, protected from light. After rinsing again in 3% BSA, all nuclei were stained with 1 µg/ml Hoechst 33342 for 5 min, the cells washed in PBS and the coverslips mounted in slides with DAKO fluorescence mounting medium, as previously described (Morte *et al.* 2013). EdU-positive cells from 3-6 independent experiments were counted in 10 images per sample, acquired in an Axio Imager Z2 microscope (Zeiss, Oberkochen, Germany).

2.2.4. Cell migration

Regarding cell migration *in vitro*, three different methods were used: radial migration, wound healing assays and time-lapse imaging.

2.2.4.1. Radial migration

In the radial migration method, neurospheres of similar sizes were plated on 0.1 mg/ml poly-L-lysine- and 10 µg/ml laminin-coated glass coverslips, in supplemented medium. The cells were fixed with 4% paraformaldehyde/4% sucrose after 4 h, and the coverslips were mounted in slides with DAKO fluorescence mounting medium. Phase contrast images from 3 independent experiments were acquired in an Axio Imager Z2 microscope (Zeiss, Oberkochen, Germany) and the migration radia of the 5 furthest cells from 20 neurospheres (Fig. 2.3) in each sample were measured (Gu *et al.* 2009), using the AxioVision Rel. 4.8 software (Zeiss, Oberkochen, Germany).

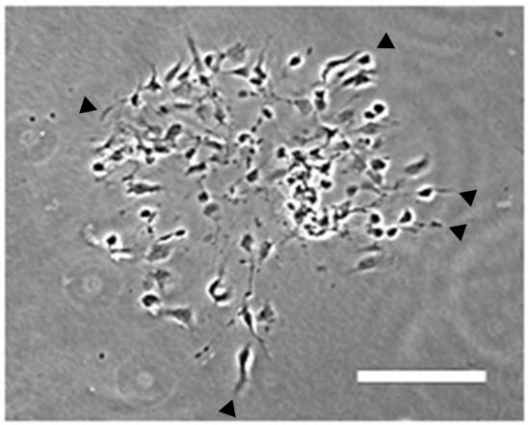

Figure 2.3 - SVZ-derived cells migrating out of a neurosphere in the radial migration assay. Cells isolated from the SVZ of post-natal mice, 4 h after being plated as a neurosphere on a poly-L-lysine- and laminin-coated coverslip. The 5 furthest cells are indicated with the black arrowheads. Scale bar: 100 µm.

2.2.4.2. Wound healing assay

For the wound healing assay, the cells were dissociated and plated on poly-L-lysine- and laminin-coated dishes, in supplemented medium, until completely confluent. A layer of cells was then removed in the middle, by scratching the bottom of the dish with a 10 µl pipette tip (Liang *et al.* 2007), across the dish (Fig. 2.4), and the area of cells that migrated towards the gap was measured in 4-6 independent experiments after 3 and 6 h, in phase contrast images acquired in an Axiovert 40 CFL microscope (Zeiss, Oberkochen, Germany).

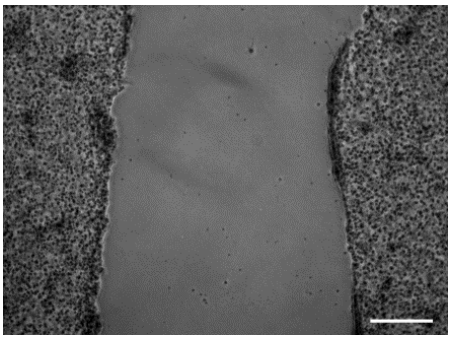

Figure 2.4 - Gap of confluent NSCs in the wound healing assay. Confluent cells isolated from the SVZ of post-natal mice, plated on a poly-L-lysine- and laminin-coated dish, with a cell layer removed in the middle, for the wound healing assay. Scale bar: 200 µm.

2.2.4.3. Time-lapse imaging

Lastly, for the time-lapse imaging assay, neurospheres were plated on poly-L-lysine- and laminin-coated chambered wells, in Krebs media (2 M NaCl, 500 mM KCl, 1 M $MgCl_2$, 1 M $CaCl_2$, 500 mM glucose, 500 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4). Images from 3 independent experiments were taken every 3 minutes for 4 h, at 37ºC, using a DeltaVision® Core inverted microscope (GE Healthcare, Little Chalfont, UK) (Fig. 2.5). Cells belonging to the first batch of cells migrating out of 3 neurospheres (38-52 cells each) per sample were tracked (Machado *et al.* 2015) and the migration speed and frequencies distributions were determined,

using the ImageJ (version 1.47v, National Institutes of Health, Bethesda, MD, USA) plugin MTrackJ.

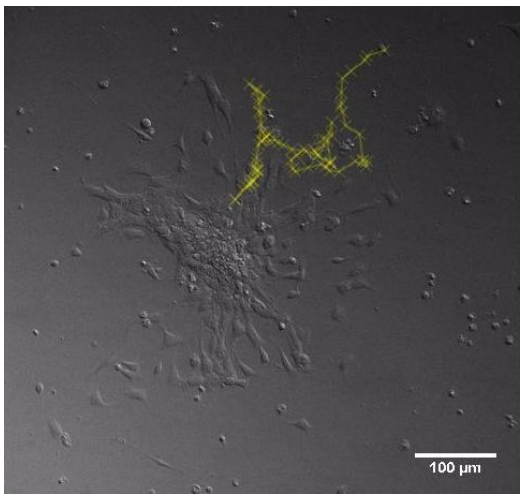

Figure 2.5 - SVZ-derived cells migrating out of a neurosphere in the time-lapse imaging assay. Cells isolated from the SVZ of post-natal mice, plated as a neurosphere on a poly-L-lysine- and laminin-coated chambered well. Example of the tracking of one cell in yellow. Scale bar: 100 µm.

2.3. *In vivo* experiments

2.3.1. Presence of CAST and CAPN2 in hippocampal lysates

To obtain hippocampal lysates, mice were sacrificed by cervical displacement, the brains removed and the hippocampi isolated and rapidly frozen in liquid nitrogen. Lysis buffer (50 mM Tris-HCl, 0.5% triton X-100, pH 7.4), supplemented with 1x PhosSTOP phosphatase inhibitors, 1x cOmplete, Mini protease inhibitors and 1 μM dithiothreitol, was added and the samples were homogenized in a Mini Potter tissue grinder (Jencons Scientific Ltd., Leighton Buzzard, UK), on ice. The lysates were then sonicated 4 times with 2-second pulses, 6 seconds apart, on ice, using a Vibra-cell™ VCX130 ultrasonic processor (Sonics, Newtown, CT, USA), and centrifuged for 10 min, 15,800 g, at 4ºC, to isolate the cytosolic fraction. Determination of protein concentration and Western blot analysis were performed similarly to section 2.2.2. Equal amounts of protein (7 μg for CAST and 15 μg for CAPN2) were separated in 10% bis-acrilamide resolving gels, and the primary antibodies used were rabbit anti-CAST 1:200, rabbit anti-CAPN2 1:1,000 and mouse anti-GAPDH 1:5,000 (loading control), followed by the correspondent horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or anti-mouse 1:2,000).

2.3.2. Hippocampal neurogenesis and behavior under pathological conditions

2.3.2.1. Kainic acid (KA) treatment

For the studies of post-injury neurogenesis, 3 month old WT and hCAST mice were used. Hippocampal damage was induced by administering kainic acid (KA) subcutaneously (s.c., 25 mg/kg, in a saline (SAL) solution of 0.9% NaCl), as previously described by our group (Carreira *et al.* 2010). After KA administration, the animals went through several stages, according to a well defined scale (Schauwecker & Steward 1997): immobility (I), tail/forelimb extension / rigid posture (II), repetitive movements / head bobbing (III), rearing and falling (IV), continuous rearing and falling (V), severe tonic-clonic seizures (VI). Only mice that reached stage V or higher were used in this study. SAL-treated animals were used as controls.

2.3.2.2. NSC proliferation

Cell proliferation after KA or SAL treatment was assessed as shown in Fig. 2.6. WT and hCAST mice were given either KA or SAL subcutaneously and, on day 5, when a peak of proliferation was seen to occur in the dentate gyrus (DG) after KA treatment (Carreira *et al.* 2010), BrdU (5-bromo-2'-deoxyuridine) was administered intraperitoneally (i.p., 4 doses of 50 mg/kg, 2 h apart), up to 12 h before sacrifice by transcardial perfusion.

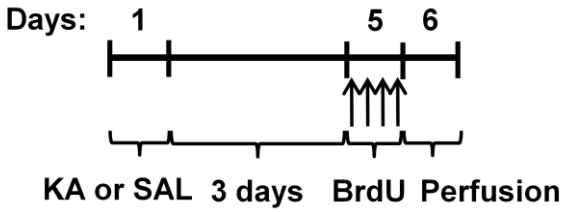

Figure 2.6 - Experimental procedure for the study of NSC proliferation after KA or SAL treatment. WT and hCAST mice were treated with either SAL or KA (25 mg/kg, s.c.) and on day 5 were given BrdU (4 doses of 50 mg/kg, i.p.), up to 12 h before sacrifice.

2.3.2.3. Neuroblast migration

In order to investigate neuroblast migration after lesion, WT and hCAST mice were given either KA or SAL subcutaneously and sacrificed by transcardial perfusion, after 14 days, when an increase in cell migration is known to occur in the DG after KA treatment (Carreira *et al.* 2015) (Fig. 2.7).

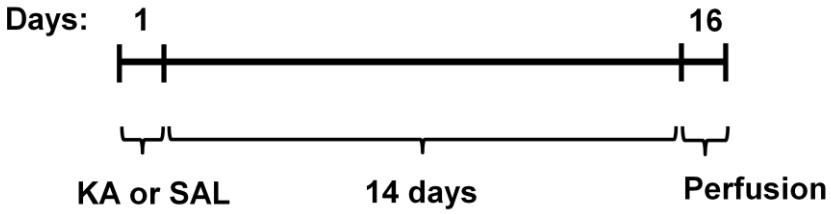

Figure 2.7 - Experimental procedure for the study of neuroblast migration after KA or SAL treatment. WT and hCAST mice were treated with either SAL or KA (25 mg/kg, s.c.) and sacrificed after 14 days.

2.3.2.4. Neuronal differentiation and behavior analysis

To assess neuronal differentiation and long-term behavioral recovery after lesion, WT and hCAST mice were treated as shown in Fig. 2.8. EdU was administered to WT and hCAST mice on days 3, 4 and 5 after KA or SAL treatment, and the animals were sacrificed by transcardial perfusion after 8 weeks, when new neurons are fully mature (Aimone *et al.* 2014). During the last week, three different behavioral tests were performed: object recognition, using open field as the habituation step, water maze and fear conditioning, as explained further in more detail. For the studies of short-term behavioral recovery, the same behavioral tests were performed on WT and hCAST mice, 2 weeks after KA or SAL treatment (Fig. 2.9).

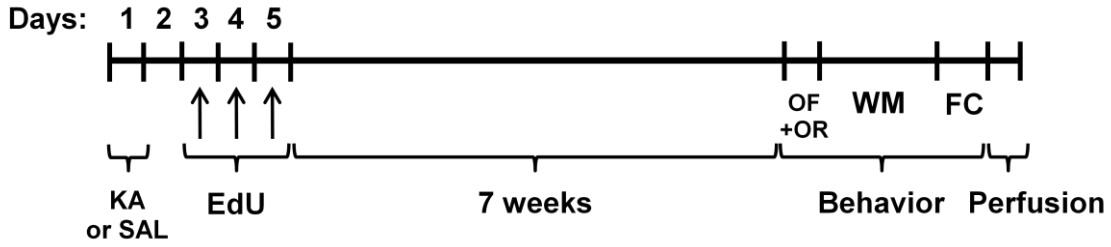

Figure 2.8 - Experimental procedure for the study of neuronal differentiation and long-term behavioral recovery after KA or SAL treatment. WT and hCAST mice were treated with either SAL or KA (25 mg/kg, s.c.) and, on days 3, 4 and 5, were given EdU (50 mg/kg, i.p.), 8 weeks before sacrifice. During the last week, the cognitive behavior of the animals was tested. On the first day, open field (OF) and object recognition (OR) performance were tested. Water maze (WM) trials were performed on the next 3 days and the final test, without the platform, was on day 4. On the next day, fear conditioning (FC) trials were performed. One day later, the animals were at first left on the same context and after 3 h the context was completely modified and the cued test performed. The animals were sacrificed on the next day.

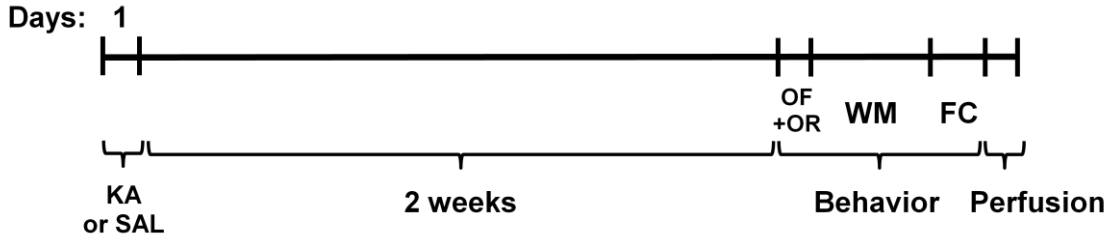

Figure 2.9 - Experimental procedure for the study of short-term behavioral recovery after KA or SAL treatment. WT and hCAST mice were treated with either SAL or KA (25 mg/kg, s.c.) and, after 2 weeks, the cognitive behavior of the animals was tested. On the first day, open field (OF) and object recognition (OR) performance were tested. Water maze (WM) trials were performed on the next 3 days and the final test, without the platform, was on day 4. On the next day, fear conditioning (FC) trials were performed. One day later, the animals were at first left on the same context and after 3 h the context was completely modified and the cued test performed. The animals were sacrificed on the next day.

2.3.2.4.1. Open field and object recognition

For the study of recognition memory, the object recognition test (Bevins & Besheer 2006) was used (Fig. 2.10). Mice were presented to two similar objects (Fig. 2.10B), following habituation to the apparatus (40x40x40 cm acrylic box) (Fig. 2.10A), after which one of the objects was replaced by a novel one (Fig. 2.10C). Each stage of the test had the duration of 5 min, 2 h apart, and the apparatus and objects were cleaned with 10% ethanol between animals. In the habituation period, we also took the opportunity to perform the open field test (Gould *et al.* 2009), by dividing the base of the apparatus into nine equal squares and observing the overall locomotor activity (line crossings and rearing episodes) and anxiety behavior (central entries and time in center) of these animals. In the presence of the objects, the percentage of time spent with each object (totalizing 100%) was measured. The analysis was done in videos acquired with a GoPro Hero (GoPro, Inc., San Mateo, CA, USA) during testing, of 12-16 independent experiments in the long-term group and 3-5 independent experiments in the short-term group, using the Any-maze software (version 4.99, Stoelting Co., Wood Dale, IL, USA).

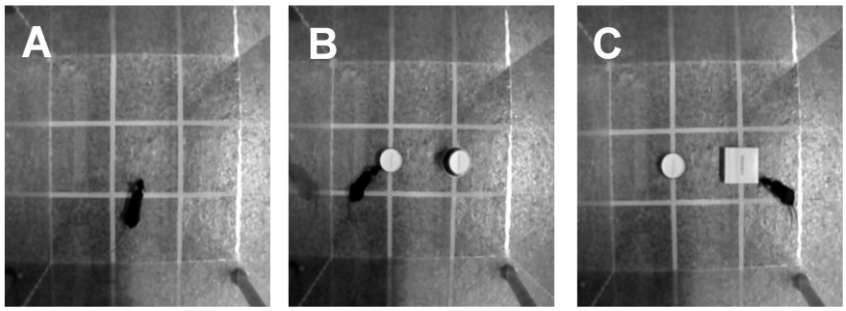

Figure 2.10 - Object recognition test. Representative images showing the apparatus (40x40x40 cm acrylic box) for the object recognition test. Each stage had the duration of 5 min, 2 h apart. (**A**) Habituation/open field. (**B**) First object presentation. (**C**) Presentation to novel object.

2.3.2.4.2. Water maze

To evaluate spatial learning and memory, the water maze test (Morris *et al.* 1982) was performed. During the first 3 days, the animals were trained to find a platform (10 cm

diameter, carved to increase grip) hidden 0.5 cm below the water surface level in a circular pool (1.52 m diameter), in a fixed location. Visual cues on the walls around the pool were provided to help the mice locating the platform and the water temperature was maintained at 22ºC ± 1ºC. The pool was divided into four quadrants (Fig. 2.11) and, on each day of training, the animals were placed in a different quadrant, always facing the border of the pool, under the course of four 60 s trials, 30 min apart. The latency to escape was measured as the time each animal took to find and climb onto the platform. If the animal did not reach the platform within the course of the trial, the latency to escape was considered 60 s and the animal was left on the platform for 10 s, in order to learn the platform location. On the last day of testing, the platform was removed and the mice were placed in the quadrant opposite to the target quadrant (where the platform was previously located), facing the border of the pool. The time spent on each quadrant and the number of crossings over the previous platform location were measured for 60 s, to see if the animals remembered where the platform was located during training. All animals were dried under an infrared light after each trial. The analysis was done in videos acquired with a GoPro Hero (GoPro, Inc., San Mateo, CA, USA) during testing, of 11-16 independent experiments in the long-term group and 4-5 independent experiments in the short-term group, using the Any-maze software (version 4.99, Stoelting Co., Wood Dale, IL, USA).

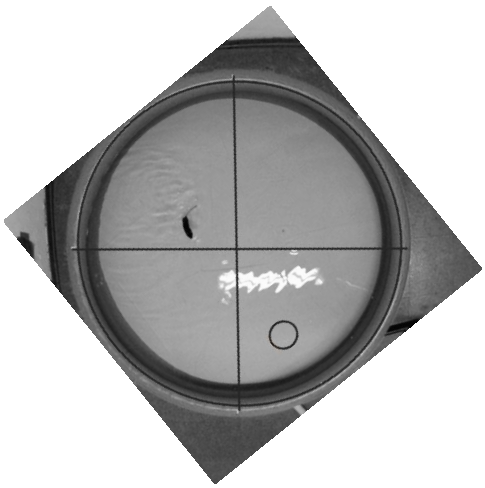

Figure 2.11 - Water maze test. Representative image showing the apparatus for the water maze test (1.52 m diameter pool). The platform remained on the same location during trials (3 days, 4 trials of 60 s per day, 30 min apart) and was removed on the fourth day (60 s test).

2.3.2.4.3. Fear Conditioning

Lastly, associative learning and memory were tested by performing the fear conditioning test (Wehner & Radcliffe 2004). The first day of testing consisted of learning to associate a cue tone (80 dB) with a footshock (0.7 mA). For this part of the test, mice were placed in a chamber (17x17x25 cm) with a grid floor inside a soundproof box (Stoelting Co., Wood Dale, IL, USA), with a background white noise, 70% ethanol scent and 100% lux lighting (context A), for 2 min (acquisition/habituation), after which the tone was played for 30 s and the footshock given during the last 2 s of tone. This was repeated 2 more times, after a 60 s rest. On the next day, the association of the context with the footshock was tested, by placing the mice inside the chamber in the same conditions as during learning for 5 min, without presentation of tone or footshock. After 3 h, we tested the association of the footshock with the tone (cued test), by placing the animals in a completely different environment for 3 min (habituation to new context) and then playing the tone for the last 3 min. The new context consisted of a background fan noise, vanilla scent, 10% lux lighting, and the previously used chamber was altered by using striped black and white walls and a white floor covering the grid (context B). During the whole fear conditioning testing, the chamber was cleaned with 10% ethanol between animals, and the fear behavior measured by calculating the percentage of freezing time, in 9-16 independent experiments in the long-term group and 3-5 16 independent experiments in the short-term group, using the Any-maze software (version 4.99, Stoelting Co., Wood Dale, IL, USA).

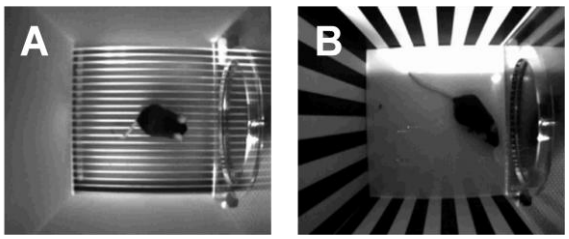

Figure 2.12 - Fear conditioning test. Representative images showing the apparatus for the fear conditioning test (17x17x25 cm chamber). (**A**) Context for trials (30 s 80 dB cue tone with a 0.7 mA shock on the last 2 s of tone, x3, 60 s apart, after 2 min habituation) and context test (5 min, on the next day). (**B**) Context for cued test (3 min habituation followed by 3 min tone, 3 h after context).

2.3.3. Early hippocampal neurogenesis under physiological conditions

To study the influence of calpains on neurogenesis in physiological conditions, WT, *Capn1* KO, *Capn2* cKO, *Capn1&2* cDKO and Cre Ctrl mice were treated with BrdU (50 mg/kg, i.p., twice, daily) for 3 days and, after 7 days, with EdU (50 mg/kg, i.p., twice, 2 h apart) (Fig. 2.13). The animals were sacrificed by transcardial perfusion on the next day, in order to access cell proliferation and migration (Machado *et al.* 2015).

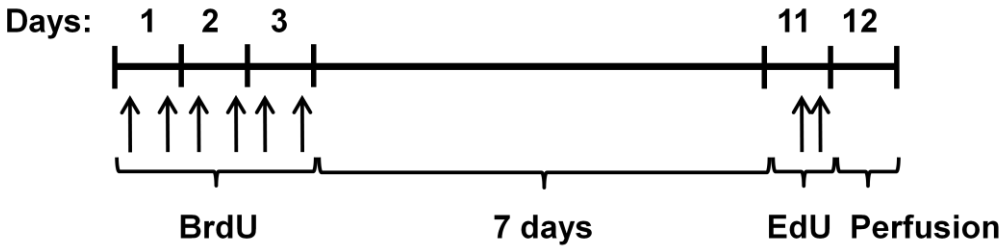

Figure 2.13 - Experimental procedure for the study of NSC proliferation and neuroblast migration. WT, *Capn1* KO, *Capn2* cKO, *Capn1&2* cDKO and Cre Ctrl mice were treated with 50 mg/kg BrdU (i.p., twice, daily) for 3 days and, after 7 days, with 50 mg/kg EdU (i.p., twice, 2 h apart). The animals were sacrificed on day 12.

2.3.4. EdU detection and immunohistochemistry

Brains used for immunohistochemistry and EdU detection were obtained after transcardial perfusion of the mice with 0.9% NaCl and 4% paraformaldehyde, following anesthesia with Avertin (12.5 mg/ml 2,2,2-tribromoethanol, 2.5% 2-methyl-2-butanol). The brains were removed and kept in 4% paraformaldehyde overnight for further fixation and then dehydrated in 20% sucrose in phosphate buffer for at least one day, all at 4ºC. Coronal sections from the hippocampal region were cryosectioned (30 µm thick, in 6-series) using a CryoStar NX50 cryostat (Thermo Fisher Scientific, Waltham, MA, USA) and stored in an antifreeze solution (30% ethylene glycol and 30% glycerol in phosphate buffer), at 4ºC. Free-floating brain sections from one of the series were labeled against CAST, BrdU, doublecortin (DCX), BrdU/DCX, EdU or EdU/NeuN (neuronal nuclei), as previously described (Machado *et al.* 2015, Morte *et al.* 2013). EdU labeling was performed using a commercially available kit (Click-iT® EdU Alexa Fluor® 488 HCS Assay). After rinsing with 3% BSA, the sections were permeabilized

with 0.5% triton X-100 for 45 min, rinsed again and then incubated with a reaction cocktail (reaction buffer, $CuSO_4$, Alexa Fluor 488 and reaction buffer additive) for 1 h, protected from light, all at room temperature. For immunohistochemistry, sections were rinsed with PBS and then blocked for 1 h, at room temperature, in 5% blocking solution in 0.25% triton X-100, using normal goat or horse serum, according to the secondary antibodies used. After blocking, the sections were kept with the primary antibodies (rabbit anti-CAST 1:250, rat anti-BrdU 1:50, goat anti-DCX 1:400, mouse anti-NeuN 1:200) for 48 h, at 4ºC. Sections were then rinsed in 2% blocking solution and incubated for 2 h, at room temperature and protected from light, with the correspondent Alexa Fluor-conjugated secondary antibodies (goat anti-rabbit 488, donkey anti-rat 488, donkey anti-goat 594, goat anti-mouse 633 and donkey anti-mouse 594; 1:200). For the labeling of BrdU, a DNA denaturation step with 1 M HCl for 20 min, at 65ºC, was performed in the beginning. In the cases where NeuN was not labeled, nuclei were stained with 2 µg/ml Hoechst 33342 for 10 min, at room temperature. When the combination of EdU and NeuN was needed, the EdU labeling was performed, followed by the immunohistochemistry, starting with the blocking step. The entire procedure was done using an orbital shaker. After a final rinsing step, the sections were mounted in gelatin-coated (0.5% gelatin with 0.5 mg/ml $CrKO_8S_2.12H_2O$) slides with DAKO fluorescence mounting medium.

2.3.5. Analysis of incorporation of BrdU and EdU

BrdU-positive and EdU-positive cells in the SGZ, the first layer of cells adjacent to the hilus, in the granular zone (GZ) or in the hilus (Fig 2.14) of 5 mid sections of the hippocampus were counted for each animal (Machado *et al.* 2015, Salazar-Colocho *et al.* 2008), directly under an epifluorescence microscope (Axio Imager Z2 microscope, Zeiss, Oberkochen, Germany). Cell counting was carried out in both upper and lower blades of the DG, in 8-14 independent experiments for the pathological studies and 5-9 independent experiments for the physiological studies.

