**Universidade do Minho**

Escola de Ciências da Saúde

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# THE ROLE OF AP2γ TRANSCRIPTION FACTOR IN THE MODULATION OF ADULT GLUTAMATERGIC NEUROGENESIS IN DEPRESSION

# O PAPEL DO FATOR DE TRANSCRIÇÃO AP2γ NA MODULAÇÃO DA NEUROGÉNESE GLUTAMATÉRGICA ADULTA EM DEPRESSÃO

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**Onde quer que estejas, a ti te dedico esta tese**

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*"Take my hand, Let's see where we wake up tomorrow..."*

# ABSTRACT

Major depressive disorder (MDD) is a multidimensional psychiatric disease, considered by the World Health Organization as one of the leading causes of disability. Despite the importance of this disease in modern societies and the large investment of resources already made in its study, the processes underlying its pathophysiology remain poorly understood. Several hypotheses have been proposed to clarify the neurobiological mechanisms underlying this psychiatric disorder, being the link between adult hippocampal neurogenesis and MDD a central topic in the past decades. Previous studies have identified AP2γ as a key regulator of adult hippocampal neurogenesis in mice, being expressed in a subpopulation of adult transient amplifying progenitors, and acting as a regulator of basal progenitors, promoting proliferation and glutamatergic neuronal differentiation. Thus, we wanted to further explore the impact of AP2γ in brain neurophysiology and behavior during development and at adult stages, dissecting also its mechanisms both in healthy and depressive states. With this study, we were able to understand the impact of AP2γ in post-natal development and during juvenile age, through the AP2γ constitutive knockout (KO) model. In the developmental milestones assessment we did not find any major impairment in the behavioral performance of AP2γ KO mice, since all parameters analyzed, including the ones where we found differences, were within the typical range for appearance of the developmental milestones. However, in the juvenile behavior assessment and in the hippocampal glutamatergic neurogenesis process, impairments were found, since AP2γ KO mice showed anxious-like behavior and decreased proliferation of immature neurons. To study the impact of modulating the transcription factor AP2γ in depression we exposed both constitutive and conditional KO animal models to a chronic stress protocol, which efficiently induced core depressive-like symptoms. Through the conditional AP2γ KO mice, we were able to elucidate the impact of deleting AP2γ on behavior and neurogenesis in depressive-like conditions specifically in adult age, without the interference of potential functions of the gene during early development that may appear in the constitutive AP2γ model. Through a multidimensional behavioral analysis, we observed that both models presented similar results in the three most affected behavioral dimensions in depression, namely anxiety, mood and cognition. Regarding anxiety and mood no major differences were found between genotypes in both animal models. Moreover, AP2γ KO mice presented cognitive deficits in basal conditions, but when exposed to chronic mild stress no detrimental effects of deletion of the gene were observed. In this work, we also identified, through a broad analysis of the dentate gyrus neurogenic niche, alterations of epigenetic regulators in the AP2γ constitutive KO mice after uCMS exposure. The reported results not only support the involvement of AP2γ in the transcriptional network that modulates the juvenile and adult neurogenic process, but also highlight the potential of this molecule as a future therapeutical tool in neuropsychiatric disorders, in which neurogenesis is impaired.