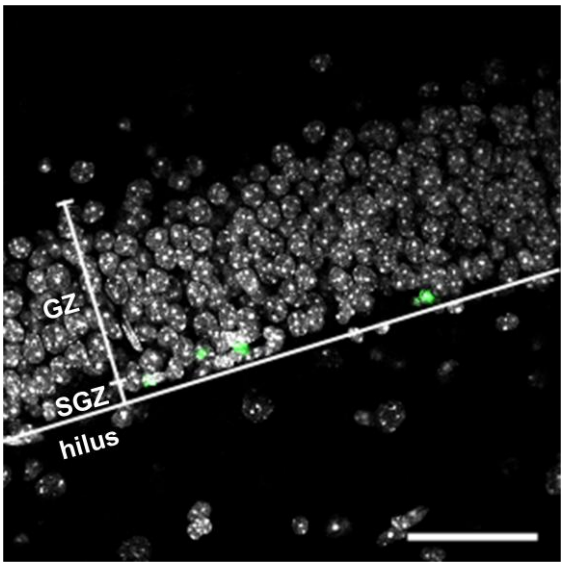

Figure 2.14 - Section of the DG from a hippocampal brain slice. Representative image of a section of the DG, showing the division into GZ, SGZ and hilus. EdU in green and nuclei, stained for Hoechst 33342, in grey. Scale bar: 50 µm.

2.3.6. DCX immunoreactivity and migration measurements

DCX immunoreactivity in the DG and migration distances of BrdU-positive/DCX-positive cells in the GZ were determined in images acquired in a laser scanning microscope (LSM710, Zeiss, Jena, Germany), in 6-9 independent experiments. The quantification of the DCX-positive area was performed in ImageJ (version 1.47v, National Institutes of Health, Bethesda, MD, USA), using a threshold analysis in 5 mid sections of the hippocampus of each animal. This consisted in defining the optimal staining threshold and calculating the area labeled with DCX (Komitova *et al.* 2005, Machado *et al.* 2015). The migration measurements, in turn, were performed in a total of at least 33 cells per group, from 5 images acquired for each animal, by determining the distance between the nucleus of the cell and the boundary between the SGZ and the hilus, perpendicularly to that delimitation (Machado *et al.* 2015), in 5-7 independent experiments, using the ZEN 2011 software (version 7.1, Zeiss, Jena, Germany).

2.3.7. Neuronal differentiation analysis

For the quantification of newborn neurons, the percentage of cells labeled for both EdU and NeuN in the DG was determined in a total of up to 50 EdU-positive cells (Carreira *et al.* 2015), in 8 independent experiments, in images (orthogonal reconstructions of projections from 0.73 µm z-stacks) acquired in a laser scanning microscope (LSM710, Zeiss, Jena, Germany).

2.4. Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined using a two-tailed t-test, a one-way or two-way analysis of variance (ANOVA, with Bonferroni's post-test), as appropriate and indicated in the figure legends, using the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered significant when $p<0.05$.

2.5. Materials

QIAamp DNA mini kit was acquired from Quiagen (Hilden, Germany), CAST KO primers from TIB MOLBIOL GmbH (Berlin, Germany), and NZY Tissue gDNA Isolation kit, the remaining primers, NZYTaq 2x Green Master Mix, agarose, GreenSafe Premium, Tris, NaCl, dithiothreitol, bis-acrilamide, tetramethylethylenediamine, ammonium persulfate, ECL and BSA were all bought from NZYTech (Lisboa, Portugal). Platinum® Taq DNA Polymerase, dNTP Mix, gentamicin, tripsin-EDTA, D-MEM/F-12 with GlutaMAX™-I, penicillin/streptomycin, B27, BCA protein kit, EdU, Click-iT® EdU Alexa Fluor® 488 HCS Assay Kit, Hoechst 33342, all Alexa Fluor-conjugated secondary antibodies, slides and glass coverslips were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Ethidium bromide, HBSS, poly-L-lysine, ethylene glycol tetraacetic acid, $MgCl_2$, glycerol, bicine, N-cyclohexyl-3-aminopropanesulfonic acid, methanol, tween-20, BrdU, paraformaldehyde, $CaCl_2$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2,2,2-tribromoethanol, 2-methyl-2-butanol, ethylene glycol, HCl and $CrKO_8S_2.12H_2O$ were bought from Sigma Aldrich (St Louis, MO, USA). The matrix used was from Stoelting Co. (Wood Dale, IL, USA), EGF and FGF were

from PeproTech (London, UK), A23187 from Enzo Life Sciences, Inc. (Farmingdale, NY, USA) and low-fat dry milk from Nestlé (Vevey, Switzerland). Triton X-100, bromophenol blue, sucrose, KCl, glucose and gelatin were all obtained from Merck (Darmstadt, Germany), and SDS, another ECL and the primary antibody rat anti-BrdU were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). PhosSTOP phosphatase inhibitors, cOmplete, Mini protease inhibitors and laminin were obtained from Roche Applied Science (Penzberg, Germany), DAKO fluorescence mounting medium from Agilent Technologies (Glostrup, Denmark), Calpeptin from Tocris Bioscience (Bristol, UK), KA from Ocean Produce International (Shelburne, Canada), and normal goat and horse serums from Vector Laboratories Inc. (Burlingame, CA, USA). Polyvinylidene difluoride membranes and the primary antibodies mouse anti-αII-spectrin, mouse anti-GAPDH and mouse anti-NeuN were acquired from Merck Millipore (Billerica, MA, USA), the horseradish peroxidase-conjugated secondary antibodies and the primary antibody rabbit anti-CAPN2 from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies rabbit anti-CAST and goat anti-DCX were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and rat anti-BrdU was acquired from (Oxford, UK). The pool used for the behavior experiments was from Bestway Inflatables & Material Corp. (Shanghai, China), the platform and acrylic box were made to measure at University of Algarve, the objects used were commonly used cardboard coffee cups and the lid from a small cardboard box, and the vanilla scent was diluted commercially available vanilla essence (Auchan, Croix, France). Ethanol was acquired from Panreac Química S.L.U. (Barcelona, Spain).

Chapter 3

Effects of calpastatin modulation on physiological or post-injury neurogenesis and behavior

3.1. Summary

In the adult mammalian brain, new neurons continue to be produced throughout life, in two main regions in the brain, the subgranular zone in the hippocampus and the subventricular zone in the walls of the lateral ventricles. Neural stem cells proliferate in these niches, and migrate as neuroblasts, to further differentiate in locations where new neurons are needed, either in normal or pathological conditions. However, this endogenous attempt of brain repair is not very efficient, since newborn cells may be lost or fail to integrate correctly in the existing neuronal network. Calpains are proteases known to be involved in the neuronal damage that occurs after brain injury and calpain inhibition has proven to be able to afford neuroprotection. These proteases are involved in cell proliferation, migration and differentiation of several cell types, though their effects on neurogenesis are not well known. Previous work by our group has shown that the absence of calpastatin, the endogenous inhibitor of calpains, impairs early stages of neurogenesis. In the present work, we show that impairment of NSC proliferation in these mice can be reversed with a calpain inhibitor, corroborating their relevance to the process, and the potential of calpain inhibition for the enhancement of neurogenesis. We further evaluated whether calpastatin overexpression would help improve efficiency of post-injury neurogenesis and cognitive recovery, in a model of seizure-induced hippocampal lesion. Enhancement of post-injury neurogenesis was mostly maintained in these mice, though results from cognitive recovery seem to corroborate the neuroprotection granted by calpain inhibiton.

3.2. Introduction

Adult mammalian neurogenesis is known to occur in two main regions in brain, the subventricular zone (SVZ), in the walls of the lateral ventricles, and the subgranular zone (SGZ), in the dentate gyrus (DG) of the hippocampus (Bond et al. 2015). Neural stem cells (NSCs) from the SVZ migrate long distances, through a rostral migratory stream (RMS), into the olfactory bulb, differentiating into interneurons, while NSCs from the SGZ migrate shorter distances into the granular zone (GZ), where they fully mature into granule neurons after 2 months (Aimone *et al*. 2014, Jin 2016). Functions of these newborn cells from both regions have been consistently associated with learning and memory (Deng *et al.* 2010, Lazarini & Lledo 2011).

When a brain lesion occurs, adult neurogenesis can become altered. In neurodegenerative diseases, it is mostly hampered, but in acute disorders, such as temporal lobe epilepsy, NSC proliferation can increase, in a possible attempt to repair the damage. However, this repair is limited by impaired cell migration or decreased survival of new neurons (Kaneko & Sawamoto 2009, Ma *et al.* 2009). Discovering how to increase the efficiency of post-injury neurogenesis may therefore be beneficial for brain repair.

Another feature of brain damage is excitotoxicity, which leads to an increase of cellular levels of calcium, activating several proteases, including calpains (Neumar *et al.* 2001). Calpains are ubiquitously expressed calcium-activated proteases, with only one known natural endogenous inhibitor, a protein called calpastatin (CAST) (Murachi 1984). In the CNS, calpain inhibition has been shown to afford neuroprotection, limiting neuronal damage and showing improved neuronal function in several brain disorders (Saez et al. 2006). In other systems, calpains were seen to be involved in cell proliferation, migration and differentiation (Kashiwagi *et al.* 2010, Kuchay *et al.* 2012, Lokuta *et al.* 2003, Parnaud *et al.* 2005, Qiu *et al.* 2006, Rock *et al.* 2000, Shimada *et al*. 2008, Yajima & Kawashima 2002), though little is known about their contribution to neurogenesis.

Previous studies by our group show that increased calpain activity, through lack of CAST (*Cast* KO), can impair early stages of neurogenesis (Machado *et al.* 2015), and also that the kainic acid (KA) model of seizure-induced hippocampal lesion presents excitotoxic damage mediated by calpains (Araujo et al. 2008) and is characterized by a pathologic increase of hippocampal neurogenesis (Carreira *et al.* 2010). Thus, we proposed to assess whether calpain inhibition could reverse the impairment of early

stages of neurogenesis in the absence of CAST, and also whether overexpression of CAST (hCAST) could improve neurogenesis and cognitive recovery after KA-induced hipppocampal lesion.

3.3. Results

3.3.1. Effects of CAST depletion on early neurogenesis *in vitro*

3.3.1.1. CAST depletion translates into increased calpain activity in NSCs

For the studies of the effects of CAST depletion on early neurogenesis, *Cast* KO mice were genotyped (Fig. 3.1A) and the absence of CAST in these mice was confirmed in brain sections, by comparison with wild-type (WT) (Fig. 3.1B,D). Negative controls (Fig. 3.1C,E) corroborate the lack of CAST staining in *Cast* KO brain sections.

Since calpains cleave αII-spectrin into specific fragments of 145 kDa and 150 kDa, distinguishable from caspase 3 activation, which generates a specific 120 kDa fragment (Wang 2000), we checked whether CAST depletion translated into increased calpain activity by treating NSCs with the calcium ionophore A23187 and observing the levels of αII-spectrin and its breakdown products. In the absence of the calcium ionophore, basal calpain activity does not seem to be increased in NSCs from *Cast* KO mice, since αII-spectrin breakdown products are not observed.. However, when treated with A23187, these cells presented increased levels of calpain-specific αII-spectrin breakdown products, in conditions that do not increase calpain activity in WT cells (Fig. 3.2).

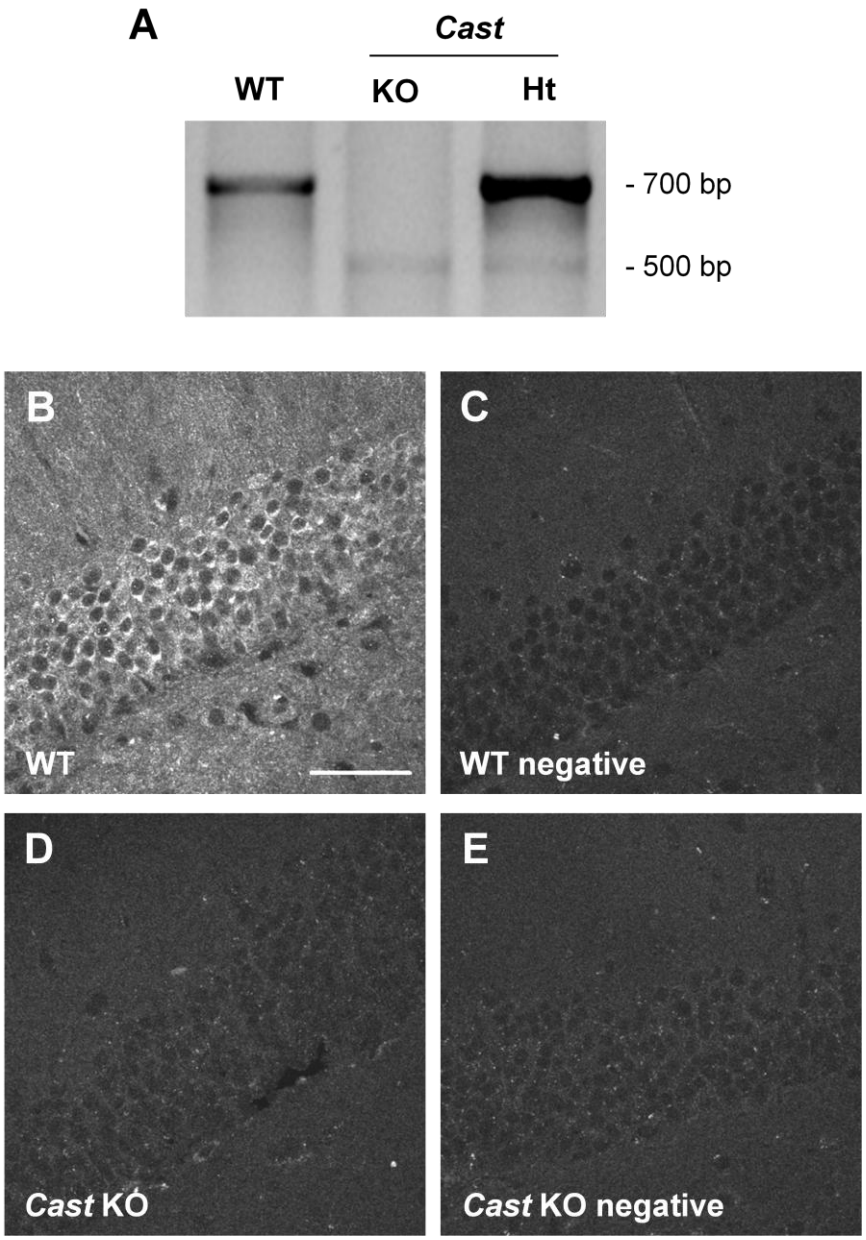

Figure 3.1 - Genotyping profiles and absence of CAST in *Cast* KO mice. Genotyping profiles of WT, *Cast* KO and *Cast* Ht mice (**A**). Representative images from hippocampal brain sections of WT (**B**) and *Cast* KO (**D**) mice, and respective negative controls (**C**, **E**), showing CAST in grey. Scale bar: 50 µm.

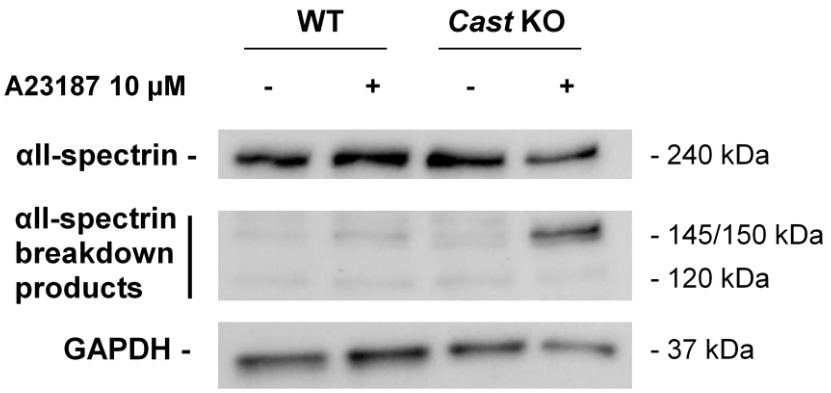

Figure 3.2 - CAST depletion translates into increased calpain activity after challenging NSCs with A23187. Western blot analysis of lysates of SVZ-derived NSCs from WT and *Cast* KO mice after treatment with A23187 10 µM for 1h30, to detect levels of full-length αII-spectrin and αII-spectrin breakdown products. GAPDH was used as a loading control.

3.3.1.2. CAST-depleted NSCs have impaired proliferation, which can be rescued by calpain inhibition

To analyze the effects of CAST depletion on cell proliferation, NSCs were treated with 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analogue that incorporates the DNA of dividing cells (Chehrehasa *et al.* 2009). We observed that cell proliferation, assessed by the percentage of cells positive for EdU, was decreased in *Cast* heterozygous cells (*Cast* Ht; 17.0 ± 1.4%, $p<0.05$), and was even more impaired in *Cast* KO cells (9.2 ± 1.0%, $p<0.001$), when compared with cells from WT mice (23.9 ± 2.3%) (Fig. 3.3), indicating a progressive decrease of cell proliferation with increased calpain activity.

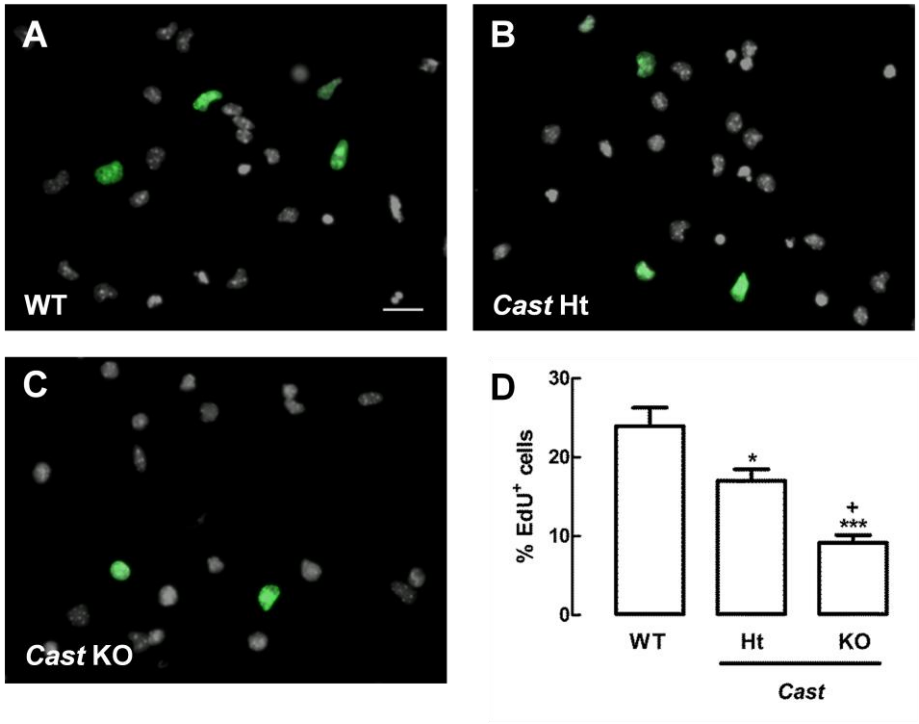

Figure 3.3 - CAST deficiency impairs NSC proliferation. SVZ-derived NSCs from post-natal WT, *Cast* Ht or *Cast* KO mice were plated on poly-L-lysine-coated glass coverslips and kept with EdU for 4 h. Representative images of cells from WT (**A**), *Cast* Ht (**B**) and *Cast* KO (**C**) mice, showing EdU-positive cells in green and nuclei, labeled for Hoechst 33342, in grey. Scale bar: 20 µm. Cell proliferation was calculated from 10 images acquired for each sample and is presented as the percentage of living cells positive for EdU (**D**). Means ± SEM of 5-6 independent experiments were analyzed by a one-way ANOVA (Bonferroni's post-test), *$p<0.05$ and ***$p<0.001$, significantly different from WT; +$p<0.05$ significantly different from *Cast* Ht.

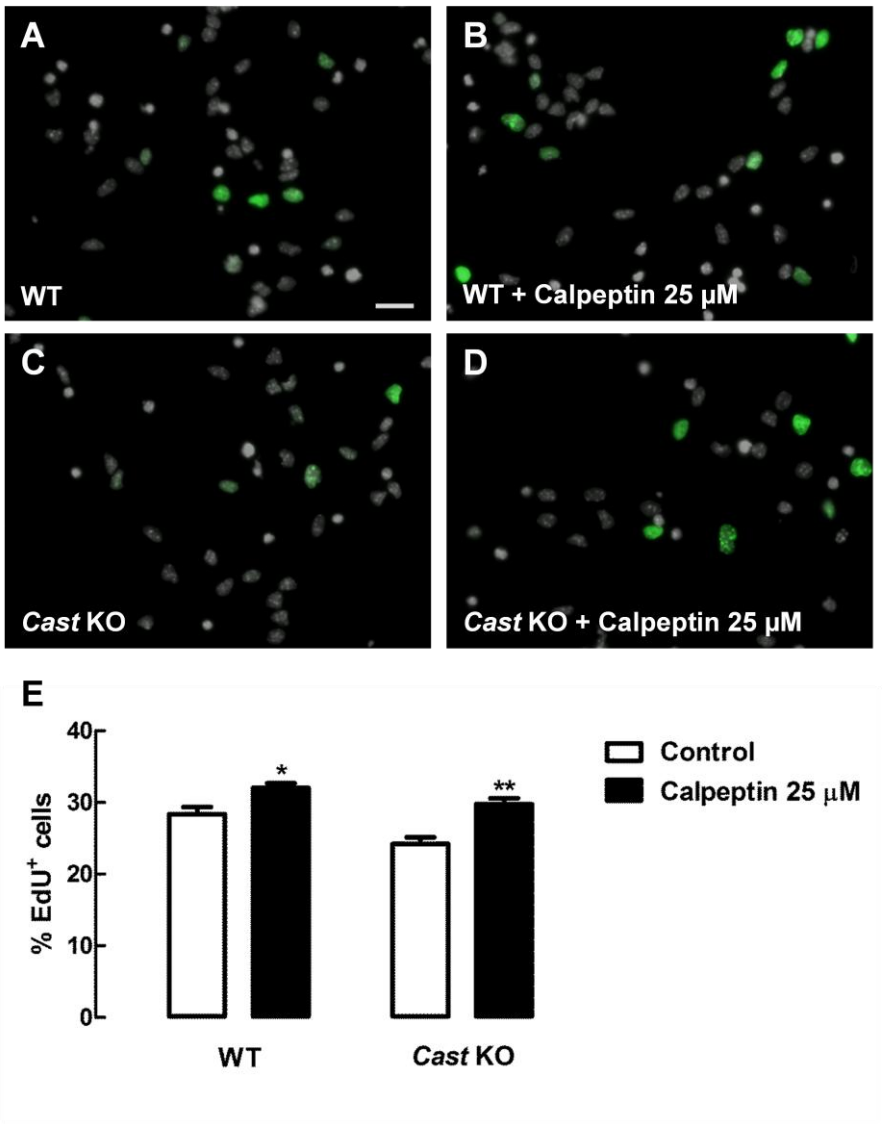

Figure 3.4 - Calpain inhibition increases NSC proliferation of WT cells and rescues the proliferation of *Cast* KO cells. SVZ-derived NSCs from post-natal WT or *Cast* KO mice were plated on poly-L-lysine-coated glass coverslips, treated with calpeptin 25 µM for 6 h and kept with EdU for 4 h. Representative images of cells from WT (**A**, **B**) and *Cast* KO (**C**, **D**) mice, either untreated (**A**, **C**) or treated with calpeptin 25 µM (**B**, **D**), showing EdU-positive cells in green and nuclei, labeled for Hoechst 33342, in grey. Scale bar: 20 µm. Cell proliferation was calculated from 10 images acquired for each sample and is presented as the percentage of living cells positive for EdU (**E**). Means ± SEM of 3 independent experiments were analyzed by a two-way ANOVA (Bonferroni's post-test), *$p<0.05$ and **$p<0.01$, significantly different from control.

Moreover, the effect of calpain inhibition on cell proliferation was investigated, by treating NSCs from WT or *Cast* KO mice with the pharmacological calpain inhibitor calpeptin (25 µM). Calpain inhibition was shown to enhance NSC proliferation in both WT (32.0 ± 0.7%, $p<0.05$) and *Cast* KO (29.7 ± 0.8%, $p<0.01$) cells when compared to untreated cells (control; WT: 28.4 ± 1.0%, *Cast* KO: 24.2 ± 0.9%), thus reverting the proliferation impairment of cells lacking CAST.

3.3.1.3. CAST depletion does not affect cell migration

Regarding cell migration, three different approaches were used: radial migration analysis, wound-healing assay and time-lapse imaging. In the radial migration analysis, NSCs were plated as neurospheres and, after 4 h, the average migration radia of *Cast* KO cells leaving the neurospheres was similar to those of WT cells (262.1 ± 4.5 µm, $p>0.05$) (Fig. 3.5).

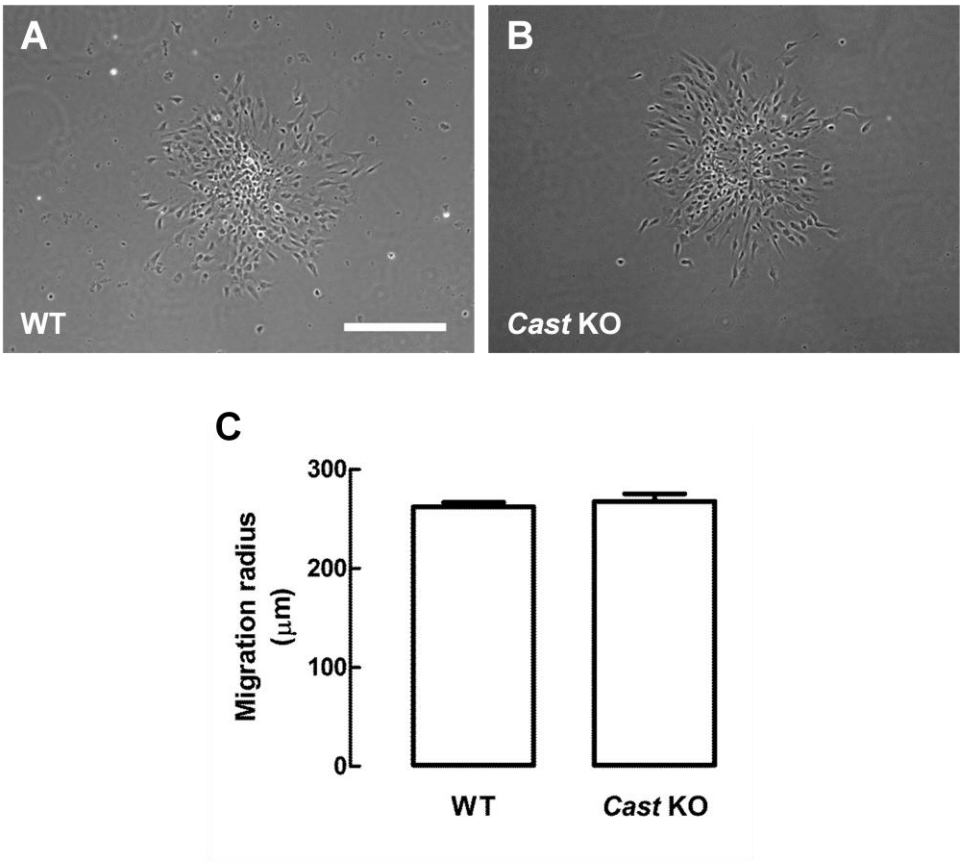

Figure 3.5 - Depletion of CAST maintains cell migration (radial migration). SVZ-derived NSCs from post-natal WT or *Cast* KO mice were plated as neurospheres on

poly-L-lysine and laminin-coated glass coverslips, for 4 h. Representative images of cells from WT (**A**) and *Cast* KO (**B**) mice, migrating out of the neurospheres. Scale bar: 200 µm. Migration radia of 5 cells from 20 neurospheres per sample were measured in images acquired by phase contrast microscopy (**C**). Means ± SEM of 3 independent experiments were analyzed by a two-tailed t test, p>0.05.

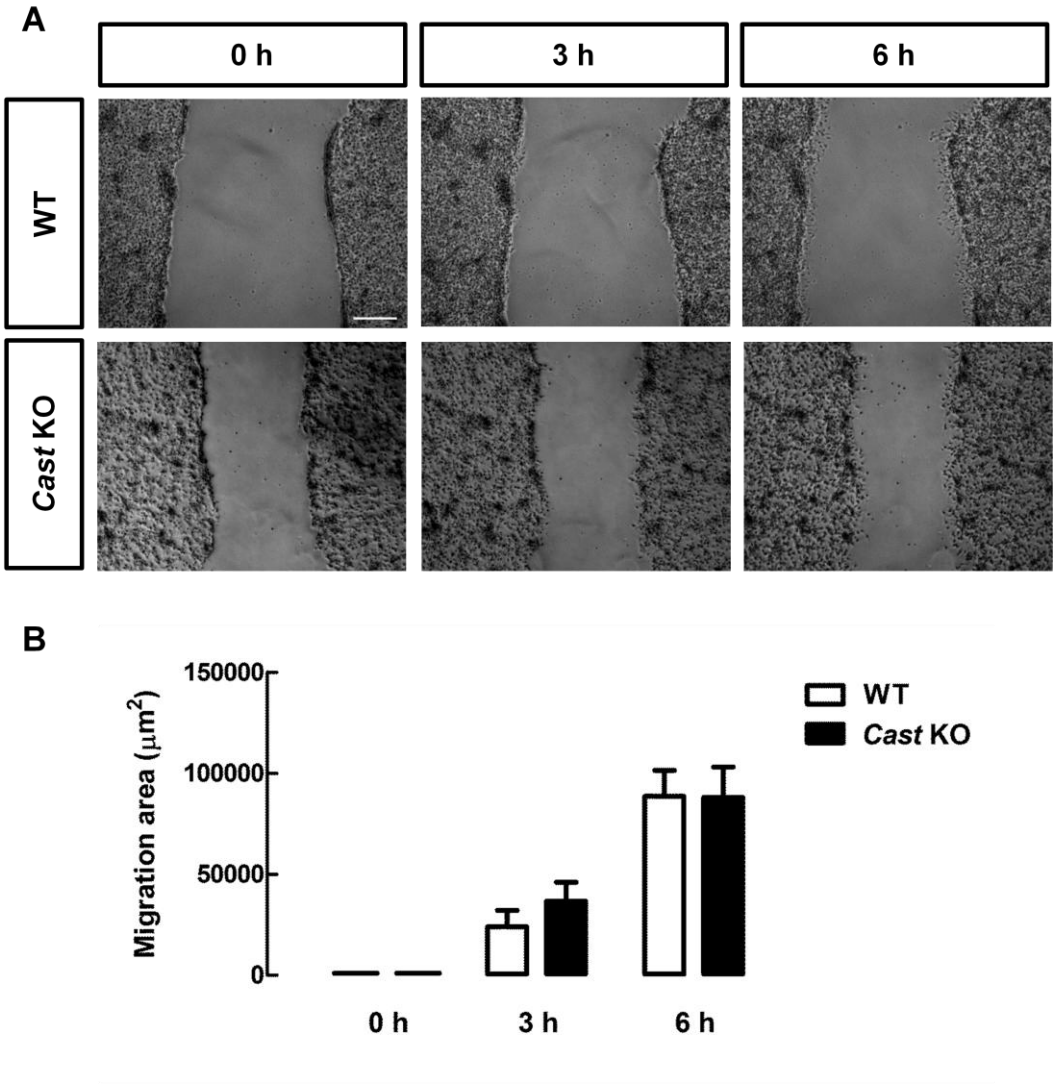

Figure 3.6 - Depletion of CAST maintains cell migration (wound-healing assay). SVZ-derived NSCs from post-natal WT or *Cast* KO mice were plated on poly-L-lysine and laminin-coated dishes until completely confluent, after which a layer of cells was removed. Representative images of the cell gaps after 0 h, 3 h and 6 h, for both genotypes (**A**). Scale bar 200 µm. Migration area was measured from images acquired by phase contrast microscopy, at 0, 3 and 6 h (**B**). Means ± SEM of 4-6 independent experiments were analyzed by a two-way ANOVA, p>0.05 between genotypes.

In the wound-healing assay, the area migrated by cells lacking CAST into the created gap was also similar to WT cells, at both time-points tested (3 h: 23,895.1 ± 8,241.4 μm^2, 6 h: 88,580.8 ± 12,926.2 μm^2, $p>0.05$) (Fig. 3.6).

Lastly, cells migrating out of plated neurospheres were tracked after time-lapse imaging (Fig. 3.7A), and the migration speed and frequency distributions were determined. Migration speed (Fig. 3.7B) also seemed to be maintained in CAST-depleted cells in comparison to WT cells (50.5 ± 10.1 µm/h, $p>0.05$), as well as the frequency distributions of cells migrating at different paces (Fig. 3.7C).

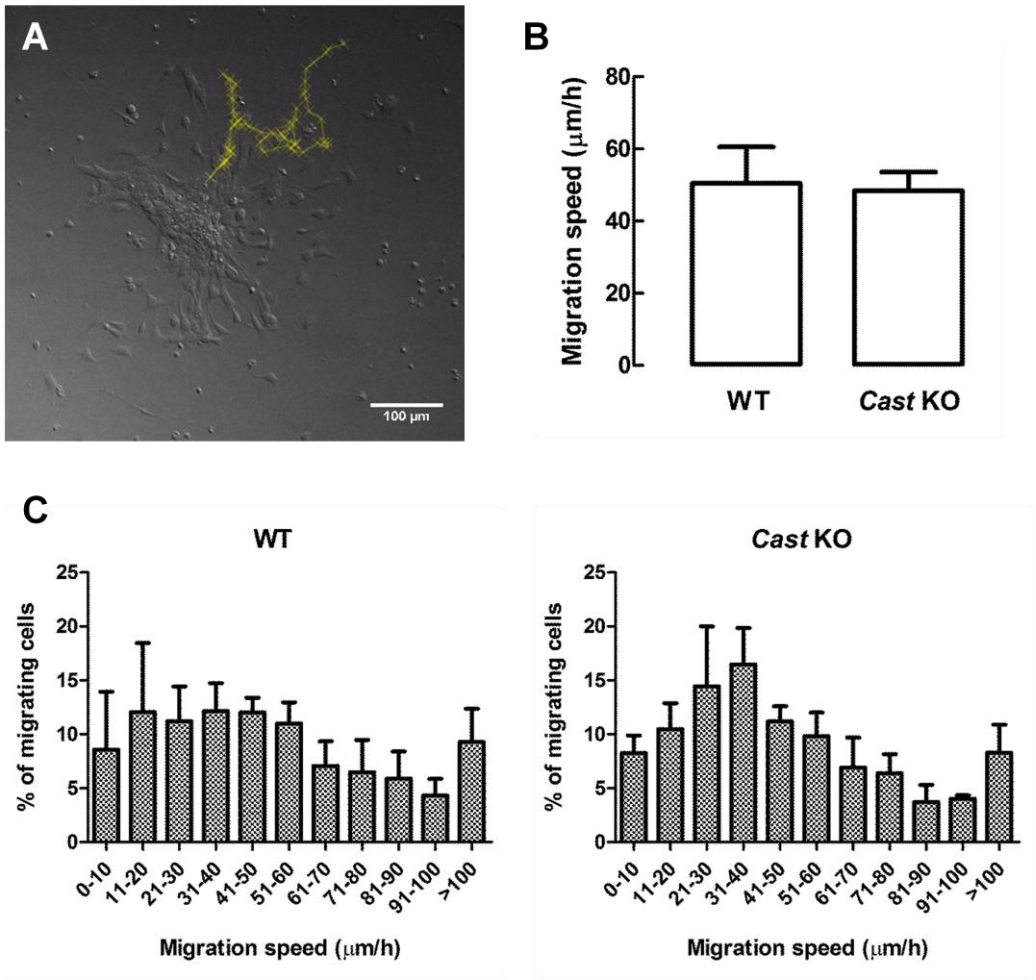

Figure 3.7 - Depletion of CAST maintains cell migration (time-lapse imaging). SVZ-derived NSCs from post-natal WT or *Cast* KO mice were plated as neurospheres on poly-L-lysine and laminin-coated wells and live imaged for 4 h, every 3 min. Representative image of the tracking of a cell migrating out of a neurosphere (**A**). Scale bar: 100 µm. Migration speed (**B**) and frequency distributions (**C**) were measured in cells belonging to the first batch of cells migrating out of 3 neurospheres (38-52 cells

each) per sample. Means ± SEM of 3 independent experiments were analyzed by a two-tailed t test (migration speed) or a one-way ANOVA (frequency distributions), $p>0.05$.

3.3.2. Effects of CAST overexpression on post-injury hippocampal neurogenesis and behavioral recovery

3.3.2.1. CAST overexpression maintains enhancement of early hippocampal neurogenesis after KA treatment

For the work in post-injury neurogenesis, hCAST mice were genotyped (Fig. 3.8A) and the overexpression of CAST in these mice was confirmed in hippocampal lysates, by comparison with WT. Levels of CAST from WT lysates were too low for detection, which was not the case for hCAST lysates, reflecting the increased levels of CAST in these mice (Fig. 3.1B).

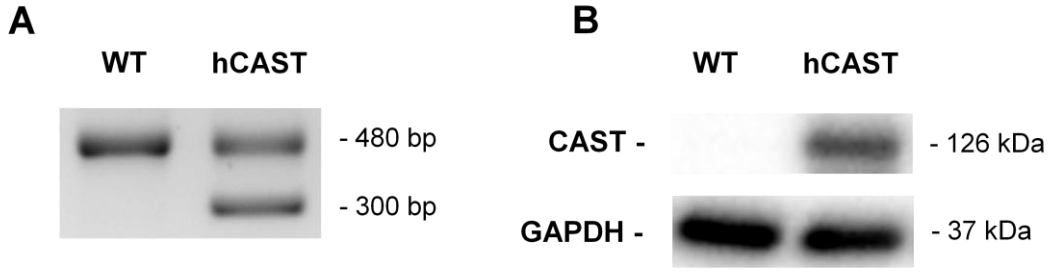

Figure 3.8 - Genotyping profiles and overexpression of CAST in hCAST mice. Genotyping profiles for WT and hCAST mice (**A**). Western blot analysis of hippocampal lysates from WT and hCAST mice (**B**), to detect overexpresison of CAST. GAPDH was used as a loading control.

In order to study the effects of CAST overexpression on the early stages of hippocampal neurogenesis after a brain insult, NSC proliferation and neuroblast migration were analyzed in the DG of adult mice, after treatment with kainic acid. Cell proliferation was assessed by incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) into the DNA of dividing NSCs from the SGZ of the hippocampus. We observed that cell proliferation was greatly enhanced in mice overexpressing CAST after KA treatment (73.1 ± 8.0 cells/section), when compared to saline(SAL)-treated mice (19.2 ± 1.5 cells/section, $p<0.001$), similarly to what was seen in WT mice (KA: 75.6 ± 9.7 cells/section, SAL: 25.0 ± 2.5 cells/section, $p<0.001$) (Fig. 3.9).

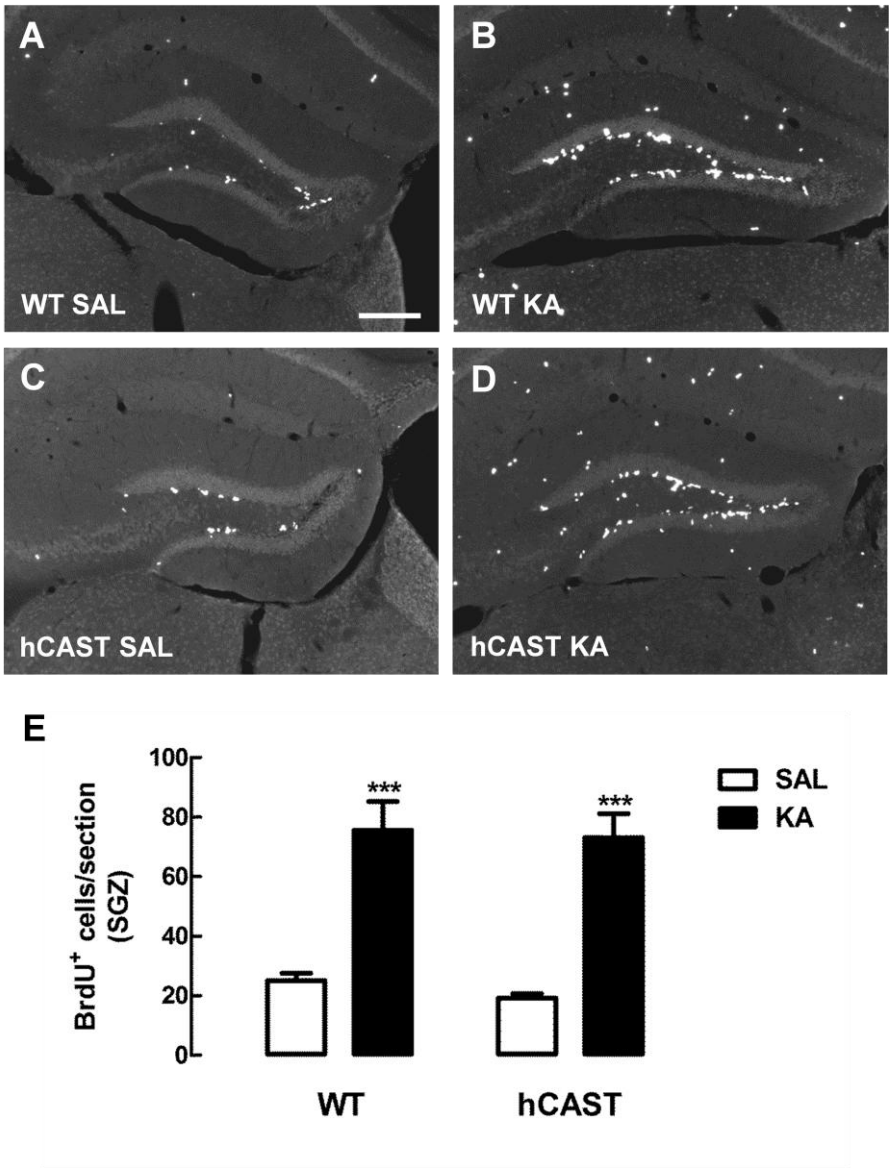

Figure 3.9 - KA-treated mice present increased NSC proliferation in the DG. WT and hCAST mice were treated with either SAL or KA and were given BrdU on the day before sacrifice, to assess cell proliferation. Representative images from hippocampal brain sections of SAL (**A**) and KA-treated (**B**) WT mice and SAL (**C**) and KA-treated (**D**) hCAST mice, showing BrdU-positive cells in white. Scale bar: 200 µm. BrdU-positive cells were counted in the SGZ of 5 mid hippocampal sections for each animal (**E**). Means ± SEM of 9-14 independent experiments were analyzed by a two-way ANOVA (Bonferroni's post-test), ***p<0.001, significantly different from SAL.

With regard to neuroblast migration, as assessed by doublecortin (DCX) staining in the DG, we also observed a significant increase in KA-treated mice overexpressing CAST (6.1 ± 0.6%), when compared to SAL-treated mice (1.9 ± 0.2%, p<0.001). Although cell

migration in the DG of KA-treated WT mice (8.5 ± 0.3%) was also enhanced in comparison with SAL (2.1 ± 0.2%, $p<0.001$), CAST overexpression seemed to attenuate this effect (Fig. 3.10).

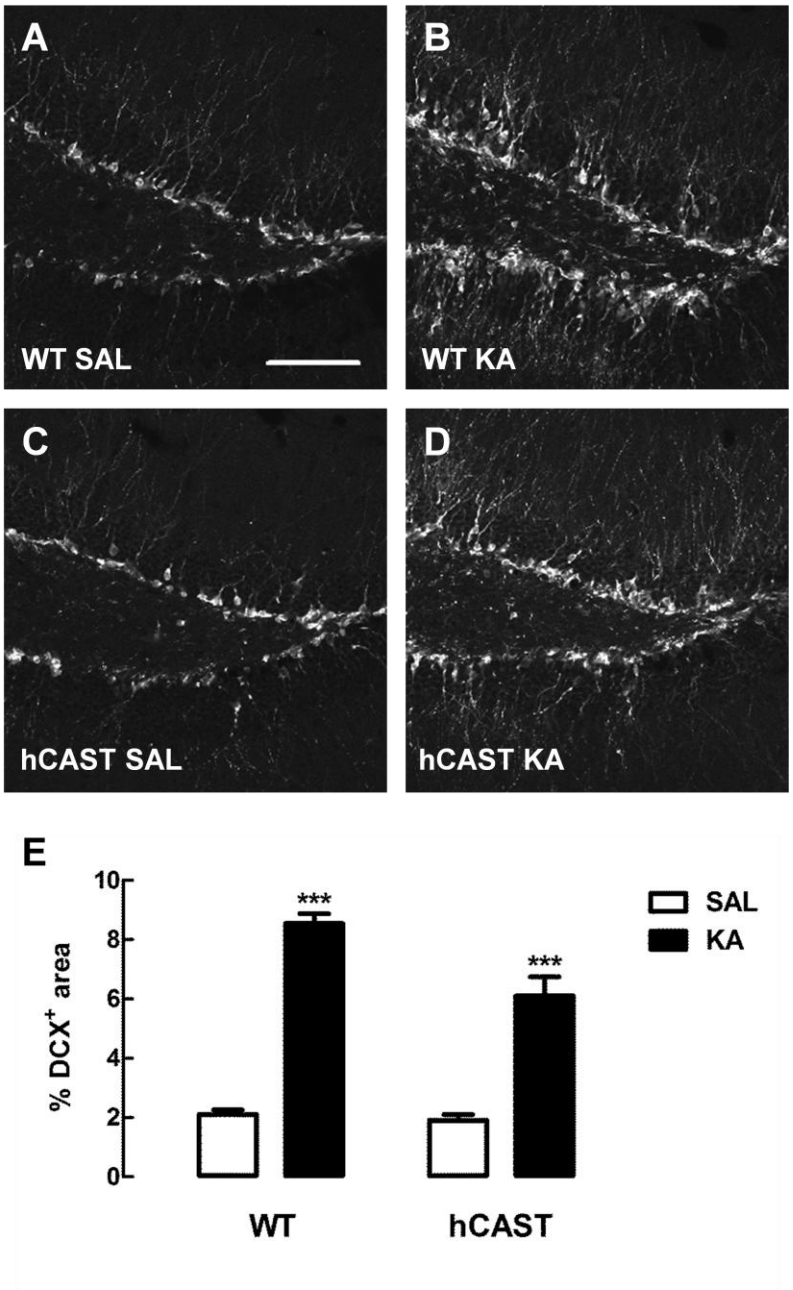

Figure 3.10 - KA treatment enhances neuroblast migration in the DG. WT and hCAST mice were treated with either SAL or KA and sacrificed after 14 days, to assess cell migration. Representative images from hippocampal brain sections of SAL (**A**) and KA-treated (**B**) WT mice and SAL (**C**) and KA-treated (**D**) hCAST mice, showing migrating neuroblasts, labeled for DCX, in white. Scale bar: 100 µm. Percentage of DCX-positive area was determined in the DG of 5 mid hippocampal sections for each

animal (**E**). Means ± SEM of 6-9 independent experiments were analyzed by a two-way ANOVA (Bonferroni's post-test), ***p<0.001, significantly different from SAL.

3.3.2.2. CAST overexpression maintains decrease in neuronal differentiation in the DG after KA treatment

Besides cell proliferation and migration, we were also interested in investigating differentiation of newborn cells after treatment with KA. For that purpose, mice were treated with EdU, and the number of EdU-positive cells remaining in the DG after 8 weeks was counted. We observed that the number of newborn cells still surviving in the DG was enhanced with KA-treatment, both in WT (SGZ+GZ: 9.7 ± 1.8 cells/section, hilus: 1.6 ± 0.3 cells/section) and hCAST (SGZ+GZ: 9.2 ± 1.1 cells/section, hilus: 1.6 ± 0.3 cells/section) mice, when compared to SAL treatment (SGZ+GZ, WT: 1.8 ± 0.1 cells/section, hCAST: 1.6 ± 0.3 cells/section, p<0.001; hilus, WT: 0.3 ± 0.1 cells/section, p<0.01, hCAST: 0.6 ± 0.2 cells/section, p<0.05) (Fig. 3.11).

However, when assessing neuronal differentiation specifically, by determining the percentage of those cells that colocalized with a neuronal marker (NeuN, neuronal nuclei), we could observe that treatment with KA reduced the amount of new neurons in the DG, both in mice overexpressing CAST (47.0 ± 4.1%) and in WT mice (52.5 ± 8.0%), when compared to SAL-treated animals (WT: 71.6 ± 4.4%, hCAST: 67.2 ± 4.2%, p<0.05) (Fig. 3.12), indicating that KA decreases neuronal survival.

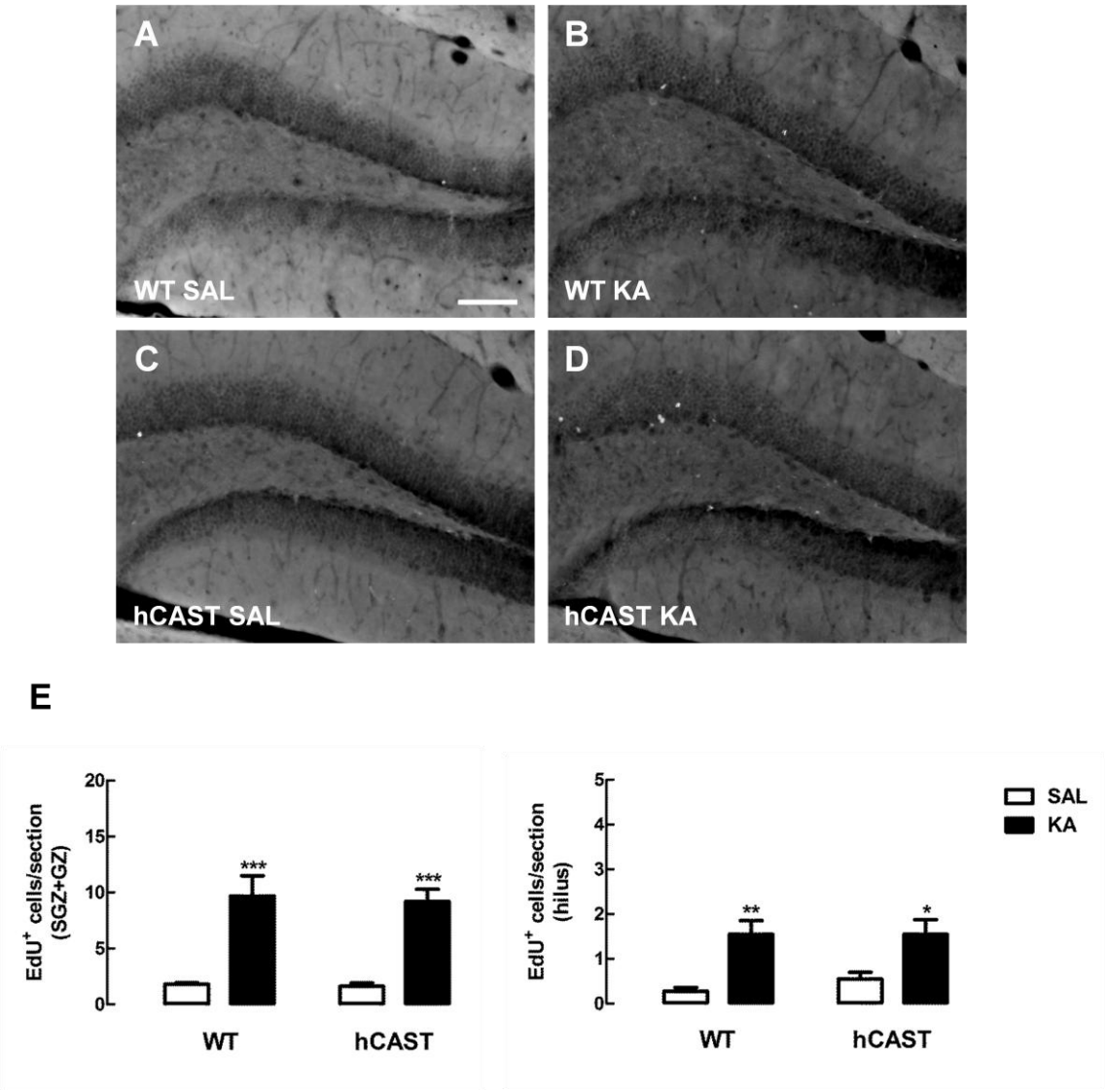

Figure 3.11 - Mice treated with KA present higher number of new cells remaining in the DG after 8 weeks. WT and hCAST mice were treated with either SAL or KA and sacrificed 8 weeks after EdU administration. Representative images from hippocampal brain sections of SAL (**A**) and KA-treated (**B**) WT mice and SAL (**C**) and KA-treated (**D**) hCAST mice, showing EdU-positive cells in white. Scale bar: 200 µm. EdU-positive cells were counted in the SGZ, GZ and hilus of 5 mid hippocampal sections for each animal (**E**). Means ± SEM of 8 independent experiments were analyzed by a two-way ANOVA (Bonferroni's post-test), *p<0.05, **p<0.01 and ***p<0.001, significantly different from SAL.