# RESUMO

O transtorno depressivo persistente é uma doença psiquiátrica multidimensional, considerada pela Organização Mundial de Saúde como uma das principais causas de incapacidade. Apesar da importância desta doença na sociedade moderna, e do largo investimento de recursos já feitos no seu estudo, os processos subjacentes à sua patofisiologia continuam pouco percebidos. Várias hipóteses foram propostas para clarificar os mecanismos neurobiológicos implícitos nesta doença psiquiátrica, tendo sido o vínculo entre a neurogénese hipocampal adulta e a depressão um tópico central nas décadas passadas. Estudos anteriores identificaram o AP2γ como um regulador chave da neurogénese hipocampal adulta em ratinhos, sendo expresso numa subpopulação de células progenitoras de rápida amplificação adultas, e atuando como regulador de progenitores basais, promovendo a proliferação e a diferenciação neuronal glutamatérgica. Deste modo, propusemos continuar a explorar o impacto do AP2γ na neurofisiologia cerebral e no comportamento, durante a fase de desenvolvimento e na idade adulta, procurando entender também os seus mecanismos tanto no estado saudável como em depressão. Com este trabalho, fomos capazes de entender o impacto do AP2γ no desenvolvimento pós-natal e em idade juvenil, a partir do modelo animal de deleção constitutiva do AP2γ. Na avaliação dos marcos de desenvolvimento, não encontramos nenhuma alteração no desempenho comportamental nos animais com deleção de AP2γ, visto que todos os parâmetros analisados, incluindo os que encontramos alguma diferença, se encontravam dentro dos intervalos típicos de aparecimento dos marcos de desenvolvimento. Contudo, na avaliação do comportamento juvenil e no processo de neurogénese glutamatérgica hipocampal observamos défices, visto que os animais com deleção de AP2γ apresentaram comportamento ansioso e uma diminuição da proliferação de neurónios imaturos. Para estudar o impacto da modulação do fator de transcrição AP2γ em depressão expusemos tanto o modelo animal com deleção constitutiva bem como o modelo animal condicional do gene a um protocolo de stress crónico, o qual eficientemente induziu sintomas primários de depressão. Através do modelo animal condicional do AP2γ, conseguimos compreender o impacto da deleção do AP2γ na modulação do comportamento e neurogénese em condições depressivas especificamente em idade adulta, sem interferência das potenciais funções do gene durante o período de desenvolvimento dos animais, que poderão surgir no modelo animal constitutivo do AP2γ. Através, de uma análise comportamental multidimensional, observamos que ambos os modelos apresentaram resultados similares nas dimensões comportamentais mais afetadas na depressão, nomeadamente a ansiedade, o humor e a cognição. Relativamente à ansiedade e ao humor não encontramos grandes diferenças entre genótipos em ambos os modelos animais. Além disso, os modelos animais de deleção do AP2γ apresentaram défices cognitivos em condições basais, mas após exposição ao stress crónico não foram observados os efeitos prejudiciais da deleção do gene. Neste trabalho, também identificamos, através de uma análise abrangente do nicho neurogénico girus denteado, um reguladores epigenéticos alterados no animal constitutivo do AP2γ. Os resultados apresentados não só suportam o envolvimento do AP2γ na rede transcripcional responsável pela modulação do processo neurogénico juvenil e adulto, como também destacam o potencial desta molécula em abordagens terapêuticas futuras em doenças neuropsiquiátricas, nas quais a neurogénese se encontra afetada.