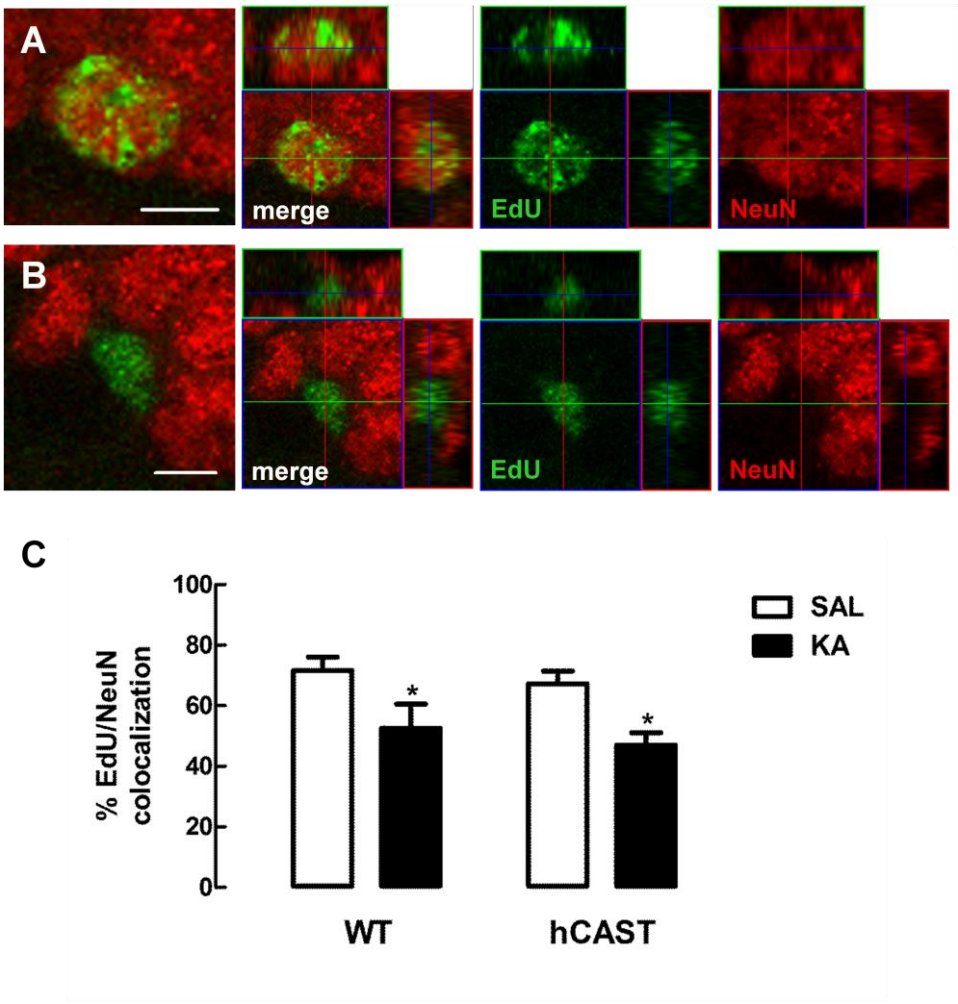

Figure 3.12 - Neuronal differentiation is impaired with KA treatment. WT and hCAST mice were treated with either SAL or KA and sacrificed 8 weeks after EdU administration, to assess neuronal differentiation. Representative images from hippocampal brain sections of EdU-positive/NeuN-positive (**A**) and EdU-positive/NeuN-negative (**B**) cells in the DG, showing cells labeled for EdU in green and cells labeled for NeuN in red. Scale bar: 5 µm. The percentage of cells labeled for both EdU and NeuN in the DG (**E**) was determined in a total of up to 50 EdU-positive cells. Means ± SEM of 8 independent experiments were analyzed by a two-way ANOVA (Bonferroni's post-test), *p<0.05, significantly different from SAL.

3.3.2.3. Short-term impairment in object recognition memory after KA treatment is not present in mice overexpressing CAST

Furthermore, to evaluate whether CAST overexpression would allow for a better and/or faster recovery after brain injury, we performed several cognitive behavioral tests on

WT and hCAST mice, on the 3rd (short-term) or 8th (long-term) week after treatment with either KA or SAL. We first tested the animals for object recognition memory, and took the opportunity, during habituation to the apparatus, to compare the overall locomotor activity and anxiety behavior of these mice. We observed that KA-treated mice presented similar levels of locomotor activity, as assessed by the number of line crossings and rearing behavior, when compared to SAL-treated animals in both short-term recovery (line crossings, WT: 122.4 ± 17.4, hCAST: 110.0 ± 11.5; rearing, WT: 30.0 ± 4.8, hCAST: 14.2 ± 3.4, p>0.05) (Fig. 3.13A) and long-term recovery (line crossings, WT: 126.7 ± 10.1, hCAST: 128.0 ± 9.1; rearing, WT: 26.4 ± 4.3, hCAST: 25.1 ± 1.7, p>0.05) (Fig. 3.13B).

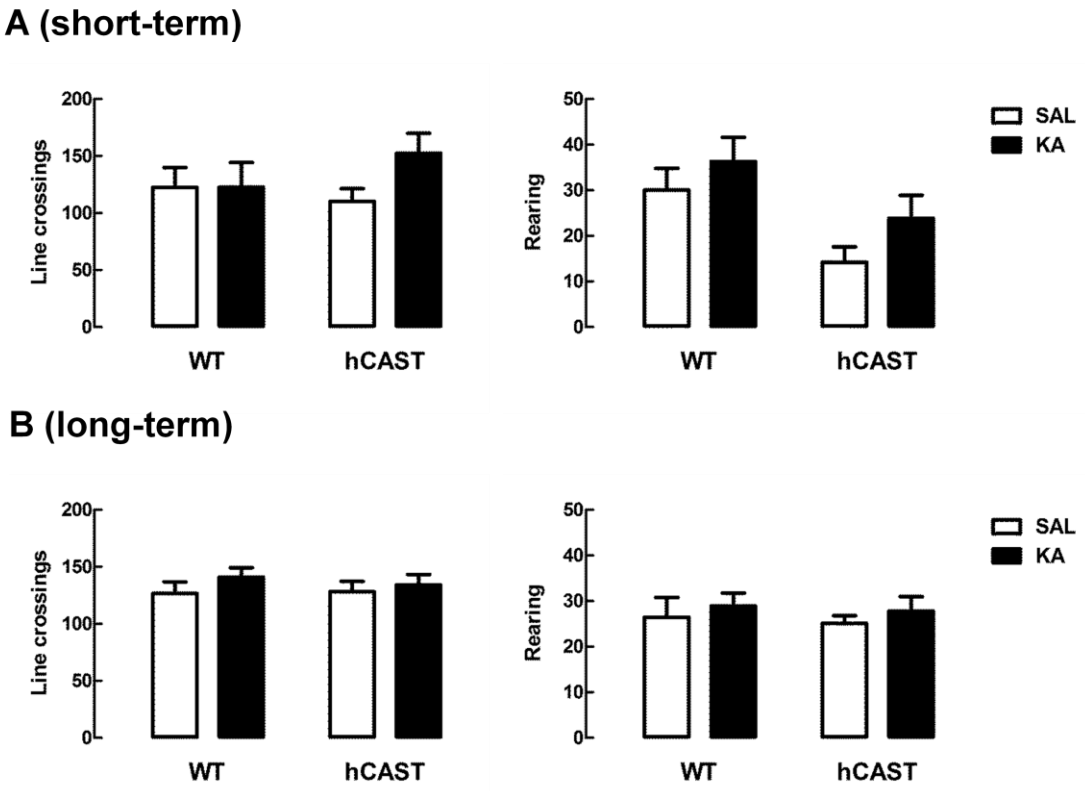

Figure 3.13 - Locomotor activity is similar between SAL and KA treatments, for both WT and hCAST mice and recovery periods. WT and hCAST mice behavior was tested on the 3rd (short-term) or 8th (long-term) week after treatment with either SAL or KA. Locomotor activity was assessed in both short-term (**A**) and long-term (**B**) groups, by counting the number of line crossings and rearing episodes, during the open field test. Means ± SEM of 4-5 independent experiments (for short-term recovery) or 12-16 independent experiments (for long-term recovery) were analyzed by a two-way ANOVA, p>0.05 between treatments.

Moreover, the anxiety behavior, measured by the number of central entries and time in the center, was also maintained after KA-treatment, in comparison with SAL, in both short-term recovery (central entries, WT: 14.8 ± 3.5, hCAST: 14.6 ± 2.3; time in center, WT: 19.1 ± 5.4 s, hCAST: 20.9 ± 5.8 s, $p>0.05$) (Fig. 3.14A) and long-term recovery (central entries, WT: 15.5 ± 1.6, hCAST: 14.5 ± 1.4; time in center, WT: 17.3 ± 2.9 s, hCAST: 16.2 ± 1.5 s, $p>0.05$) (Fig. 3.14B).

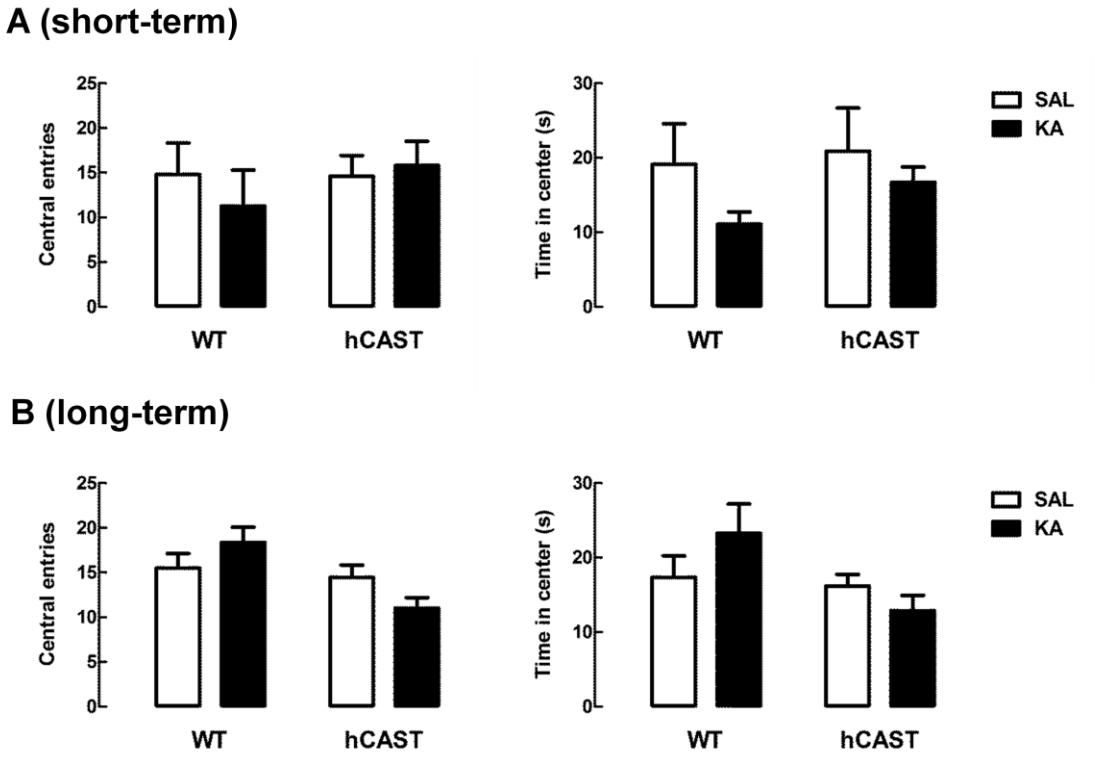

Figure 3.14 - Anxiety behavior is similar between SAL and KA treatments, for both WT and hCAST mice and recovery periods. WT and hCAST mice behavior was tested on the 3rd (short-term) or 8th (long-term) week after treatment with either SAL or KA. Anxiety behavior was assessed in both short-term (**A**) and long-term (**B**) groups, by counting the number of entries into the central area and the time spent in the center, during the open field test. Means ± SEM of 4-5 independent experiments (for short-term recovery) or 12-16 independent experiments (for long-term recovery) were analyzed by a two-way ANOVA, $p>0.05$ between treatments.

Regarding the object recognition test, we observed that, for both time periods, WT mice treated with SAL spent more time exploring the novel object (short-term recovery: 58.3

± 3.7%, long-term recovery: 62.9 ± 3.3%) than the familiar object, that they had already explored previously (short-term recovery: 41.7 ± 3.7%, $p<0.05$, long-term recovery: 37.1 ± 3.3%, $p<0.001$). However, after KA treatment, only WT mice from the long-term recovery group were able to distinguish the novel object (58.9 ± 4.5%), from the familiar object (41.1 ± 4.5%, $p<0.01$) (Fig. 3.15, left panels), indicating impairment in recognition memory in the short-term group. On the other hand, all mice overexpressing CAST maintained the recognition memory for the novel object, in both short-term recovery (SAL, novel object: 61.5 ± 5.0%, familiar object: 38.5 ± 5.0%, $p<0.01$; KA, novel object: 61.1 ± 1.3%, familiar object: 34.9 ± 1.3%, $p<0.001$) and long-term recovery (SAL, novel object: 62.9 ± 3.8%, familiar object: 37.1 ± 3.8%, $p<0.001$; KA, novel object: 59.7 ± 3.6%, familiar object: 40.3 ± 3.6%, $p<0.001$) (Fig. 3.15, right panels).

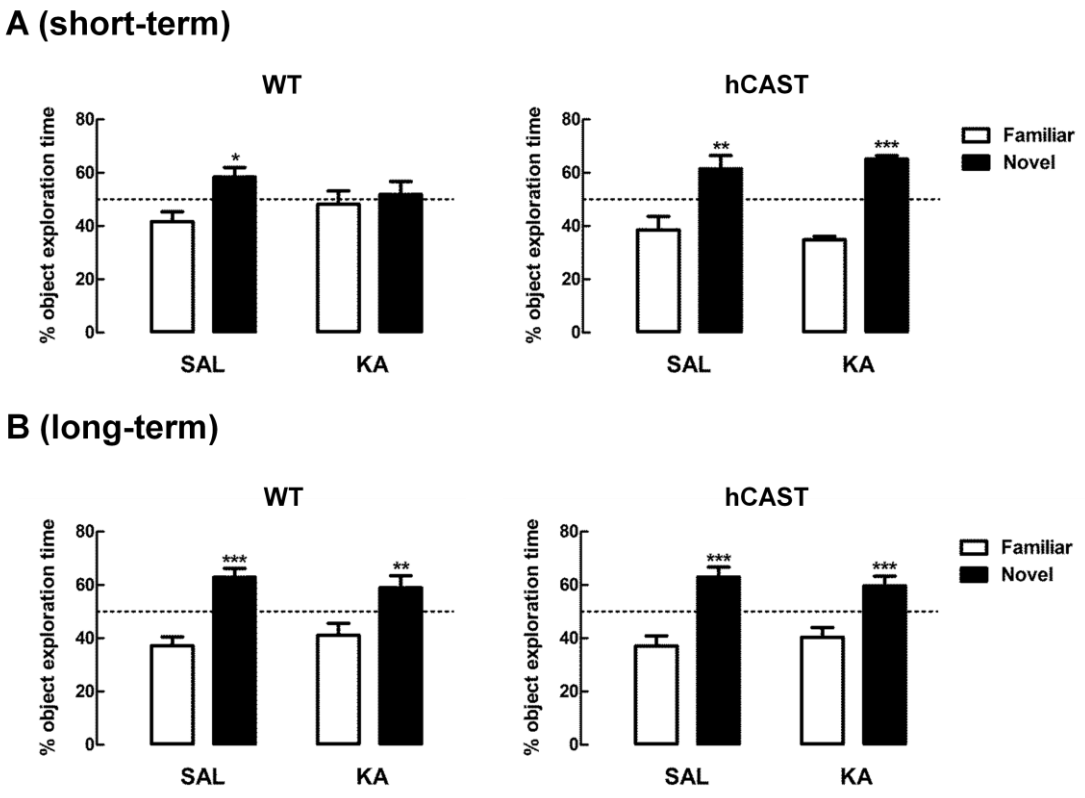

Figure 3.15 - KA-treated WT mice present short-term impairment in recognition memory, but not hCAST mice. WT and hCAST mice behavior was tested on the 3rd (short-term) or 8th week (long-term) after treatment with either SAL or KA. Recognition memory was assessed in both short-term (**A**) and long-term (**B**) groups, by measuring the percentage of exploration time during the presentation of a novel object, in the object recognition test. Means ± SEM of 3-5 independent experiments (for short-term

recovery) or 12-15 independent experiments (for long-term recovery) were analyzed by a two-way ANOVA (Bonferroni's post-test), *p<0.05, **p<0.01 and ***p<0.001, significantly different from familiar object.

3.3.2.4. Long-term impairment in spatial memory for an exact location during the water maze test after KA treatment is not present in mice overexpressing CAST

In order to study spatial learning and memory, the Morris water maze test was performed. During trials, we observed that KA-treated mice overexpressing CAST in the short-term recovery group learned more slowly the platform location, since the escape latency time on Day 2 (42.6 ± 10.2 s) was higher than in SAL (15.5 ± 3.6 s, p<0.05). However, on the beginning and by the end of the trials, escape latency was similar to SAL (Day 1: 39.4 ± 4.1 s, Day 3: 14.2 ± 4.7 s, p>0.05) (Fig. 3.16A). KA-treated WT mice from this group learned the platform location similarly to SAL (Day 1: 49.3 ± 7.3 s, Day 2: 36.3 ± 10.0 s, Day 3: 24.2 ± 10.9 s, p>0.05) on all days, as well as all KA-treated mice in the long-term recovery group (Day 1, WT: 38.4 ± 2.7 s, hCAST: 47.9 ± 2.7 s; Day 2, WT: 26.4 ± 3.9 s, hCAST: 30.9 ± 5.3 s; Day 3, WT: 17.4 ± 4.9 s, hCAST: 20.7 ± 2.1 s, p>0.05) (Fig. 3.16). All mice showed improved spatial learning over the course of trials.

After learning the platform location, the platform was removed and the spatial memory evaluated by calculating the percentage of time spent in the target quadrant (where the platform previously was) and by counting the number of crossings through the exact previous platform location. Time spent in the target quadrant was maintained with KA treatment in both time periods, by comparison with SAL (short-term recovery, WT: 60.4 ± 3.7%, hCAST: 49.0 ± 5.7%, p>0.05; long-term recovery, WT: 56.4 ± 4.1%, hCAST: 49.0 ± 4.3%, p>0.05) (Fig. 3.17, left panels), indicating that all animals remembered the relative position of the platform. The number of platform crossings, in turn, also seemed to be maintained with KA treatment in the short-term recovery period when comparing to SAL (WT: 4.3 ± 1.6, hCAST: 3.8 ± 0.9, p>0.05) (Fig. 3.17A, right panel). However, in the long-term recovery period, the number of platform crossings is impaired in KA-treated WT mice (2.4 ± 0.4) by comparison with SAL treatment (4.5 ± 0.7, p<0.05), indicating increased difficulty in remembering the exact platform location, which is not observed in mice onverexpressing CAST (SAL: 3.0 ± 0.6, p>0.05) (Fig. 3.17B, right panel).

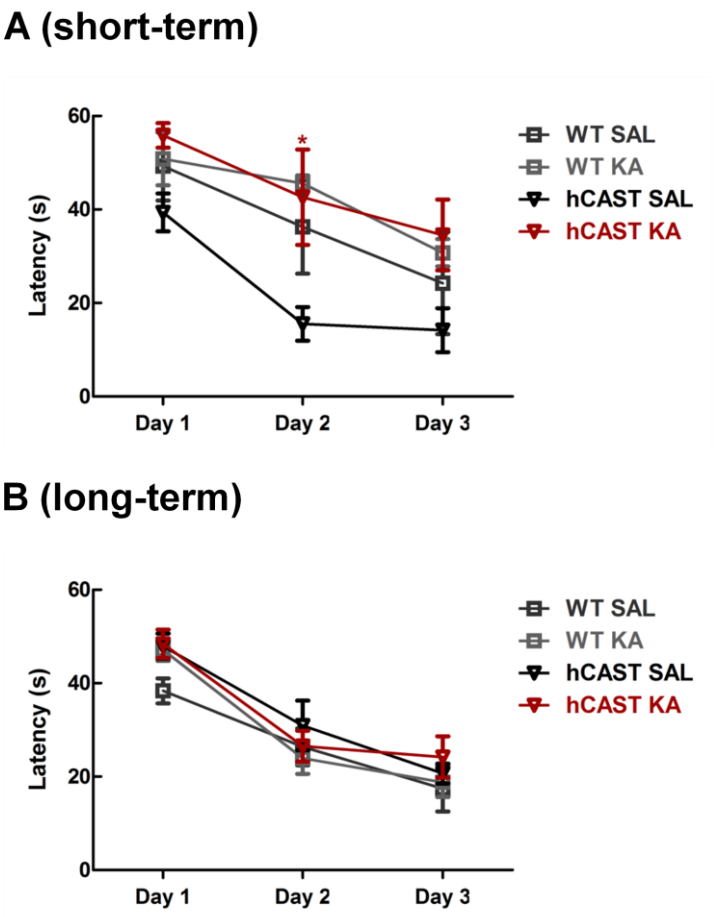

Figure 3.16 - Escape latency during spatial learning improves slower with training in KA-treated mice overexpressing CAST from the short-term recovery group. WT and hCAST mice behavior was tested on the 3rd (short-term) or 8th (long-term) week after treatment with either SAL or KA. Spatial learning was assessed in both short-term (**A**) and long-term (**B**) groups, by measuring the escape latency on the first 3 days of water maze (4 trials per day, latency of 60 s if the platform was not reached). Means ± SEM of 4-5 independent experiments (for short-term recovery) or 11-16 independent experiments (for long-term recovery) were analyzed by a two-way ANOVA (Bonferroni's post-test), *p<0.01, significantly different from hCAST SAL.

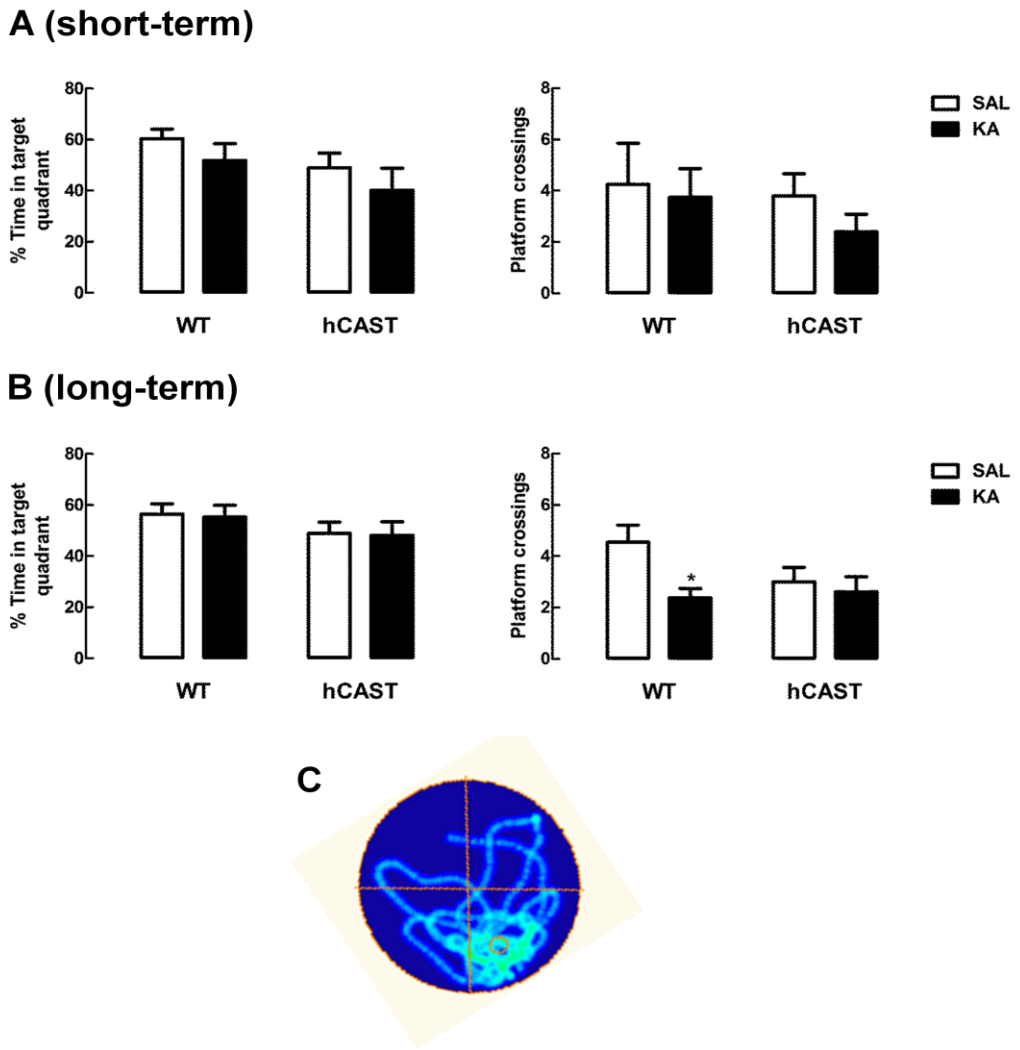

Figure 3.17 - Spatial memory for the exact platform location is impaired in KA-treated WT mice from the long-term recovery group. WT and hCAST mice behavior was tested on the 3rd (short-term) or 8th (long-term) week after treatment with either SAL or KA. Spatial memory was assessed in both short-term (**A**) and long-term (**B**) groups, by measuring the percentage of time spent on the target quadrant and the number of crossings in the exact location the platform was during trials, on the last day of water maze. Means ± SEM of 4-5 independent experiments (for short-term recovery) or 11-16 independent experiments (for long-term recovery) were analyzed by a two-way ANOVA (Bonferroni's post-test), *$p<0.05$, significantly different from SAL. Example of an occupancy track plot, showing the route chosen by the animal in light blue (**C**).

3.3.2.5. Associative fear memory is preserved after KA treatment

Lastly, associative learning and memory was evaluated, by performing the fear conditioning test. By the end of the trials, on the first day, all mice successfully learned to associate the cue tone with a footshock, by spending nearly half the time of the last hearing of the tone freezing, both in the short-term recovery (WT SAL: 42.7 ± 5.4%, hCAST SAL: 51.5 ± 5.3%, p>0.05) and in the long-term recovery (WT SAL: 48.9 ± 6.8%, hCAST SAL: 47.1 ± 4.5%, p>0.05) (Fig. 3.18).

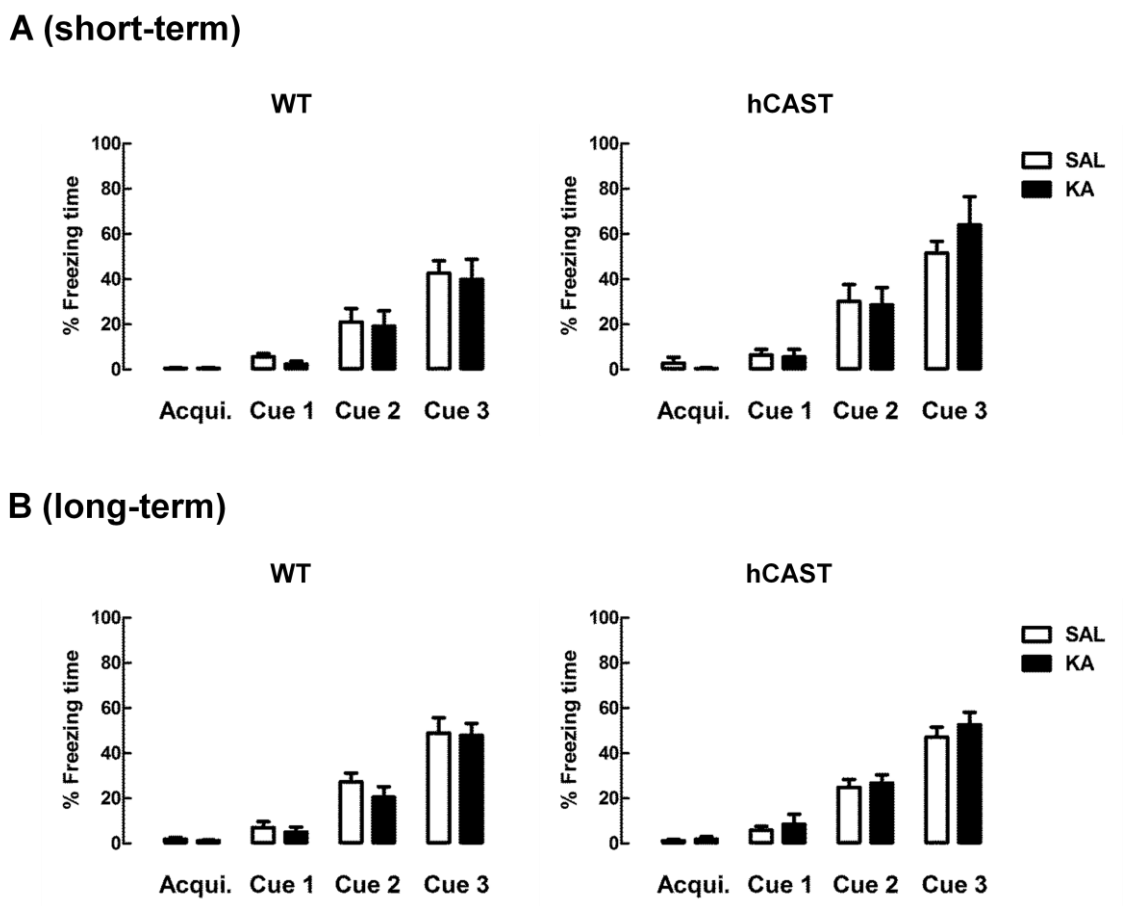

Figure 3.18 - Associative learning was achieved similarly in all groups. WT and hCAST mice behavior was tested on the 3rd (short-term) or 8th (long-term) week after treatment with either SAL or KA. Associative learning was assessed in both short-term (**A**) and long-term (**B**) groups, by measuring the percentage of freezing time during fear conditioning trials, with the delivery of a footshock by the end of each cue tone. Means ± SEM of 4-5 independent experiments (for short-term recovery) or 12-16 independent

experiments (for long-term recovery) were analyzed by a two-way ANOVA, $p>0.05$ between treatments. Acqui. (acquisition).

When tested for associative memory to context (context A), all KA-treated mice seemed to recognize the context similarly to SAL, both in the short-term recovery (WT: 48.9 ± 6.8%, hCAST: 64.2 ± 10.8%, $p>0.05$) and in the long-term recovery (WT: 51.5 ± 6.0%, hCAST: 69.4 ± 2.6%, $p>0.05$) (Fig. 3.19, upper panels). When in the context meant for the cued test (context B), hCAST mice from the short-term recovery group treated with KA spent significantly more time freezing than with SAL treatment (SAL: 37.4 ± 7.2%, KA: 63.4 ± 9.8%, $p<0.05$), which was not observed in KA-treated WT mice, when compared to SAL (23.1 ± 4.5%, $p>0.05$) (Fig. 3.19A, lower left panel). In the long-term recovery group, the percentage of freezing time was more similar to SAL in both WT and hCAST mice (WT: 25.4 ± 4.4%, hCAST: 33.8 ± 4.4%, $p>0.05$) (Fig. 3.19B, lower left panel). Moreover, all KA-treated mice seemed to maintain the associative memory to the cued stimulus by comparison with SAL, both in the short-term recovery (WT: 56.7 ± 3.9%, hCAST: 75.5 ± 5.2%, $p>0.05$) and in the long-term recovery (WT: 69.1 ± 6.5%, hCAST: 75.1 ± 3.2%, $p>0.05$) (Fig. 3.19, lower right panels).

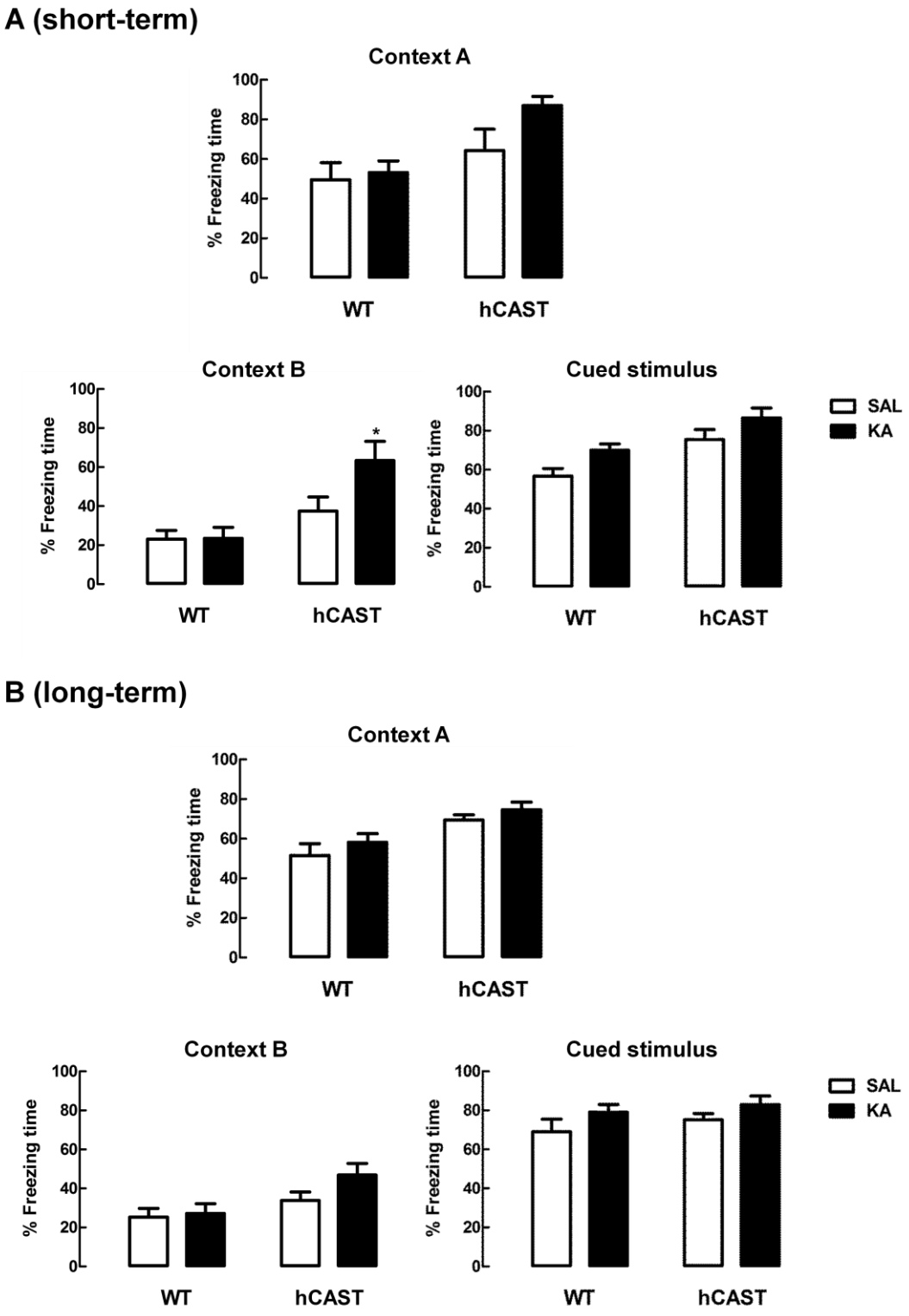

Figure 3.19 - KA treatment enhances associative memory to context B in hCAST mice from the short-term recovery period. WT and hCAST mice behavior was tested on the 3rd (short-term) or 8th (long-term) week after treatment with either SAL or KA. Associative memory to the context and cue tone were assessed in both short-term (**A**) and long-term (**B**) groups, by measuring the percentage of freezing time during the

fear conditioning context test (context A) and cued test, in a new context (context B). Means ± SEM of 3-5 independent experiments (for short-term recovery) or 9-16 independent experiments (for long-term recovery) were analyzed by a two-way ANOVA (Bonferroni's post-test), *$p<0.05$, significantly different from SAL.

3.5. Discussion

The realization that the adult mammalian brain is not an immutable organ and that new neurons are actually able to form and thrive in the neuronal circuitry has opened new doors for the putative treatment of neurodegenerative disorders and brains lesions. This is due to fact that NSCs not only give origin to new neurons in the healthy brain, but are also known to increase the formation of new cells after a lesion, travelling as neuroblasts into regions where neurons were lost as a consequence of brain damage, in what seems like an endogenous attempt to replace those lost neurons. However, this process is not very efficient, as most of the new cells die, fail to integrate the networks, or do it erroneously (Kaneko & Sawamoto 2009, Ma *et al.* 2009). While cell transplantation into the sites of injury, for example, also seems promising, increasing endogenous neurogenesis would offer a less invasive approach for the treatment of brain damage. Our group has been interested in the study of the effects of calpains in this process, since they are proteases involved in neurodegeneration and their inhibition has been shown to be neuroprotective in several diseases (Saez *et al.* 2006). Furthermore, calpains have also been associated with cell proliferation, migration and differentiation in other systems (Kashiwagi *et al.* 2010, Lokuta *et al.* 2003, Paulhe *et al.* 2001, Qiu *et al.* 2006, Raimbourg *et al.* 2013, Yajima & Kawashima 2002). One of the approaches we can use to study calpains is by modulating their selective endogenous inhibitor, CAST. We had previously seen that the absence of CAST impairs NSC proliferation in one of the neurogenic niches of the adult brain, the DG of the mouse hippocampus, and also hinders neuroblast migration, both in the DG and in the RMS, whose cells originate from another neurogenic niche, the SVZ. Moreover, calpain inhibition was shown to increase cell migration in explants from the SVZ (Machado *et al.* 2015). Work by Santos *et al.* has shown that calpain inhibition decreased cell proliferation in a NSC line, but also that it increased neuronal differentiation (Santos *et al.* 2012). However, this NSC line was originated from fetal brain, thus the mechanisms involved in these processes may be different from the ones occurring in the post-natal brain. Nonetheless, taken together, these evidences point to an involvement of calpains in the regulation of neurogenesis, so we were interested in investigating it further. SVZ-derived cells from the post-natal brain allow for the maintenance of numerous NSCs in culture, and provide for a model suitable for the analysis of neurogenesis *in vitro*, while analyzing SVZ neurogenesis *in vivo* may be more complicated. On the contrary, the low amount of NSCs in the DG make the analysis easier in brain sections, but these cells are difficult to isolate and maintain in culture.

The hippocampus is also a main target in several neurological disorders that affect mankind, making for a more attractive target for human disease modeling *in vivo*, rather than the SVZ. For that reason, we used SVZ-derived NSCs for the studies *in vitro*, and analyzed hippocampal neurogenesis in the mouse brain for the rest of the work.

To complement the previous results seen with *Cast* KO mice, in which we observed an impairment of the early stages of hippocampal neurogenesis (Machado *et al.* 2015), we wanted to investigate whether calpain inhibition would reverse the impairment on NSC proliferation and neuroblast migration in these CAST-depleted mice. Since we were testing the pharmacological calpain inhibitor, calpeptin, *in vitro*, we first checked if the impairment in cell proliferation was maintained in cultured NSCs lacking CAST. In fact, as expected, we saw a progressive decrease in cell proliferation with the decrease of CAST levels, i.e., NSC proliferation in *Cast* Ht was impaired when compared to WT and, in turn, it was impaired in *Cast* KO, not only by comparison with WT, but also with *Cast* Ht. We then proceeded to treating WT and *Cast* KO cells with calpeptin, and verified that the impairment in NSC proliferation in mice lacking CAST was rescued by calpain inhibition. Concerning calpain activity in *Cast* KO mice, we also verified that cells from mice lacking CAST presented higher calpain activity in the presence of a calcium ionophore, by observing an increase of calpain-specific αII-spectrin breakdown products. We can therefore assume that the impairment in NSC proliferation observed is associated with increased activation of calpains, usually prevented by CAST, further involving them in this process. Moreover, calpain inhibition was also able to increase the proliferation of WT NSCs. Together with the previous observation of the enhancement of cell migration by calpeptin in SVZ explants, this indicates calpain inhibition as a possibility for increasing endogenous neurogenesis.

Regarding cell migration when CAST is absent, however, the results obtained were not in accordance with our previous observations *in vivo*, since it was maintained in all of the approaches used. We measured radial migration and migration speed of cells leaving plated neurospheres, and also the area migrated by dissociated NSCs. Since mice lacking CAST show impairment in cell migration *in vivo*, both in the DG and the RMS, and since cells from the RMS are also derived from the SVZ, it seems unlikely that the fact that we did not observe these differences *in vitro* was due to the type of cells used. Lack of sensitivity of the approaches used to distinguish these variations could also be a possibility, but taking into account that we tried three different methods, and that one of them involved live tracking of migrating cells, this also does not seem to be the case. The most plausible hypothesis is the fact that one of the biggest problems

presented by *in vitro* studies is the difficulty to replicate the exact conditions of the environment that the cells encounter *in vivo*. This means that, for cell migration, something else in the environment might be helping calpains interfere with the process. Although we were not able to verify the involvement of calpains on cell migration *in vitro*, their clear involvement in NSC proliferation and the enhancement of cell proliferation and migration by calpain inhibition led us to investigate the effects of calpain inhibition after neuronal damage. For that purpose, we used mice overexpressing CAST and the KA model of seizure-induced hippocampal lesion.

KA is a strong agonist of non-NMDA ionotropic glutamate receptors that induces excitotoxicity and consequent cell death, by activating ionotropic receptors highly abundant in the hippocampus (Carta *et al.* 2014, Wang *et al.* 2005). The KA model of brain lesion is thus often used for research involving hippocampal damage, especially in studies of temporal lobe epilepsy, since it mimics other features of this disease (Levesque & Avoli 2013). It is also a model recurrently used by our group for the investigation of hippocampal injury and its consequences (Araujo *et al.* 2008, Carreira *et al.* 2010, Carreira *et al.* 2015). Like with other types of brain damage, an enhancement of endogenous neurogenesis has also been reported after seizures. Independently of the model chosen to induce seizures in rodents, NSC proliferation is specially shown to be largely increased in the DG after just a few days (Gray & Sundstrom 1998, Huttmann *et al.* 2003, Jessberger *et al.* 2007a, Mohapel *et al.* 2004, Nakagawa et al. 2000, Parent et al. 1997, Sierra *et al.* 2015). Interestingly, evidence of increased cell proliferation has also been observed in the hippocampus of patients with temporal lobe epilepsy (Blumcke *et al.* 2001, Crespel *et al.* 2005). In the model used by our group, the peak of cell proliferation in the DG was shown to occur on day 5 after seizure induction, with a significant increase in neuroblast migration after 14 days (Carreira *et al.* 2015), results that we were able to replicate in the present work. This heavy enhancement of early neurogenesis indicates that the microenvironment in the hippocampus not long after seizures is extremely pro-neurogenic, which makes it difficult for changes due to altered levels of calpain activity to be observed. Nonetheless, since we have shown that increased calpain activity can impair NSC proliferation and migration of neuroblasts, it is important to exclude whether, in this case, calpain inhibition, by overexpression of CAST, increases cell proliferation and migration to alarming levels. An even more extreme rise in cell proliferation could originate unwanted cellular masses of undifferentiated cells, and more migrating cells could also mean more cells migrating into ectopic regions, interfering with the normal neuronal networks (Parent *et al.* 2006). Regarding cell proliferation, the enhancement

observed after KA treatment was similar in WT mice and in mice overexpressing CAST. Curiously, however, while the increase in neuroblast migration in KA-treated WT mice was around 4-fold, it dropped to 3-fold with the overexpression of CAST. Brain damage is closely associated with an increase in calpain activity, and it is known that calpain inhibition can be neuroprotective (Saez et al. 2006). This means that the fact that CAST was already overexpressed when seizures were induced may have attenuated the damage. Less damage could therefore also mean a less dramatic increase in neurogenesis, which in the long term could be favorable, proven that more cells would integrate correctly in the existing networks. Cell proliferation in the DG was shown by another group to substantially rise even after less severe seizures, with no further increase with seizure severity, indicating a threshold event (Mohapel *et al.* 2004), which might explain why we observed a similar increase in cell proliferation even with the neuroprotective effect CAST overexpression might have provided during seizures.

This increase in neurogenesis after lesion, however, is only efficient if the new cells ultimately differentiate, survive and integrate the neuronal circuitry correctly. After seizures, this is usually not the case. Despite the enhancement in the early stages of neurogenesis, most of the new cells end up differentiating into astrocytes, with relatively less neurons surviving 1-2 months after seizures (Carreira *et al.* 2015, Hattiangady & Shetty 2010, Sierra *et al.* 2015). Moreover, several newly formed neurons resembling granule cells are found ectopically in the hilus, and have also been reported to occur in patients with temporal lobe epilepsy (Parent *et al*. 2006, Parent *et al.* 1997). These ectopic granule-like hilar cells are hyperexcitable, contributing to a disruption of the existing neuronal networks and possibly aggravating the outcome of the disease (Dashtipour *et al.* 2001, Cameron *et al.* 2011). By overexpressing CAST, we were hoping to attenuate the survival impairment of new neurons in the DG after severe seizures and possibly reduce the presence of ectopic hilar cells, thus improving the efficiency of post-injury neurogenesis. Our findings with WT mice are consistent with what has been reported. After a 2-month period, there were significantly more new cells found in the hilus of mice treated with KA, as well as an increase in the number of new cells present in the SGZ and GZ of the DG. Since the percentage of new neurons was decreased in these mice, the overall increase in the number of new cells might be justifiable by the increased differentiation into astrocytes that occurs after seizures. The results obtained with mice overexpressing CAST were very similar, indicating that suppression of calpain activity is not enough to compensate for the impairment of neuronal differentiation in the DG. Although calpain inhibition was shown to increase

neuronal differentiation *in vitro*, in a cell line from fetal NSCs (Santos *et al.* 2012), we cannot exclude that in the developed brain calpains may not be involved in the process, or that other mechanisms may compensate for the decrease in calpain activity under a certain threshold, or even help in maintaining levels of calpain activity too elevated, especially in a pathologic environment. The inflammatory environment, e.g., may have some detrimental effects. Nitric oxide from inflammatory origin, for example, has been implicated in the neuronal impairment observed after seizures, in a different study performed by our group (Carreira *et al.* 2015). This multifactorial feature of brain pathology must thus be taken into account when developing new strategies for brain repair.

Nonetheless, even though the percentage of neurons formed after seizures is not ameliorated by CAST overexpression, the ones that thrive may still be more functional and better integrated in the hippocampal neuronal networks. Since the hippocampus is the main structure involved in learning and memory, it is not surprising that treatment with KA in rodents can induce cognitive impairment (Stubley-Weatherly *et al.* 1996), which has also been reported in patients with temporal lobe epilepsy (Hattiangady & Shetty 2008). With this in mind, we were especially interested in verifying whether mice overexpressing CAST would show differences in cognitive recovery on the 3rd (short-term) or 8th week (long-term) after KA treatment. In the short-term group, neurons born a few days after the brain insult are still immature and just starting to project their axons and dendrites and integrating the neuronal networks, while in the long-term group those neurons are already fully mature and integrated (Deng *et al.* 2010). Electrophysiology studies may also be performed to directly assess the functional behavior of the new neurons, but this was not addressed in our work. Although we were more interested in investigating the mice cognitive behavior, it is important to exclude the effects of general locomotor behavior and anxiety in these studies, especially since the hippocampus has also been related to anxiety (Barkus *et al.* 2010). Differences in locomotor activity and anxiety in these mice could lead to differences not related to cognition in the performance of the other tests, possibly giving rise to incorrect interpretation of the results. Due to its simplicity, the open field test is the most used for a general assessment of locomotor activity and anxiety behavior in rodents (Gould *et al.* 2009). Since it could be performed in the same apparatus, the habituation step of the object recognition test was therefore availed for that purpose. Overall, we did not find significant differences in locomotor activity or anxiety with KA treatment, suggesting that the brain areas related to these behaviors were relatively preserved after lesion. Therefore, any differences observed in the other tests should be related to

cognition *per se*. We then tested the mice for three different types of cognitive behavior: recognition memory (object recognition), spatial learning and memory (water maze) and associative learning and memory (fear conditioning). The object recognition test makes use of the natural exploratory behavior of rodents. In the presence of a familiar and a novel object, rats and mice will normally spend relatively more time exploring the novel one, in a process shown by several studies to implicate the hippocampus (Cohen & Stackman 2015). It was thus not a surprise that KA treatment impaired recognition of the previously explored object in WT mice from the short-term recovery group. In the long-term group, however, this impairment seemed to be overcome, suggesting that the newly formed cells ultimately succeeded in restoring the networks necessary for object recognition. Interestingly, CAST overexpression prevented impairment after KA treatment even in the short-term group. Having in mind that in this group the newly formed neurons are still immature, it is unlikely that they were already able to restore lost connections. This might thus mean that the regions involved in recognition memory were less affected when CAST was overexpressed, in accordance with the aforementioned neuroprotective effect afforded by calpain inhibition.

Spatial memory, in turn, has long been known to require the hippocampus, being one of the most affected by hippocampal lesions (Best *et al.* 2001, Martin & Clark 2007). In a fundamental work by Richard Morris (Morris *et al*. 1982), e.g., rats with hippocampi removed took longer to learn to reach an escape platform hidden in a pool, but not a visible platform, and also had more difficulty in distinguishing the exact previous escape location when the platform was removed from the pool, indicating the importance of the hippocampal integrity in navigating through space using only distal environmental cues. In our study, we used this Morris water maze test to investigate whether impairment in spatial memory was also seen in our model and if CAST overexpression could be beneficial. In the short-term group, we observed that, even though KA-treated mice seemed to take a bit longer to learn to reach the escape platform, by the end of the trials they performed similarly to the SAL-treated controls. Moreover, all animals spent a high percentage of time in the target quadrant when platform was removed, and there seemed to be no differences in the number of crossings over the exact previous platform location. However, the low number of animals used in the short-term group seemed to affect this cognitive test the most, and the increased variability especially during trials and platform crossings makes it difficult to take reliable conclusions from this group. Nonetheless, when assessing the results from the long-term group, we could clearly see that all animals were able to learn to

reach the platform during trials, and also spent a high percentage of time in the target quadrant when platform was removed. The number of crossings over the exact previous platform location, however, was impaired in KA-treated WT mice in comparison with SAL, which is not seen in mice overexpressing CAST. Interestingly, certain neurons in the hippocampus, the place cells, have the peculiarity of firing only when the animal enters a specific position in space, and different place cells respond to different locations of the same environment, allowing the brain to create a spatial map of that particular environment (O'Keefe & Dostrovsky 1971). Additionally, a study on the pattern of place-cell activity during a water maze test in rats has shown that the percentage of place cells firing maximally was more than doubled near the platform when compared to other locations in the pool (Hollup *et al.* 2001), indicating a great contribution of these cells to the correct mental positioning of the platform in this particular task. Our results may suggest a loss of place cells or correct place-cell activity in WT mice treated with KA, since they seemed to have only a general idea of the platform whereabouts, but failed to determine exactly where it was, as compared to SAL-treated mice. This notion is further corroborated by a study showing that seizure-induced hippocampal lesion can indeed affect the activity of place cells several weeks later, making them less precise and less stable (Liu *et al.* 2003). The existing place cells in mice overexpressing CAST seem therefore to be less affected by KA treatment, suggesting that calpain inhibition may also limit the seizure-induced damage of these cells.

Finally, the fear conditioning test was the last to be performed, since it was the most stressful for the animals, due to the footshock they received during learning. This test is based on the association of an aversive stimulus with a tone and with the context where it occurred, which is measured by the time the animal spends freezing, i.e., when the only observed movement is from breathing, a known indicative of fear in rodents (Blanchard & Blanchard 1969, Wehner & Radcliffe 2004). As with the other performed cognitive tests, the fear conditioning test also has the involvement of the hippocampus, but only in relation to context, being the response to the cued stimulus dependent on the integrity of the amygdala (Phillips & LeDoux 1992), reiterating the relationship of the hippocampus with spatial features. For that reason, during learning, we were expecting all mice to associate the cued tone with the aversive stimulus, with progressively more time spent freezing with further tone presentations. Indeed, by the last time the tone was played, all animals spent around half of the time freezing, in contrast with virtually no freezing that was observed when they were first placed in the chamber. On the following day, the animals were placed in the exact same context,

without presentation of tone or footshock, and SAL-treated animals presented associative memory to this context, by presenting freezing behavior for most of the time spent inside the chamber, as expected. However, KA-treatment failed to impair the time spent freezing, which may be linked with the relative resistance of the used strain to KA-induced cell death (McKhann *et al.* 2003). Even though differences in associative memory to the context where the mice received the aversive stimulus might have been too subtle for detection, freezing to the modified context was significantly increased in KA-treated mice overexpressing CAST from the short-term recovery group. Unlike all other mice, that seemed to understand the context was different, these mice seemed to assimilate that they were in a similar situation as before and missed the change of context in the experiment cage, suggesting hippocampal function impairment. One interesting feature of the aforementioned place cells is that they can be involved in the mechanisms of pattern separation, the ability to discriminate between very similar memories, and pattern completion, the ability to recall a previous memory in full based only on some of the components that composed that memory (Sahay *et al.* 2011). If these mechanisms are not balanced, there is an alteration in the perception of the environment. For instance, if pattern completion is relatively enhanced, there is generalization of the current environment, which means that several contexts may be linked together, even if they present reduced similarities (Sahay *et al.* 2011). This imbalance may thus be what caused KA-treated hCAST mice from the short-term group to present higher freezing in the modified context, since the small similarities between both contexts seemed to be enough for them to recall the aversive stimulus. A possible explanation for this may relate to the findings that young granule cells (immature neurons) seem to be involved with pattern separation and older granule cells with pattern completion (Nakashiba *et al.* 2012). Given the neuroprotection provided by calpain inhibition, less neurons are expected to be lost after KA-treatment in mice overexpressing CAST. Assuming that old neurons are more resistant, relatively more younger ones would die (Spalding *et al.* 2013). We show previously in this chapter that KA enhances the early stages of neurogenesis, so an increased number of younger granule cells is expected to exist in the hippocampus of mice from the short-term group. At first, the new cells do not appear to be enough, or functional enough, to compensate for the relative higher number of older cells, though later on they seem to regain balance, since the differential behavioral response to the new context of hCAST mice treated with KA from the short-term recovery group is lost in the long-term group. On the other hand, assuming that without neuroprotection younger and older neurons are lost more or less equally in KA-treated WT mice, and since the balance between younger and older granule cells seems to be maintained, by observation of memory

association to the new context, we suggest that the new young neurons may not be fully functional. Electrophysiology studies would help to evaluate if this is in fact the case. Lastly, like with learning, we were expecting, and observed, associative memory to the cue tone to be maintained in all animals, since the hippocampus does not seem to be involved in this process.

These results seem to corroborate the neuroprotective effect of calpain inhibition, though the fact that CAST was already overexpressed prior to lesion may have masked potential benefits of calpain inhibition on post-injury neurogenesis, since the injury on CAST mice may have been smaller. Moreover, the fact that the absence of CAST and, consequently, increased calpain activity, can impair early hippocampal neurogenesis, does not necessarily mean that reduced or lack of calpain activity can enhance it, which is addressed in the next chapter of this thesis.

Chapter 4

Effects of calpain depletion on early neurogenesis in the adult hippocampus

4.1. Summary

In the brain, calpains have mostly been related with neuronal damage, but the information regarding how they may affect neurogenesis is limited. Absence of the endogenous inhibitor of calpains, calpastatin, has been shown to impair NSC proliferation and neuroblast migration in the hippocampus of adult mice. In this work, we wanted to evaluate whether decreased calpain activity could enhance these early stages of hippocampal neurogenesis. For that purpose, we used mice lacking one or both of the most abundant calpain isoforms, calpain 1 and calpain 2, and evaluated the effects on this process. We thus used mice lacking calpain 1, conditional neural knockout mice for calpain 2 and double-knockout mice for both calpain 1 and calpain 2, resulting from crossing the previous two animals, and evaluated neural stem cell proliferation and neurobast migration in the dentate gyrus of the hippocampus of adult mice. We observed that the lack of calpains did not alter early stages of hippocampal neurogenesis, which, taken together with our previous reports, suggest that calpains only seem to be involved in hippocampal neurogenesis when their activity is over a certain threshold, which is usually prevented by the inhibitory effect of calpastatin.

4.2. Introduction

Neurogenesis is now currently accepted to occur in the adult mammalian brain, in the walls of the lateral ventricles, as well as in the hippocampus (Bond *et al.* 2015). Several factors can affect adult neurogenesis, including brain pathology. Interestingly, the brain seems to have an endogenous attempt to repair itself. Early stages of neurogenesis can be increased after lesion, though the efficiency of the process is limited by impaired cell migration or decreased survival of new neurons (Kaneko & Sawamoto 2009, Ma *et al.* 2009). Changes in the environment after a pathological event may partially affect the efficiency of adult neurogenesis, and understanding how these changes affect the process could contribute to a better outcome for brain repair.

One of the changes observed in a pathological environment is the increased activity of some proteases, including calpains (Neumar *et al*. 2001). Calpains are known to cleave a plethora of substrates and therefore act on several cellular processes, including cell proliferation and migration (Goll *et al.* 2003). Absence of the endogenous inhibitor of calpains, calpastatin (CAST), impairs early stages of hippocampal neurogenesis, involving increased calpain activity in the process (Machado *et al*. 2015).

We therefore proposed to evaluate whether the lack of one or both of the most abundant calpain isoforms, calpain 1 and calpain 2, could increase early stages of hippocampal neurogenesis. We thus used mice lacking calpain 1 (*Capn1* KO), conditional neural knockout mice for calpain 2 (*Capn2* cKO) and double-knockout mice for both calpain 1 and calpain 2, resulting from crossing the previous two animals (*Capn1&2* cDKO), and evaluated neural stem cell (NSC) proliferation and neuroblast migration in the dentate gyrus (DG) of the hippocampus of adult mice. Conditional knockout mice must be used for calpain 2, since conventional knockout mice for this protease are embryonically lethal (Dutt *et al.* 2006, Takano *et al.* 2011).

4.3. Results

4.3.1. Calpain depletion maintains early neurogenesis in the adult hippocampus

To study the effects of calpain depletion on early neurogenesis, the presence of *Cre*, *Capn1* and loxp sites for *Capn2* in the mice DNA were verified by genotyping (Fig. 4.1A). Presence of CAPN2 was checked in hippocampal lysates (Fig. 4.1B) and, since we were not able to acquire a reliable antibody to verify CAPN1 expression by Western blot or immunohistochemistry, we trusted the validation work published on these animals (Azam et al. 2001, Takano et al. 2011).

In order to evaluate NSC proliferation in the subgranular zone (SGZ) of the DG, mice were given 5-ethynyl-2'-deoxyuridine (EdU) on the day before sacrifice. We observed that depletion of calpains, as well as the presence of Cre, used as controls of Cre expression (Cre Ctrl), maintained NSC proliferation, by comparison with wild-type (WT, 17.7 ± 1.9 cells/section, $p>0.05$), as assessed by the number of cells that incorporated EdU in the SGZ (Fig. 4.2).

Furthermore, mice were also given 5-bromo-2'-deoxyuridine (BrdU) one week prior to sacrifice, and the evaluation of cell migration in the DG was performed by three different approaches: distribution of newborn cells, overall neuroblast migration and distance of migration of newborn cells into the granular zone (GZ). The distribution of newborn cells in the DG, as assessed by BrdU incorporation, seemed to be preserved in the DG of mice lacking calpains, as well as in the presence of Cre, when compared to WT mice (SGZ: 47.0 ± 3.5 cells/section, GZ: 7.9 ± 0.7 cells/section, $p>0.05$) (Fig. 4.3).

Overall neuroblast migration was assessed by doublecortin (DCX) labeling, and was also shown to be maintained when calpains are depleted, as well as in the presence of Cre, by comparison with WT (3.4 ± 0.2%, $p>0.05$) (Fig. 4.4).

Similarly to what was seen with the other approaches used to study cell migration in the DG of mice lacking calpains, as well as in the presence of Cre, the distance of migration of newborn cells into the GZ was also maintained, when compared to WT (18.2 ± 1.3 µm, $p>0.05$) (Fig. 4.5).

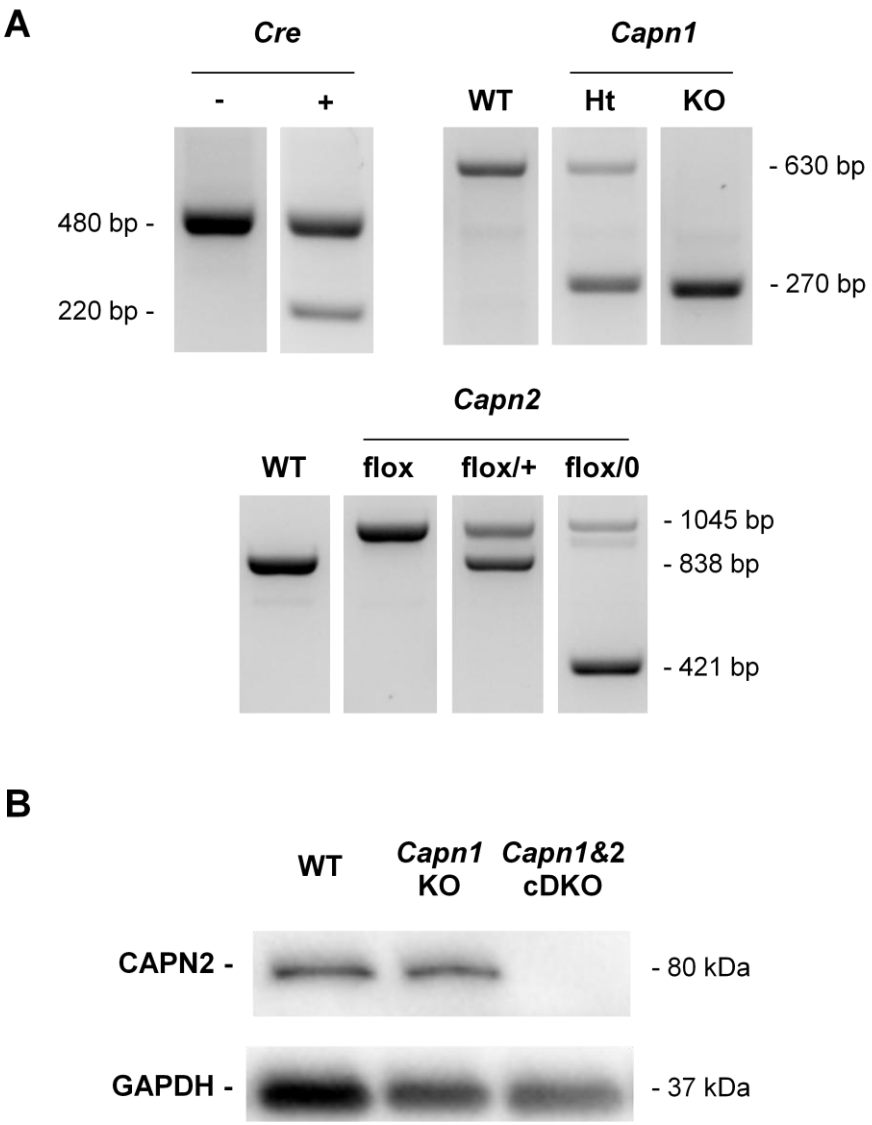

Figure 4.1 - Genotyping profiles and detection of CAPN2. Genotyping profiles for Cre, *Capn1* and *Capn2* (**A**). Western blot analysis of hippocampal lysates to detect presence of CAPN2. GAPDH was used as a loading control (**B**).

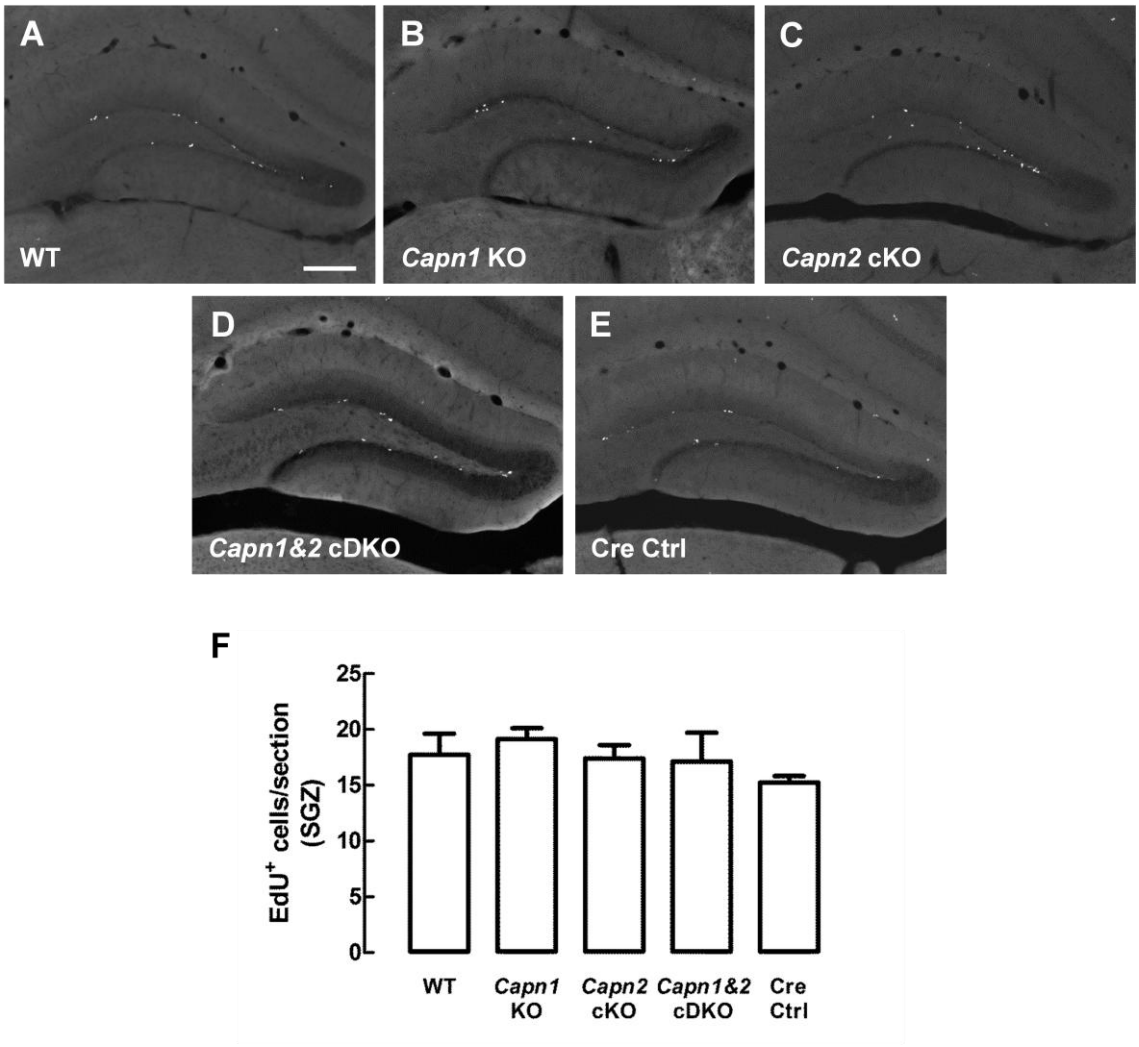

Figure 4.2 - Absence of calpains maintains NSC proliferation in the DG. Mice were given EdU on the day before sacrifice, to assess cell proliferation. Representative images from hippocampal brain sections of WT (**A**), *Capn1* KO (**B**), *Capn2* cKO (**C**), *Capn1&2* cDKO (**D**) and Cre Ctrl (**E**) mice, showing EdU-positive cells in white. Scale bar: 200 µm. EdU-positive cells were counted in the SGZ of 5 mid hippocampal sections for each animal (**F**). Means ± SEM of 6-9 independent experiments were analyzed by a one-way ANOVA, $p>0.05$.

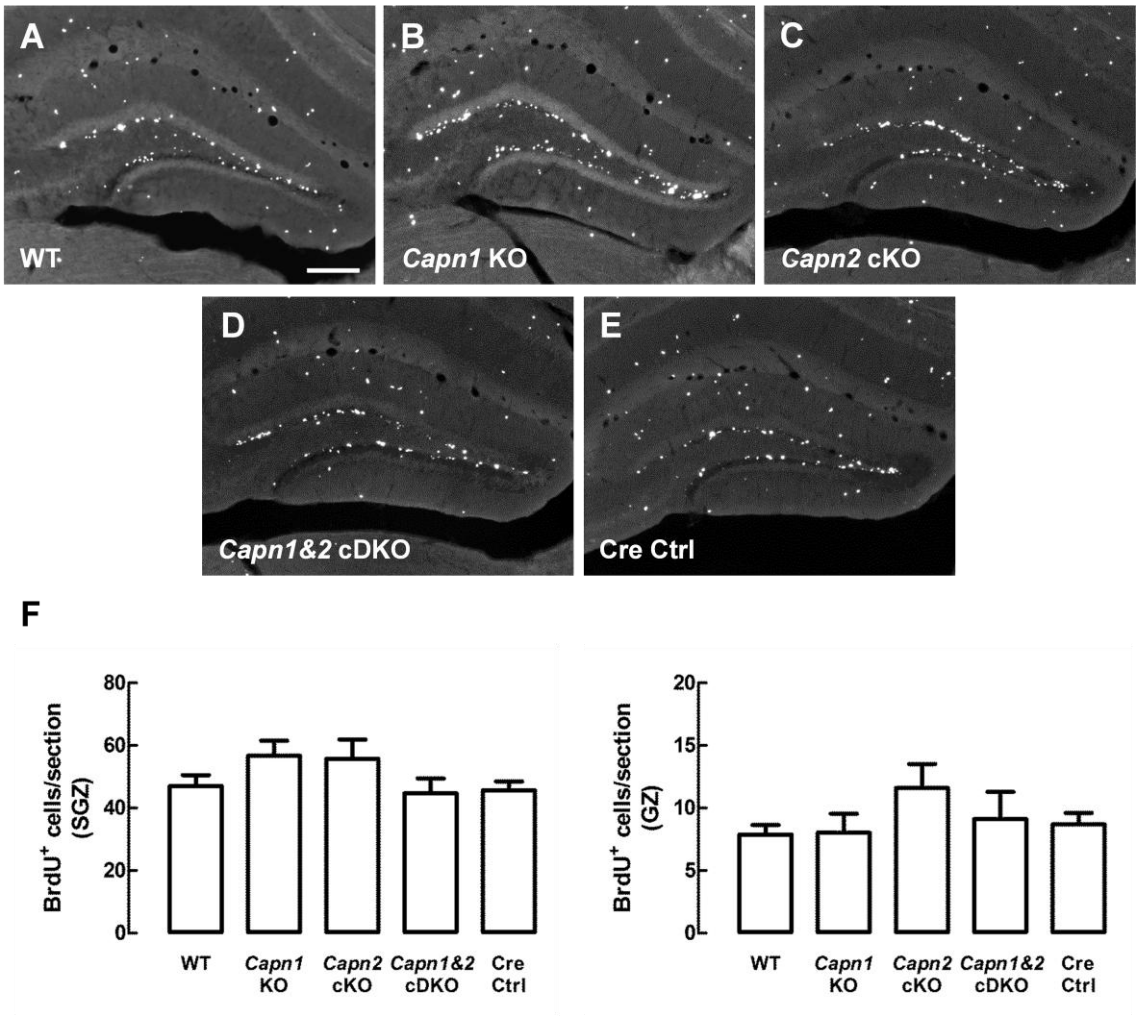

Figure 4.3 - Lack of calpains preserves distribution of BrdU-positive cells in the DG. Mice were given BrdU one week before sacrifice, to assess cell migration. Representative images from hippocampal brain sections of WT (**A**), *Capn1* KO (**B**), *Capn2* cKO (**C**), *Capn1&2* cDKO (**D**) and Cre Ctrl (**E**) mice, showing BrdU-positive cells in white. Scale bar: 200 µm. BrdU-positive cells were counted in the SGZ and GZ of 5 mid hippocampal sections for each animal (**F**). Means ± SEM of 5-7 independent experiments were analyzed by a one-way ANOVA, $p>0.05$.

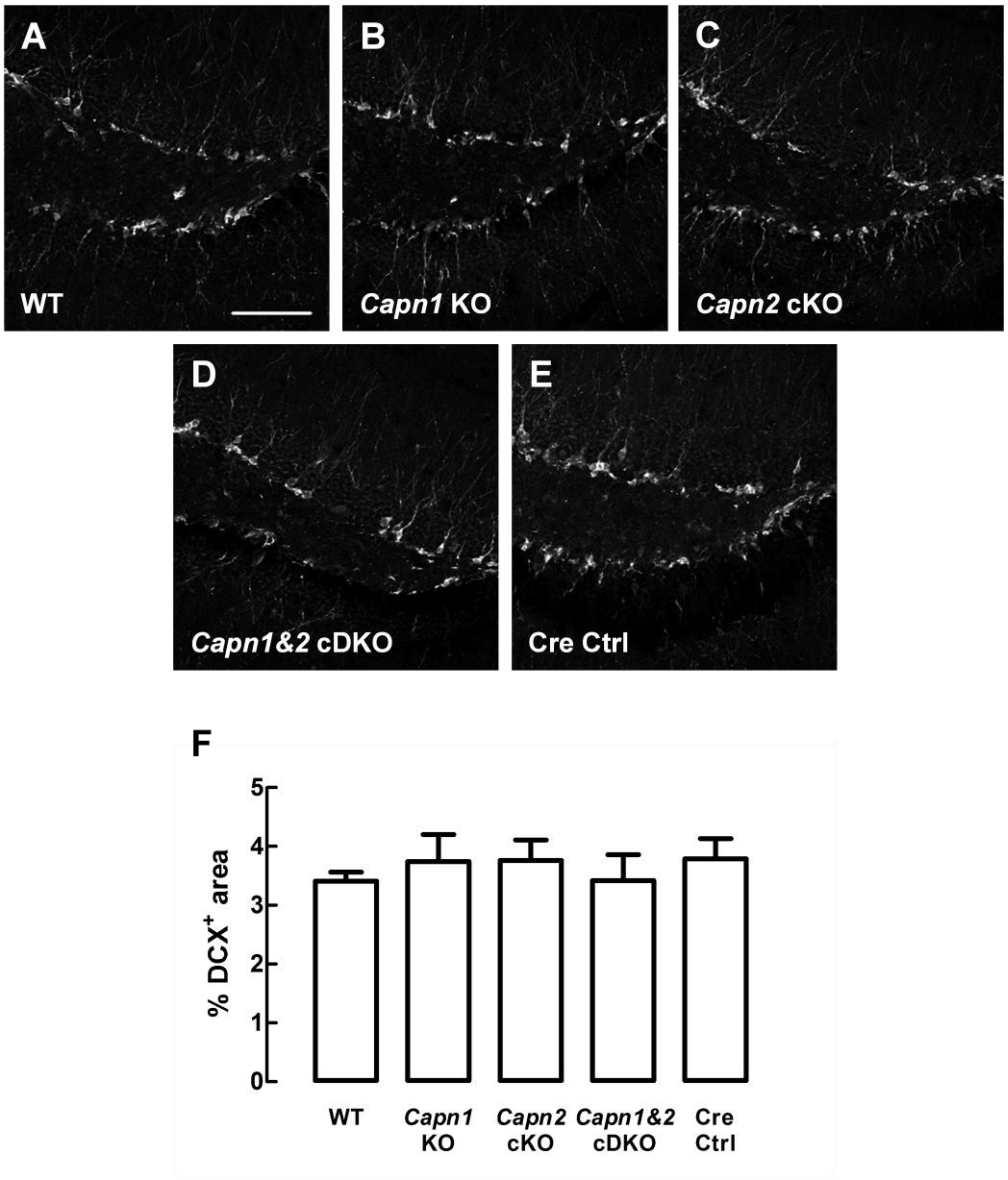

Figure 4.4 - Overall neuroblast migration in the DG is preserved when calpains are depleted. DCX immunoreactivity was used to assess cell migration. Representative images from hippocampal brain sections of WT (**A**), *Capn1* KO (**B**), *Capn2* cKO (**C**), *Capn1&2* cDKO (**D**) and Cre Ctrl (**E**) mice, showing migrating neuroblasts, labeled for DCX, in white. Scale bar: 100 µm. Percentage of DCX-positive area was determined in the DG of 5 mid hippocampal sections for each animal (**F**). Means ± SEM of 6-9 independent experiments were analyzed by a one-way ANOVA, $p>0.05$.

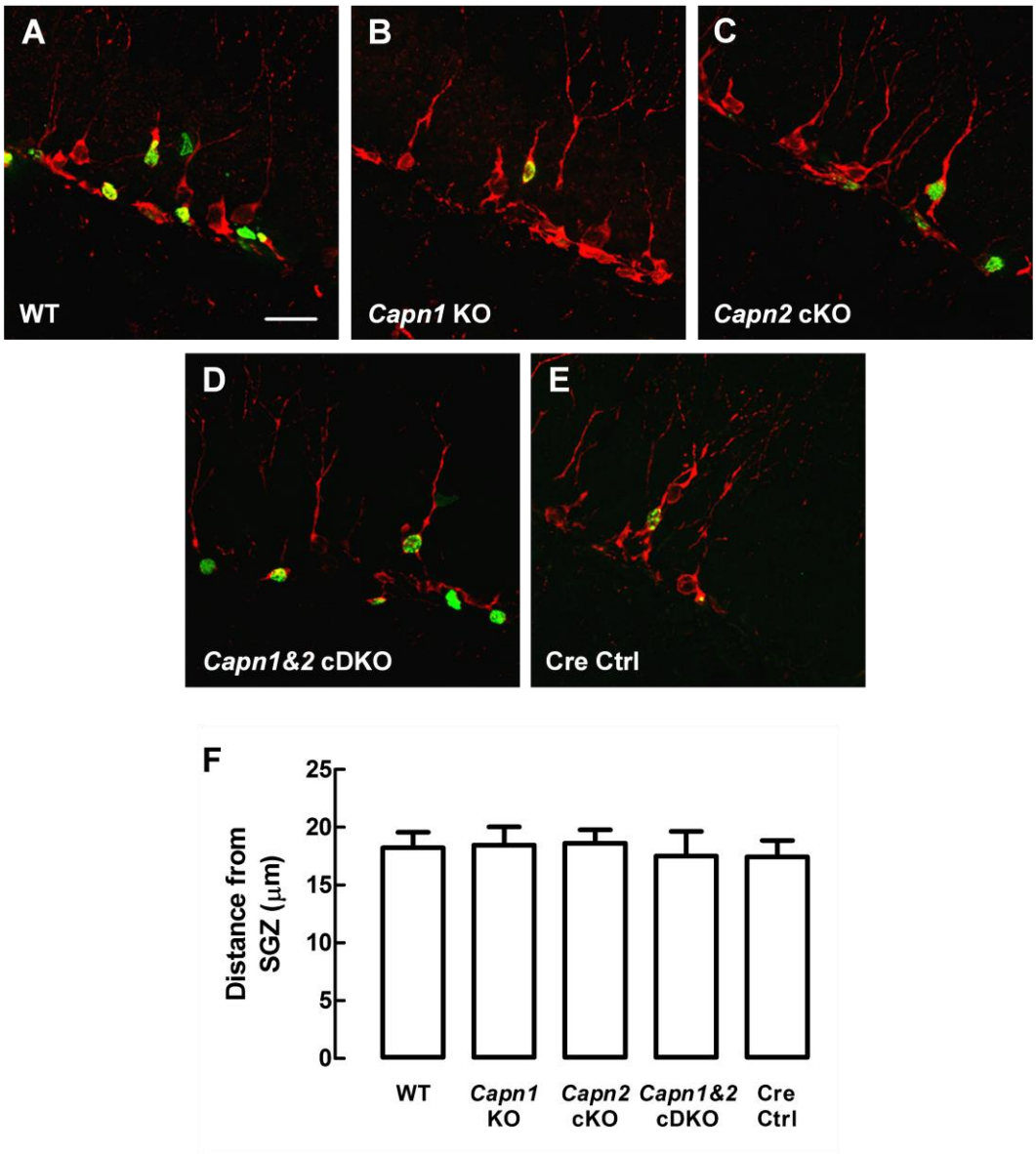

Figure 4.5 - Calpain deficiency maintains the migration distance of newborn cells into the GZ of the DG. Mice were given BrdU one week before sacrifice, to assess cell migration. Representative images from hippocampal brain sections of WT (**A**), *Capn1* KO (**B**), *Capn2* cKO (**C**), *Capn1&2* cDKO (**D**) and Cre Ctrl (**E**) mice, showing BrdU-positive cells in green and migrating neuroblasts, labeled for DCX, in red. Scale bar: 20 μm. Distance migrated from the SGZ into the GZ by BrdU-positive/DCX-positive cells were measured in a total of at least 33 cells per group, from 5 images acquired for each animal (**F**). Means ± SEM of 5-7 independent experiments were analyzed by a one-way ANOVA, $p>0.05$.

4.4. Discussion

Calpains are key players in the neuronal damage that occurs after a brain lesion, which makes it not surprising that inhibition of these proteases is shown to be neuroprotective in a variety of models of neurodegenerative diseases and other types of brain damage (Saez et al. 2006). On the other hand, brain damage has also been associated with an enhancement of endogenous neurogenesis, the process that culminates in the birth of new neurons. Following a lesion, NSCs present in neurogenic niches increase proliferation, and migrating neuroblasts are then re-routed into the affected regions, where new neurons differentiate, but very few end up successfully replacing the lost neurons (Kaneko & Sawamoto 2009, Ma et al. 2009). Since calpains can cleave a plethora of substrates, it is also not surprising that they can affect several cellular processes, including cell proliferation, migration and proliferation (Kashiwagi et al. 2010, Lokuta et al. 2003, Paulhe et al. 2001, Qiu et al. 2006, Raimbourg et al. 2013, Yajima & Kawashima 2002), which are key stages in the process of neurogenesis. However, the involvement of calpains in these processes in the brain remains elusive, but could potentially shed new light into how endogenous neurogenesis can be modulated. We have previously seen in our group that increased calpain activity can indeed affect adult neurogenesis, since the lack of CAST, the endogenous calpain inhibitor, impaired both NSC proliferation and neuroblast migration in mice (Machado *et al.* 2015). Nevertheless, in the event of a brain lesion, successfully enhancing neurogenesis would be more relevant. Inhibition of calpains could thus potentially help with neuroregeneration, in addition to being neuroprotective. As seen in the previous chapter, however, overexpressing CAST seemed to more or less maintain the neurogenesis pattern observed after kainic acid (KA)-induced hippocampal lesion, but its influence on post-injury neurogenesis may have been masked by the neuroprotective effect of calpain inhibition. Thus, we could not clearly conclude whether calpain inhibition had the potential to enhance endogenous neurogenesis. Since CAST can be modulated by other factors *in vivo*, we decided to use knockout mice for one or both of the most abundant calpain isoforms in the brain, calpain 1 and calpain 2 (Ono & Sorimachi 2012), in order to evaluate the possible effects on early hippocampal neurogenesis. Mice lacking calpain 1 are viable (Azam *et al.* 2001), but mice lacking calpain 2 or the small regulatory subunit, associated with both isoforms, present embryonic lethality (Dutt et al. 2006, Takano et al. 2011). However, the establishment of conditional knockout mice for calpain 2 prevented the embryonic lethality (Takano *et al.* 2011) and conditional knockout mice in which calpain 2 is absent mostly in cells

from the central and peripheral nervous system interested us for the study on hippocampal neurogenesis. We therefore evaluated NSC proliferation and neuroblast migration in the hippocampus of conventional knockout mice for calpain 1 (CAPN1 KO), conditional knockout mice for calpain 2 (CAPN2 cKO) and knockout mice for both calpains 1 and 2 (CAPN1&2 cDKO), in comparison with WT mice. Mice expressing both calpain 1 and calpain 2 normally, but that also expressed Cre recombinase in the same conditions as CAPN2 cKO and CAPN1&2 cDKO were used as controls (Cre Ctrl mice), to exclude any influence of Cre itself on the evaluated parameters.

Considering that the absence of CAST impairs the early stages of hippocampal neurogenesis (Machado *et al.* 2015), we were expecting an enhancement of this process with the absence of calpains. For the assessment of NSC proliferation, the thymidine analogue EdU was administered to the mice, which were sacrificed on the following day. Cells that incorporated EdU would therefore be the ones in the proliferative stage. Contrary to what we were expecting, cell proliferation in the SGZ of the hippocampus was maintained in all animals. Nonetheless, calpains can also affect cell migration, and some of the studies on the subject have shown that reduced calpain activity can enhance the migration ability of certain cell types (Lokuta *et al.* 2003, Kuchay *et al.* 2012, Noma *et al.* 2009, Katsube *et al.* 2008, Raimbourg *et al.* 2013). In our study, we evaluated the effects of the lack of calpains on neuroblast migration in the mouse hippocampus, using three different approaches, as previously reported (Machado *et al*. 2015). Another thymidine analogue, BrdU, was administered to the mice one week prior to sacrifice, to give cells that incorporated BrdU time to migrate. The first approach used was BrdU distribution in the DG. NSCs from the DG proliferate in the SGZ and then migrate as neuroblasts into the GZ, where they later differentiate into new granule neurons (Aimone *et al.* 2014). If neuroblast migration is affected, differences in the migration of newborn cells into the GZ should be observed. However, distribution of BrdU cells seemed to be preserved in the DG of all mice. This was further corroborated by the other two approaches used, consisting in the evaluation of the overall area of migrating neuroblasts and distance of migration of newborn cells into the GZ, which also seemed to be preserved in all animals. Moreover, the observed results with CAPN2 cKO and CAPN1&2 cDKO mice were not influenced by the presence of Cre, since Cre Ctrl mice presented results similar to WT. The maintenance of NSC proliferation and neuroblast migration in mice lacking either calpain 1 or calpain 2 could have been due to a possible mutual compensatory effect of these isoforms. However, mice lacking both calpains presented similar results, discarding that hypothesis. Since increased calpain activity impairs these early stages of neurogenesis

(Machado *et al.* 2015), another explanation can be that calpains only influence physiologic hippocampal neurogenesis when their activity rises over a certain threshold, and not when the activity is diminished. However, calpain inhibition is able to enhance cell proliferation and migration *in vitro*, in cells derived from the subventricular zone (Machado *et al.* 2015), which means that calpain inhibition may still hold promise in enhancing subventricular zone neurogenesis.

Chapter 5

General Discussion and Conclusion

With the present work, we aimed at further investigating how modulation of calpain activity could affect the process of neurogenesis. Calpain inhibition is known to be neuroprotective and calpains also play a role in the processes of cell proliferation, migration and differentiation, which are key stages on the formation of new neurons. However, very little is known about the effects of calpains on neural stem cells (NSC) and the overall process of neurogenesis. Finding a way to modulate calpain activity in order to help improve the outcome of post-injury neurogenesis, the endogenous attempt to restore neurons lost as a consequence of brain damage, would highly benefit patients with neurodegenerative diseases or other types of brain insults.

We have previously seen that increased calpain activity impaired, *in vivo*, NSC proliferation in one of the neurogenic niches of the adult brain, the dentate gyrus (DG) of the hippocampus, and also neuroblast migration in the DG and the rostral migratory stream, which originates from another highly neurogenic niche in the brain, the subventricular zone (SVZ). In this work, we show that increased calpain activity can also impair NSC proliferation *in vitro*, in cultures derived from the SVZ of post-natal mice, and that this impairment is rescued by calpain inhibition. Moreover, calpain inhibition was also able to increase wild-type (WT) NSC proliferation and, in previous results by our group, cell migration in explants from the SVZ. Calpain inhibition seemed thus a promising approach for the enhancement of neurogenesis after brain lesion.

We also investigated the effects of calpain inhibition on post-injury neurogenesis. We used mice overexpressing calpastatin (CAST), the endogenous inhibitor of calpains, and the kainic acid model of seizure-induced hippocampal lesion, currently in use by our lab. We show that the high increase in NSC proliferation in the DG after lesion is maintained with CAST overexpression, probably due to a threshold event, in which a small lesion is able to trigger maximally the increase in NSC proliferation. However, even though neuroblast migration in the DG also increased considerably after lesion with CAST overexpression, this increase seemed to be more attenuated in these mice. We propose that, since calpain inhibition can be neuroprotective and CAST was already overexpressed in these animals before lesion, the extension of the damage was probably reduced, so the recruitment of fewer cells was needed to repair the damage. Following migration, we assessed cell differentiation in the DG, but suppression of calpain activity by overexpression of CAST did not seem to be enough to compensate for the increase of ectopic newborn cells and for the impairment in neuronal differentiation that occur after lesion. Though we cannot exclude the possibility that calpains might not be involved in neuronal differentiation in the adult brain, or at least in the hippocampus, we think that a better outcome may have been

prevented by the pathologic conditions in the environment. This pathologic environment must thus not be overlooked when developing new strategies for brain repair.

Although CAST overexpression was not able to increase the percentage of new neurons in the DG after lesion, they could still be more functional and better integrated in the neuronal circuits. Since the hippocampus is highly involved in learning and memory, we decided to investigate whether the cognitive behavior could be improved in these mice. We chose two time-points to evaluate cognitive recovery: on the 3rd week after treatment, when the newborn neurons are still immature (short-term group) and on the 8th week after treatment, when the newborn neurons are already mature and integrated in the existing networks. We found that recognition memory was only impaired in WT mice from the short-term recovery group, and not with CAST overexpression. This is another indicator of neuroprotection by calpain inhibition, since newborn neurons are still not fully mature at this stage to have been able to reverse the damage. Moreover, regarding spatial memory, calpain inhibition also seemed to either prevent damage or to have successfully allowed for the correct replacement of place cells in the hippocampus, since mice overexpressing CAST from the long-term group did not present increased difficulty in remembering an exact spatial location, in contrast to lesioned WT mice. Lastly, associative memory to a modified context after fear conditioning to an aversive stimulus seemed to only be affected in the short-term group, with overexpression of CAST. These mice apparently understood better that they were in a similar situation as when they received a footshock, even though we tried to change the context as much as possible. This led us to propose that an imbalance in the proportion of immature and mature neurons, due to possible increased neuroprotection of more mature neurons, could have enhanced the process of pattern completion in these mice, inducing the association of both contexts by the few similarities between the two. All of the cognitive results therefore corroborate the neuroprotection granted by calpain inhibiton, though it would be important to confidently be able to determine whether reduced calpain activity could in fact positively affect neurogenesis.

Having this in mind, for our last set of experiments, we decided to use mice lacking calpains, in order to assess whether absence of activity of one or both of the calpain isoforms most abundant in the brain would be able to enhance the early stages of hippocampal neurogenesis. However, neither NSC proliferation nor neuroblast migration seemed to be affected by impairment of calpain activity. Considering that increased calpain activity impairs these early stages of hippocampal neurogenesis, we propose that calpains only affect this process when their activity is over a certain

threshold, under which neurogenesis is not affected. Taken together, the results in this work clearly involve calpains in the early stages of neurogenesis.

In conclusion, even though reduced calpain activity does not seem to be able to enhance hippocampal neurogenesis, it could potentially be able to prevent the impairment in the formation of new neurons after injury, given that the levels of calpain activity could be reduced under a certain threshold. Strategies to reduce the harmful effects of the pathologic environment, such as controling neuroinflammation, for example, could also help boost the outcome of calpain inhibition in the survival of new neurons. Moreover, these observations are in regard to hippocampal neurogenesis. Cells derived from the SVZ seem to hold more promise to present enhanced proliferation and migration with calpain inhibition, encouraging further research involving these cells and calpain activity in the field of brain damage.

Chapter 6

References

Aimone, J. B., Li, Y., Lee, S. W., Clemenson, G. D., Deng, W. and Gage, F. H. (2014) Regulation and function of adult neurogenesis: from genes to cognition. *Physiol Rev,* **94,** 991-1026.

Altman, J. and Das, G. D. (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol,* **124,** 319-335.

Araujo, I. M., Carreira, B. P., Carvalho, C. M. and Carvalho, A. P. (2010) Calpains and delayed calcium deregulation in excitotoxicity. *Neurochem Res,* **35,** 1966-1969.

Araujo, I. M., Gil, J. M., Carreira, B. P., Mohapel, P., Petersen, A., Pinheiro, P. S., Soulet, D., Bahr, B. A., Brundin, P. and Carvalho, C. M. (2008) Calpain activation is involved in early caspase-independent neurodegeneration in the hippocampus following status epilepticus. *J Neurochem,* **105,** 666-676.

Ariyoshi, H., Okahara, K., Sakon, M., Kambayashi, J., Kawashima, S., Kawasaki, T. and Monden, M. (1998) Possible involvement of m-calpain in vascular smooth muscle cell proliferation. *Arterioscler Thromb Vasc Biol,* **18,** 493-498.

Arthur, J. S. and Crawford, C. (1996) Investigation of the interaction of m-calpain with phospholipids: calpain-phospholipid interactions. *Biochim Biophys Acta,* **1293,** 201-206.

Arthur, J. S., Elce, J. S., Hegadorn, C., Williams, K. and Greer, P. A. (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,** 4474-4481.

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z. and Lindvall, O. (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med,* **8,** 963-970.

Azam, M., Andrabi, S. S., Sahr, K. E., Kamath, L., Kuliopulos, A. and Chishti, A. H. (2001) Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol,* **21,** 2213-2220.

Baki, A., Tompa, P., Alexa, A., Molnar, O. and Friedrich, P. (1996) Autolysis parallels activation of mu-calpain. *Biochem J,* **318 (Pt 3),** 897-901.

Barkus, C., McHugh, S. B., Sprengel, R., Seeburg, P. H., Rawlins, J. N. and Bannerman, D. M. (2010) Hippocampal NMDA receptors and anxiety: at the interface between cognition and emotion. *Eur J Pharmacol,* **626,** 49-56.

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

Bedard, A., Cossette, M., Levesque, M. and Parent, A. (2002) Proliferating cells can differentiate into neurons in the striatum of normal adult monkey. *Neurosci Lett,* **328,** 213-216.

Berg, D. A., Belnoue, L., Song, H. and Simon, A. (2013) Neurotransmitter-mediated control of neurogenesis in the adult vertebrate brain. *Development,* **140,** 2548-2561.

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

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

Best, P. J., White, A. M. and Minai, A. (2001) Spatial processing in the brain: the activity of hippocampal place cells. *Annu Rev Neurosci,* **24,** 459-486.

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

Biebl, M., Cooper, C. M., Winkler, J. and Kuhn, H. G. (2000) Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. *Neurosci Lett,* **291,** 17-20.

Blanchard, R. J. and Blanchard, D. C. (1969) Crouching as an index of fear. *J Comp Physiol Psychol,* **67,** 370-375.

Blumcke, I., Schewe, J. C., Normann, S., Brustle, O., Schramm, J., Elger, C. E. and Wiestler, O. D. (2001) Increase of nestin-immunoreactive neural precursor cells in the dentate gyrus of pediatric patients with early-onset temporal lobe epilepsy. *Hippocampus,* **11,** 311-321.

Bond, A. M., Ming, G. L. and Song, H. (2015) Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. *Cell Stem Cell,* **17,** 385-395.

Bondolfi, L., Ermini, F., Long, J. M., Ingram, D. K. and Jucker, M. (2004) Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice. *Neurobiol Aging,* **25,** 333-340.

Cameron, M. C., Zhan, R. Z. and Nadler, J. V. (2011) Morphologic integration of hilar ectopic granule cells into dentate gyrus circuitry in the pilocarpine model of temporal lobe epilepsy. *J Comp Neurol,* **519,** 2175-2192.

Carreira, B. P., Morte, M. I., Inacio, A., Costa, G., Rosmaninho-Salgado, J., Agasse, F., Carmo, A., Couceiro, P., Brundin, P., Ambrosio, A. F., Carvalho, C. M. and Araujo, I. M. (2010) Nitric oxide stimulates the proliferation of neural stem cells bypassing the epidermal growth factor receptor. *Stem Cells,* **28,** 1219-1230.

Carreira, B. P., Santos, D. F., Santos, A. I., Carvalho, C. M. and Araujo, I. M. (2015) Nitric Oxide Regulates Neurogenesis in the Hippocampus following Seizures. *Oxid Med Cell Longev,* **2015,** 451512.

Carta, M., Fievre, S., Gorlewicz, A. and Mulle, C. (2014) Kainate receptors in the hippocampus. *Eur J Neurosci,* **39,** 1835-1844.

Chakraborti, S., Alam, M. N., Paik, D., Shaikh, S. and Chakraborti, T. (2012) Implications of calpains in health and diseases. *Indian J Biochem Biophys,* **49,** 316-328.

Chehrehasa, F., Meedeniya, A. C., Dwyer, P., Abrahamsen, G. and Mackay-Sim, A. (2009) EdU, a new thymidine analogue for labelling proliferating cells in the nervous system. *J Neurosci Methods,* **177,** 122-130.

Chirumamilla, S., Sun, D., Bullock, M. R. and Colello, R. J. (2002) Traumatic brain injury induced cell proliferation in the adult mammalian central nervous system. *J Neurotrauma,* **19,** 693-703.

Cohen, S. J. and Stackman, R. W., Jr. (2015) Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behav Brain Res,* **285,** 105-117.

Cong, J., Goll, D. E., Peterson, A. M. and Kapprell, H. P. (1989) The role of autolysis in activity of the Ca2+-dependent proteinases (mu-calpain and m-calpain). *J Biol Chem,* **264,** 10096-10103.

Cools, R., Ivry, R. B. and D'Esposito, M. (2006) The human striatum is necessary for responding to changes in stimulus relevance. *J Cogn Neurosci,* **18,** 1973-1983.

Crespel, A., Rigau, V., Coubes, P., Rousset, M. C., de Bock, F., Okano, H., Baldy-Moulinier, M., Bockaert, J. and Lerner-Natoli, M. (2005) Increased number of neural progenitors in human temporal lobe epilepsy. *Neurobiol Dis,* **19,** 436-450.

Croce, K., Flaumenhaft, R., Rivers, M., Furie, B., Furie, B. C., Herman, I. M. and Potter, D. A. (1999) Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J Biol Chem,* **274,** 36321-36327.

Curtis, M. A., Kam, M., Nannmark, U., Anderson, M. F., Axell, M. Z., Wikkelso, C., Holtas, S., van Roon-Mom, W. M., Bjork-Eriksson, T., Nordborg, C., Frisen, J., Dragunow, M., Faull, R. L. and Eriksson, P. S. (2007) Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science,* **315,** 1243-1249.

Curtis, M. A., Penney, E. B., Pearson, A. G., van Roon-Mom, W. M., Butterworth, N. J., Dragunow, M., Connor, B. and Faull, R. L. (2003) Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain. *Proc Natl Acad Sci U S A,* **100,** 9023-9027.

Dash, P. K., Mach, S. A. and Moore, A. N. (2001) Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. *J Neurosci Res,* **63,** 313-319.

Dashtipour, K., Tran, P. H., Okazaki, M. M., Nadler, J. V. and Ribak, C. E. (2001) Ultrastructural features and synaptic connections of hilar ectopic granule cells in the rat dentate gyrus are different from those of granule cells in the granule cell layer. *Brain Res,* **890,** 261-271.

Deng, W., Aimone, J. B. and Gage, F. H. (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat Rev Neurosci,* **11,** 339-350.

Dobrossy, M. D., Drapeau, E., Aurousseau, C., Le Moal, M., Piazza, P. V. and Abrous, D. N. (2003) Differential effects of learning on neurogenesis: learning increases or

decreases the number of newly born cells depending on their birth date. *Mol Psychiatry,* **8,** 974-982.

Donkor, I. O. (2015) An updated patent review of calpain inhibitors (2012 - 2014). *Expert Opin Ther Pat,* **25,** 17-31.

Dupret, D., Revest, J. M., Koehl, M., Ichas, F., De Giorgi, F., Costet, P., Abrous, D. N. and Piazza, P. V. (2008) Spatial relational memory requires hippocampal adult neurogenesis. *PLoS One,* **3,** e1959.

Dutt, P., Croall, D. E., Arthur, J. S., Veyra, T. D., Williams, K., Elce, J. S. and Greer, P. A. (2006) m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev Biol,* **6,** 3.

Elce, J. S., Hegadorn, C. and Arthur, J. S. (1997) Autolysis, Ca2+ requirement, and heterodimer stability in m-calpain. *J Biol Chem,* **272,** 11268-11275.

Emery, D. L., Fulp, C. T., Saatman, K. E., Schutz, C., Neugebauer, E. and McIntosh, T. K. (2005) Newly born granule cells in the dentate gyrus rapidly extend axons into the hippocampal CA3 region following experimental brain injury. *J Neurotrauma,* **22,** 978-988.

Emori, Y., Kawasaki, H., Imajoh, S., Imahori, K. and Suzuki, K. (1987) Endogenous inhibitor for calcium-dependent cysteine protease contains four internal repeats that could be responsible for its multiple reactive sites. *Proc Natl Acad Sci U S A,* **84,** 3590-3594.

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. and Gage, F. H. (1998) Neurogenesis in the adult human hippocampus. *Nat Med,* **4,** 1313-1317.

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

Franco, S. J. and Huttenlocher, A. (2005) Regulating cell migration: calpains make the cut. *J Cell Sci,* **118,** 3829-3838.

Gao, X., Enikolopov, G. and Chen, J. (2009) Moderate traumatic brain injury promotes proliferation of quiescent neural progenitors in the adult hippocampus. *Exp Neurol,* **219,** 516-523.

Garcia, A. D., Doan, N. B., Imura, T., Bush, T. G. and Sofroniew, M. V. (2004) GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat Neurosci,* **7,** 1233-1241.

Ge, S., Sailor, K. A., Ming, G. L. and Song, H. (2008) Synaptic integration and plasticity of new neurons in the adult hippocampus. *J Physiol,* **586,** 3759-3765.

Gil, J. M., Mohapel, P., Araujo, I. M., Popovic, N., Li, J. Y., Brundin, P. and Petersen, A. (2005) Reduced hippocampal neurogenesis in R6/2 transgenic Huntington's disease mice. *Neurobiol Dis,* **20,** 744-751.

Glading, A., Chang, P., Lauffenburger, D. A. and Wells, A. (2000) Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J Biol Chem,* **275,** 2390-2398.

Goll, D. E., Thompson, V. F., Li, H., Wei, W. and Cong, J. (2003) The calpain system. *Physiol Rev,* **83,** 731-801.

Gould, E., Reeves, A. J., Graziano, M. S. and Gross, C. G. (1999) Neurogenesis in the neocortex of adult primates. *Science,* **286,** 548-552.

Gould, T. D., Dao, D. T. and Kovacsics, C. E. (2009) The Open Field Test. In: *Mood and Anxiety Related Phenotypes in Mice: Characterization Using Behavioral Tests*, (T. D. Gould ed.), pp. 1-20. Humana Press, New York.

Gray, W. P. and Sundstrom, L. E. (1998) Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. *Brain Res,* **790,** 52-59.

Gu, W. L., Fu, S. L., Wang, Y. X., Li, Y., Lu, H. Z., Xu, X. M. and Lu, P. H. (2009) Chondroitin sulfate proteoglycans regulate the growth, differentiation and migration of multipotent neural precursor cells through the integrin signaling pathway. *BMC Neurosci,* **10,** 128.

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

Hanna, R. A., Garcia-Diaz, B. E. and Davies, P. L. (2007) Calpastatin simultaneously binds four calpains with different kinetic constants. *FEBS Lett,* **581,** 2894-2898.

Hata, S., Doi, N., Kitamura, F. and Sorimachi, H. (2007) Stomach-specific calpain, nCL-2/calpain 8, is active without calpain regulatory subunit and oligomerizes through C2-like domains. *J Biol Chem,* **282,** 27847-27856.

Hattiangady, B. and Shetty, A. K. (2008) Implications of decreased hippocampal neurogenesis in chronic temporal lobe epilepsy. *Epilepsia,* **49 Suppl 5,** 26-41.

Hattiangady, B. and Shetty, A. K. (2010) Decreased neuronal differentiation of newly generated cells underlies reduced hippocampal neurogenesis in chronic temporal lobe epilepsy. *Hippocampus,* **20,** 97-112.

Heine, V. M., Maslam, S., Joels, M. and Lucassen, P. J. (2004) Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of an age-related hypothalamus-pituitary-adrenal axis activation. *Neurobiol Aging,* **25,** 361-375.

Hilton, G. (1994) Behavioral and cognitive sequelae of head trauma. *Orthop Nurs,* **13,** 25-32.

Hitomi, K., Yokoyama, A. and Maki, M. (1998) Expression of biologically active human calpastatin in baculovirus-infected insect cells and in Escherichia coli. *Biosci Biotechnol Biochem,* **62,** 136-141.

Hoglinger, G. U., Rizk, P., Muriel, M. P., Duyckaerts, C., Oertel, W. H., Caille, I. and Hirsch, E. C. (2004) Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nat Neurosci,* **7,** 726-735.

Hollup, S. A., Molden, S., Donnett, J. G., Moser, M. B. and Moser, E. I. (2001) Accumulation of hippocampal place fields at the goal location in an annular watermaze task. *J Neurosci,* **21,** 1635-1644.

Huttmann, K., Sadgrove, M., Wallraff, A., Hinterkeuser, S., Kirchhoff, F., Steinhauser, C. and Gray, W. P. (2003) Seizures preferentially stimulate proliferation of radial glia-like astrocytes in the adult dentate gyrus: functional and immunocytochemical analysis. *Eur J Neurosci,* **18,** 2769-2778.

Imajoh, S., Kawasaki, H. and Suzuki, K. (1986) Limited autolysis of calcium-activated neutral protease (CANP): reduction of the Ca2+-requirement is due to the NH2-terminal processing of the large subunit. *J Biochem,* **100,** 633-642.

Imajoh, S., Kawasaki, H. and Suzuki, K. (1987) The COOH-terminal E-F hand structure of calcium-activated neutral protease (CANP) is important for the association of subunits and resulting proteolytic activity. *J Biochem,* **101,** 447-452.

Imayoshi, I., Sakamoto, M., Ohtsuka, T., Takao, K., Miyakawa, T., Yamaguchi, M., Mori, K., Ikeda, T., Itohara, S. and Kageyama, R. (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nat Neurosci,* **11,** 1153-1161.

Jessberger, S., Clark, R. E., Broadbent, N. J., Clemenson, G. D., Jr., Consiglio, A., Lie, D. C., Squire, L. R. and Gage, F. H. (2009) Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learn Mem,* **16,** 147-154.

Jessberger, S., Nakashima, K., Clemenson, G. D., Jr., Mejia, E., Mathews, E., Ure, K., Ogawa, S., Sinton, C. M., Gage, F. H. and Hsieh, J. (2007a) Epigenetic modulation of seizure-induced neurogenesis and cognitive decline. *J Neurosci,* **27,** 5967-5975.

Jessberger, S., Zhao, C., Toni, N., Clemenson, G. D., Jr., Li, Y. and Gage, F. H. (2007b) Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling. *J Neurosci,* **27,** 9400-9407.

Jiang, W., Wan, Q., Zhang, Z. J., Wang, W. D., Huang, Y. G., Rao, Z. R. and Zhang, X. (2003) Dentate granule cell neurogenesis after seizures induced by pentylenetrazol in rats. *Brain Res,* **977,** 141-148.

Jin, K., Minami, M., Lan, J. Q., Mao, X. O., Batteur, S., Simon, R. P. and Greenberg, D. A. (2001) Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. *Proc Natl Acad Sci U S A,* **98,** 4710-4715.

Jin, K., Wang, X., Xie, L., Mao, X. O., Zhu, W., Wang, Y., Shen, J., Mao, Y., Banwait, S. and Greenberg, D. A. (2006) Evidence for stroke-induced neurogenesis in the human brain. *Proc Natl Acad Sci U S A,* **103,** 13198-13202.

Jin, X. (2016) The role of neurogenesis during development and in the adult brain. *Eur J Neurosci*.

Kandasamy, M., Couillard-Despres, S., Raber, K. A., Stephan, M., Lehner, B., Winner, B., Kohl, Z., Rivera, F. J., Nguyen, H. P., Riess, O., Bogdahn, U., Winkler, J., von Horsten, S. and Aigner, L. (2010) Stem cell quiescence in the hippocampal neurogenic niche is associated with elevated transforming growth factor-beta signaling in an animal model of Huntington disease. *J Neuropathol Exp Neurol,* **69,** 717-728.

Kaneko, N. and Sawamoto, K. (2009) Adult neurogenesis and its alteration under pathological conditions. *Neurosci Res,* **63,** 155-164.

Kashiwagi, A., Schipani, E., Fein, M. J., Greer, P. A. and Shimada, M. (2010) Targeted deletion of Capn4 in cells of the chondrocyte lineage impairs chondrocyte proliferation and differentiation. *Mol Cell Biol,* **30,** 2799-2810.

Katsube, M., Kato, T., Kitagawa, M., Noma, H., Fujita, H. and Kitagawa, S. (2008) Calpain-mediated regulation of the distinct signaling pathways and cell migration in human neutrophils. *J Leukoc Biol,* **84,** 255-263.

Kempermann, G., Gast, D. and Gage, F. H. (2002) Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Ann Neurol,* **52,** 135-143.

Kempermann, G., Kuhn, H. G. and Gage, F. H. (1997) More hippocampal neurons in adult mice living in an enriched environment. *Nature,* **386,** 493-495.

Kokoeva, M. V., Yin, H. and Flier, J. S. (2005) Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science,* **310,** 679-683.

Komitova, M., Mattsson, B., Johansson, B. B. and Eriksson, P. S. (2005) Enriched environment increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of stroke-lesioned adult rats. *Stroke,* **36,** 1278-1282.

Kuchay, S. M., Wieschhaus, A. J., Marinkovic, M., Herman, I. M. and Chishti, A. H. (2012) Targeted gene inactivation reveals a functional role of calpain-1 in platelet spreading. *J Thromb Haemost,* **10,** 1120-1132.

Kuhn, H. G., Dickinson-Anson, H. and Gage, F. H. (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci,* **16,** 2027-2033.

Lazarini, F. and Lledo, P. M. (2011) Is adult neurogenesis essential for olfaction? *Trends Neurosci,* **34,** 20-30.

Lazic, S. E., Grote, H., Armstrong, R. J., Blakemore, C., Hannan, A. J., van Dellen, A. and Barker, R. A. (2004) Decreased hippocampal cell proliferation in R6/1 Huntington's mice. *Neuroreport,* **15,** 811-813.

Lee, H. J., Tomioka, S., Kinbara, K., Masumoto, H., Jeong, S. Y., Sorimachi, H., Ishiura, S. and Suzuki, K. (1999) Characterization of a human digestive tract-specific calpain, nCL-4, expressed in the baculovirus system. *Arch Biochem Biophys,* **362,** 22-31.

Levesque, M. and Avoli, M. (2013) The kainic acid model of temporal lobe epilepsy. *Neurosci Biobehav Rev,* **37,** 2887-2899.

Liang, C. C., Park, A. Y. and Guan, J. L. (2007) In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc,* **2,** 329-333.

Lindvall, O. and Kokaia, Z. (2010) Stem cells in human neurodegenerative disorders--time for clinical translation? *J Clin Invest,* **120,** 29-40.

Liu, X., Muller, R. U., Huang, L. T., Kubie, J. L., Rotenberg, A., Rivard, B., Cilio, M. R. and Holmes, G. L. (2003) Seizure-induced changes in place cell physiology: relationship to spatial memory. *J Neurosci,* **23,** 11505-11515.

Lokuta, M. A., Nuzzi, P. A. and Huttenlocher, A. (2003) Calpain regulates neutrophil chemotaxis. *Proc Natl Acad Sci U S A,* **100,** 4006-4011.

Ma, D. K., Bonaguidi, M. A., Ming, G. L. and Song, H. (2009) Adult neural stem cells in the mammalian central nervous system. *Cell Res,* **19,** 672-682.

Macas, J., Nern, C., Plate, K. H. and Momma, S. (2006) Increased generation of neuronal progenitors after ischemic injury in the aged adult human forebrain. *J Neurosci,* **26,** 13114-13119.

Machado, V. M., Morte, M. I., Carreira, B. P., Azevedo, M. M., Takano, J., Iwata, N., Saido, T. C., Asmussen, H., Horwitz, A. R., Carvalho, C. M. and Araujo, I. M. (2015) Involvement of calpains in adult neurogenesis: implications for stroke. *Front Cell Neurosci,* **9,** 22.

Madsen, T. M., Kristjansen, P. E., Bolwig, T. G. and Wortwein, G. (2003) Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. *Neuroscience,* **119,** 635-642.

Magnusson, J. P., Goritz, C., Tatarishvili, J., Dias, D. O., Smith, E. M., Lindvall, O., Kokaia, Z. and Frisen, J. (2014) A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. *Science,* **346,** 237-241.

Marti-Fabregas, J., Romaguera-Ros, M., Gomez-Pinedo, U., Martinez-Ramirez, S., Jimenez-Xarrie, E., Marin, R., Marti-Vilalta, J. L. and Garcia-Verdugo, J. M. (2010) Proliferation in the human ipsilateral subventricular zone after ischemic stroke. *Neurology,* **74,** 357-365.

Martin, S. J. and Clark, R. E. (2007) The rodent hippocampus and spatial memory: from synapses to systems. *Cell Mol Life Sci,* **64,** 401-431.

McKhann, G. M., 2nd, Wenzel, H. J., Robbins, C. A., Sosunov, A. A. and Schwartzkroin, P. A. (2003) Mouse strain differences in kainic acid sensitivity, seizure behavior, mortality, and hippocampal pathology. *Neuroscience,* **122,** 551-561.

Melloni, E., Averna, M., Salamino, F., Sparatore, B., Minafra, R. and Pontremoli, S. (2000) Acyl-CoA-binding protein is a potent m-calpain activator. *J Biol Chem,* **275,** 82-86.

Melloni, E., Michetti, M., Salamino, F., Minafra, R. and Pontremoli, S. (1996) Modulation of the calpain autoproteolysis by calpastatin and phospholipids. *Biochem Biophys Res Commun,* **229,** 193-197.

Melloni, E., Michetti, M., Salamino, F., Sparatore, B. and Pontremoli, S. (1998) Mechanism of action of a new component of the Ca(2+)-dependent proteolytic system in rat brain: the calpain activator. *Biochem Biophys Res Commun,* **249,** 583-588.

Minami, Y., Emori, Y., Kawasaki, H. and Suzuki, K. (1987) E-F hand structure-domain of calcium-activated neutral protease (CANP) can bind Ca2+ ions. *J Biochem,* **101,** 889-895.

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

Mirzadeh, Z., Merkle, F. T., Soriano-Navarro, M., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (2008) Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. *Cell Stem Cell,* **3,** 265-278.

Mohapel, P., Ekdahl, C. T. and Lindvall, O. (2004) Status epilepticus severity influences the long-term outcome of neurogenesis in the adult dentate gyrus. *Neurobiol Dis,* **15,** 196-205.

Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z. and Davies, P. L. (2002) A Ca(2+) switch aligns the active site of calpain. *Cell,* **108,** 649-660.

Morris, R. G., Garrud, P., Rawlins, J. N. and O'Keefe, J. (1982) Place navigation impaired in rats with hippocampal lesions. *Nature,* **297,** 681-683.

Morte, M. I., Carreira, B. P., Machado, V., Carmo, A., Nunes-Correia, I., Carvalho, C. M. and Araujo, I. M. (2013) Evaluation of proliferation of neural stem cells in vitro and in vivo. *Curr Protoc Stem Cell Biol,* **Chapter 2,** Unit 2D 14.

Mu, Y. and Gage, F. H. (2011) Adult hippocampal neurogenesis and its role in Alzheimer's disease. *Mol Neurodegener,* **6,** 85.

Murachi, T. (1984) Calcium-dependent proteinases and specific inhibitors: calpain and calpastatin. *Biochem Soc Symp,* **49,** 149-167.

Nakagawa, E., Aimi, Y., Yasuhara, O., Tooyama, I., Shimada, M., McGeer, P. L. and Kimura, H. (2000) Enhancement of progenitor cell division in the dentate gyrus triggered by initial limbic seizures in rat models of epilepsy. *Epilepsia,* **41,** 10-18.

Nakashiba, T., Cushman, J. D., Pelkey, K. A., Renaudineau, S., Buhl, D. L., McHugh, T. J., Rodriguez Barrera, V., Chittajallu, R., Iwamoto, K. S., McBain, C. J., Fanselow, M. S. and Tonegawa, S. (2012) Young dentate granule cells mediate pattern separation, whereas old granule cells facilitate pattern completion. *Cell,* **149,** 188-201.

Nakatomi, H., Kuriu, T., Okabe, S., Yamamoto, S., Hatano, O., Kawahara, N., Tamura, A., Kirino, T. and Nakafuku, M. (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell,* **110,** 429-441.

Neumar, R. W., Meng, F. H., Mills, A. M., Xu, Y. A., Zhang, C., Welsh, F. A. and Siman, R. (2001) Calpain activity in the rat brain after transient forebrain ischemia. *Exp Neurol,* **170,** 27-35.

Niu, W., Zang, T., Zou, Y., Fang, S., Smith, D. K., Bachoo, R. and Zhang, C. L. (2013) In vivo reprogramming of astrocytes to neuroblasts in the adult brain. *Nat Cell Biol,* **15,** 1164-1175.

Noma, H., Kato, T., Fujita, H., Kitagawa, M., Yamano, T. and Kitagawa, S. (2009) Calpain inhibition induces activation of the distinct signalling pathways and cell migration in human monocytes. *Immunology,* **128,** e487-496.

O'Keefe, J. and Dostrovsky, J. (1971) The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res,* **34,** 171-175.

Ono, Y. and Sorimachi, H. (2012) Calpains: an elaborate proteolytic system. *Biochim Biophys Acta,* **1824,** 224-236.

Palmer, T. D., Willhoite, A. R. and Gage, F. H. (2000) Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol,* **425,** 479-494.

Parent, J. M., Elliott, R. C., Pleasure, S. J., Barbaro, N. M. and Lowenstein, D. H. (2006) Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy. *Ann Neurol,* **59,** 81-91.

Parent, J. M., Vexler, Z. S., Gong, C., Derugin, N. and Ferriero, D. M. (2002) Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol,* **52,** 802-813.

Parent, J. M., Yu, T. W., Leibowitz, R. T., Geschwind, D. H., Sloviter, R. S. and Lowenstein, D. H. (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci,* **17,** 3727-3738.

Parnaud, G., Hammar, E., Rouiller, D. G. and Bosco, D. (2005) Inhibition of calpain blocks pancreatic beta-cell spreading and insulin secretion. *Am J Physiol Endocrinol Metab,* **289,** E313-321.

Paulhe, F., Bogyo, A., Chap, H., Perret, B. and Racaud-Sultan, C. (2001) Vascular smooth muscle cell spreading onto fibrinogen is regulated by calpains and phospholipase C. *Biochem Biophys Res Commun,* **288,** 875-881.

Phillips, R. G. and LeDoux, J. E. (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav Neurosci,* **106,** 274-285.

Qiu, K., Su, Y. and Block, E. R. (2006) Use of recombinant calpain-2 siRNA adenovirus to assess calpain-2 modulation of lung endothelial cell migration and proliferation. *Mol Cell Biochem,* **292,** 69-78.

Raimbourg, Q., Perez, J., Vandermeersch, S., Prignon, A., Hanouna, G., Haymann, J. P., Baud, L. and Letavernier, E. (2013) The calpain/calpastatin system has opposing roles in growth and metastatic dissemination of melanoma. *PLoS One,* **8,** e60469.

Rao, M. V., Mohan, P. S., Peterhoff, C. M., Yang, D. S., Schmidt, S. D., Stavrides, P. H., Campbell, J., Chen, Y., Jiang, Y., Paskevich, P. A., Cataldo, A. M., Haroutunian, V. and Nixon, R. A. (2008) Marked calpastatin (CAST) depletion in Alzheimer's disease accelerates cytoskeleton disruption and neurodegeneration: neuroprotection by CAST overexpression. *J Neurosci,* **28,** 12241-12254.

Rice, A. C., Khaldi, A., Harvey, H. B., Salman, N. J., White, F., Fillmore, H. and Bullock, M. R. (2003) Proliferation and neuronal differentiation of mitotically active cells following traumatic brain injury. *Exp Neurol,* **183,** 406-417.

Rock, M. T., Dix, A. R., Brooks, W. H. and Roszman, T. L. (2000) Beta1 integrin-mediated T cell adhesion and cell spreading are regulated by calpain. *Exp Cell Res,* **261,** 260-270.

Saez, M. E., Ramirez-Lorca, R., Moron, F. J. and Ruiz, A. (2006) The therapeutic potential of the calpain family: new aspects. *Drug Discov Today,* **11,** 917-923.

Sahay, A., Wilson, D. A. and Hen, R. (2011) Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. *Neuron,* **70,** 582-588.

Salamino, F., De Tullio, R., Mengotti, P., Viotti, P. L., Melloni, E. and Pontremoli, S. (1993) Site-directed activation of calpain is promoted by a membrane-associated natural activator protein. *Biochem J,* **290 (Pt 1),** 191-197.

Salazar-Colocho, P., Lanciego, J. L., Del Rio, J. and Frechilla, D. (2008) Ischemia induces cell proliferation and neurogenesis in the gerbil hippocampus in response to neuronal death. *Neurosci Res,* **61,** 27-37.

Santos, D. M., Xavier, J. M., Morgado, A. L., Sola, S. and Rodrigues, C. M. (2012) Distinct regulatory functions of calpain 1 and 2 during neural stem cell self-renewal and differentiation. *PLoS One,* **7,** e33468.

Saxe, M. D., Battaglia, F., Wang, J. W., Malleret, G., David, D. J., Monckton, J. E., Garcia, A. D., Sofroniew, M. V., Kandel, E. R., Santarelli, L., Hen, R. and Drew, M. R. (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci U S A,* **103,** 17501-17506.

Scharfman, H. E., Goodman, J. H. and Sollas, A. L. (2000) Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci,* **20,** 6144-6158.

Schauwecker, P. E. and Steward, O. (1997) Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. *Proc Natl Acad Sci U S A,* **94,** 4103-4108.

Schlachetzki, J. C., Grimm, T., Schlachetzki, Z., Ben Abdallah, N. M., Ettle, B., Vohringer, P., Ferger, B., Winner, B., Nuber, S. and Winkler, J. (2016) Dopaminergic lesioning impairs adult hippocampal neurogenesis by distinct modification of alpha-synuclein. *J Neurosci Res,* **94,** 62-73.

Schmidt, R. H., Scholten, K. J. and Maughan, P. H. (1999) Time course for recovery of water maze performance and central cholinergic innervation after fluid percussion injury. *J Neurotrauma,* **16,** 1139-1147.

Scott, B. W., Wojtowicz, J. M. and Burnham, W. M. (2000) Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures. *Exp Neurol,* **165,** 231-236.

Sengoku, T., Bondada, V., Hassane, D., Dubal, S. and Geddes, J. W. (2004) Tat-calpastatin fusion proteins transduce primary rat cortical neurons but do not inhibit cellular calpain activity. *Exp Neurol,* **188,** 161-170.

Shen, Q., Wang, Y., Kokovay, E., Lin, G., Chuang, S. M., Goderie, S. K., Roysam, B. and Temple, S. (2008) Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. *Cell Stem Cell,* **3,** 289-300.

Shimada, M., Greer, P. A., McMahon, A. P., Bouxsein, M. L. and Schipani, E. (2008) In vivo targeted deletion of calpain small subunit, Capn4, in cells of the osteoblast lineage impairs cell proliferation, differentiation, and bone formation. *J Biol Chem,* **283,** 21002-21010.

Shiraha, H., Glading, A., Chou, J., Jia, Z. and Wells, A. (2002) Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. *Mol Cell Biol,* **22,** 2716-2727.

Shors, T. J., Miesegaes, G., Beylin, A., Zhao, M., Rydel, T. and Gould, E. (2001) Neurogenesis in the adult is involved in the formation of trace memories. *Nature,* **410,** 372-376.

Sierra, A., Martin-Suarez, S., Valcarcel-Martin, R., Pascual-Brazo, J., Aelvoet, S. A., Abiega, O., Deudero, J. J., Brewster, A. L., Bernales, I., Anderson, A. E., Baekelandt, V., Maletic-Savatic, M. and Encinas, J. M. (2015) Neuronal hyperactivity accelerates depletion of neural stem cells and impairs hippocampal neurogenesis. *Cell Stem Cell,* **16,** 488-503.

Simpson, J. M., Gil-Mohapel, J., Pouladi, M. A., Ghilan, M., Xie, Y., Hayden, M. R. and Christie, B. R. (2011) Altered adult hippocampal neurogenesis in the YAC128 transgenic mouse model of Huntington disease. *Neurobiol Dis,* **41,** 249-260.

Smith, S. D., Jia, Z., Huynh, K. K., Wells, A. and Elce, J. S. (2003) Glutamate substitutions at a PKA consensus site are consistent with inactivation of calpain by phosphorylation. *FEBS Lett,* **542,** 115-118.

Snyder, J. S., Glover, L. R., Sanzone, K. M., Kamhi, J. F. and Cameron, H. A. (2009) The effects of exercise and stress on the survival and maturation of adult-generated granule cells. *Hippocampus,* **19,** 898-906.

Sorimachi, H., Hata, S. and Ono, Y. (2011) Calpain chronicle--an enzyme family under multidisciplinary characterization. *Proc Jpn Acad Ser B Phys Biol Sci,* **87,** 287-327.

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., Bostrom, E., Westerlund, I., Vial, C., Buchholz, B. A., Possnert, G., Mash, D. C., Druid,

H. and Frisen, J. (2013) Dynamics of hippocampal neurogenesis in adult humans. *Cell,* **153,** 1219-1227.

Spalding, K. L., Bhardwaj, R. D., Buchholz, B. A., Druid, H. and Frisen, J. (2005) Retrospective birth dating of cells in humans. *Cell,* **122,** 133-143.

Stubley-Weatherly, L., Harding, J. W. and Wright, J. W. (1996) Effects of discrete kainic acid-induced hippocampal lesions on spatial and contextual learning and memory in rats. *Brain Res,* **716,** 29-38.

Sun, D., McGinn, M. J., Zhou, Z., Harvey, H. B., Bullock, M. R. and Colello, R. J. (2007) Anatomical integration of newly generated dentate granule neurons following traumatic brain injury in adult rats and its association to cognitive recovery. *Exp Neurol,* **204,** 264-272.

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

Takano, J., Tomioka, M., Tsubuki, S., Higuchi, M., Iwata, N., Itohara, S., Maki, M. and Saido, T. C. (2005) Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. *J Biol Chem,* **280,** 16175-16184.

Tattersfield, A. S., Croon, R. J., Liu, Y. W., Kells, A. P., Faull, R. L. and Connor, B. (2004) Neurogenesis in the striatum of the quinolinic acid lesion model of Huntington's disease. *Neuroscience,* **127,** 319-332.

Tavazoie, M., Van der Veken, L., Silva-Vargas, V., Louissaint, M., Colonna, L., Zaidi, B., Garcia-Verdugo, J. M. and Doetsch, F. (2008) A specialized vascular niche for adult neural stem cells. *Cell Stem Cell,* **3,** 279-288.

Tompa, P., Emori, Y., Sorimachi, H., Suzuki, K. and Friedrich, P. (2001) Domain III of calpain is a ca2+-regulated phospholipid-binding domain. *Biochem Biophys Res Commun,* **280,** 1333-1339.

Tonchev, A. B., Yamashima, T., Zhao, L., Okano, H. J. and Okano, H. (2003) Proliferation of neural and neuronal progenitors after global brain ischemia in young adult macaque monkeys. *Mol Cell Neurosci,* **23,** 292-301.

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

van Praag, H., Shubert, T., Zhao, C. and Gage, F. H. (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. *J Neurosci,* **25,** 8680-8685.

Wang, K. K. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci,* **23,** 20-26.

Wang, Q., Yu, S., Simonyi, A., Sun, G. Y. and Sun, A. Y. (2005) Kainic acid-mediated excitotoxicity as a model for neurodegeneration. *Mol Neurobiol,* **31,** 3-16.

Wehner, J. M. and Radcliffe, R. A. (2004) Cued and contextual fear conditioning in mice. *Curr Protoc Neurosci,* **Chapter 8,** Unit 8 5C.

Xing, Y. L., Roth, P. T., Stratton, J. A., Chuang, B. H., Danne, J., Ellis, S. L., Ng, S. W., Kilpatrick, T. J. and Merson, T. D. (2014) Adult neural precursor cells from the subventricular zone contribute significantly to oligodendrocyte regeneration and remyelination. *J Neurosci,* **34,** 14128-14146.

Xu, L. and Deng, X. (2006) Protein kinase Ciota promotes nicotine-induced migration and invasion of cancer cells via phosphorylation of micro- and m-calpains. *J Biol Chem,* **281,** 4457-4466.

Xu, Y. and Mellgren, R. L. (2002) Calpain inhibition decreases the growth rate of mammalian cell colonies. *J Biol Chem,* **277,** 21474-21479.

Yajima, Y. and Kawashima, S. (2002) Calpain function in the differentiation of mesenchymal stem cells. *Biol Chem,* **383,** 757-764.

Yamashita, T., Ninomiya, M., Hernandez Acosta, P., Garcia-Verdugo, J. M., Sunabori, T., Sakaguchi, M., Adachi, K., Kojima, T., Hirota, Y., Kawase, T., Araki, N., Abe, K., Okano, H. and Sawamoto, K. (2006) Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. *J Neurosci,* **26,** 6627-6636.

Zhang, C. L., Zou, Y., He, W., Gage, F. H. and Evans, R. M. (2008) A role for adult TLX-positive neural stem cells in learning and behaviour. *Nature,* **451,** 1004-1007.

Zhang, R. L., Zhang, Z. G., Zhang, L. and Chopp, M. (2001) Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience,* **105,** 33-41.