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# LIST OF ABREVIATIONS

**#**

5caC - 5-carboxylcytosine

5fC - 5-formylcytosine

5hmC - 5-hydroxymethylcytosine

5mC - 5-methylcytosine

**A**

ACTH - Adrenocorticotropic

AD - Alzheimer's disease

ADs - Antidepressant drugs

ANPs - Amplifying neural progenitor cells

AP2γ - Activating protein 2 gamma

**B**

BDNF - Brain-derived neurotrophic factor

BrdU - 5-bromo-2-deoxyuridine

**C**

CBP - cyclic AMP-response element binding protein

CFC - Contextua fear conditioning

cm - centimeter

CMS - Chronic mild stress

CNS - Central nervous system

coREST - REST corepressor

CREB - cAMP response element binding

CSF - Cerebrospinal fluid

CUS - Chronic unpredictable stress

**D**

DAPI - 4-6-diamidino-2-phenylindole

DCX - Doublecortin

DG - Dentate gyrus

DNMTs - DNA methyltransferases

**E**

E - Embryonic day

ELISA - enzyme-linked immunossorbant assay

EPM - Elevated-plus maze

ESCs - Embryonic stem cells

**F**

FBS - fetal bovine serum

FGF - Fibroblast growth factor

FOX - Forkhead box protein

FST - Forced swimming test

**G**

GADD45β - DNA-damage-inducible protein 45β

GCL - Granular cell layer

GFAP - Glial fibrillary acidic protein

**H**

h - hours

hA - Horizontal astrocytes

HDAC - HDAC inhibitor

HDAC - Histone deacetylases

HPA - Hypothalamic-pituitary axis

**I**

i.p. - Intraperitoneally

**K**

kDa - Kilodalton

KO - Knockout

**L**

lncRNAs - Long non-coding RNAs

**M**

M - Molar

mA - milliamp

MDB1 - Methyl binding protein 1

MDD - Major depressive disorder

MeCP2 - Methly-CpG- binding protein

mg - Milligram

mg/kg - Milligram per kilo

mg/ml - Milligram per milliliter

min - minutes

miRNAs - MicroRNA

ml - Milliliter

mRNA - messenger RNA

MWM - Morris water maze

**N**

NaCl - Sodium Chloride

NeuN - Neuronal nuclei

NeuroD - Neurogenic differentiation

Ngn2 - Neurogenin 2

NOR - Novel object recognition

NPCs - Neural progenitor cells

NSCs - Neural stem cells

**O**

OB - Olfactory bulb

OF - Open field

**P**

Pax6 - Paired box gene 6

PBS - Phosphate buffered saline

PBS-T - Phosphate buffered saline-Triton

PCR - Polymerase chain reaction

PFA - Paraformaldehyde

PFC - Prefrontal cortex

PND - Postnatal day

Prox1 - Prospero homeobox 1

PSA-NCAM - Polysialylated-neural cell adhesion molecule

**Q**

QNPs - Quiescent neural progenitors

qRT-PCR - Quantitative real time - Polymerase chain reaction

**R**

rA - Radial astrocytes

REST - Repression element 1 silencing transcription factor

RMS - Rostral migratory stream

rpm - Rotations per minute

RT - Room temperature

RT-PCR - Reverse transcriptase - Polymerase chain reaction

**S**

S - seconds

SEM - Standard error of the mean

SEZ - Subependymal zone

SGZ - Subgranular zone

SOX2 - SRY-related HMG box 2

**T**

TET - Ten-eleven translocation

TGFβ - Transforming growth factor β

TST - Tail suspension test

TM - Melting temperature

Tbr - T-box brain protein

TAPs - Transient amplifying progenitor

Trx6 - Trithorax protein

**U**

uCMS - Unpredictable chronic mild stress

**V**

VPA - Valproic acid

**W**

WHO - World health organization

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CHAPTER 1

# INTRODUCTION

# 1) INTRODUCTION

## 1.1. Depression

### 1.1.1. State of the art

Major depressive disorder (MDD) is considered by the World Health Organization (WHO) as one of the world leading causes of disability, since it is estimated that around 350 million people worldwide are affected with this psychiatric disorder. It is the most disabling medical condition, in terms of years lost due to disability, and it is foreseen that by 2030 depression will be the major contributor to the global illness burden (Willner et al. 2013). Patients suffering from this disorder usually display a loss of interest for experiencing pleasurable activities (anhedonia), changes in appetite and sleep pattern, abnormal sadness states, high levels of anxiety, lack of energy and ultimately suicidal ideation. Moreover, rather than low self-esteem, depressive patients present a deeply negative view of the world and the future, showing also deficits of attention, interpretation and memory (Mathews and MacLeod 2005; Willner et al. 2013; Bergstrom and Meacham 2016).

Despite the importance of this multidimensional psychiatric disease in modern societies and the large investment of resources already made to look for efficient treatment, the processes underlying its pathophysiology remain poorly understood. It is accepted that this complex disorder involves gene-environment interactions, but the genetic and environmental subtracts are largely unknown. Even though there is little knowledge regarding the real causes for the precipitation of MDD, vulnerability or predisposition to develop depression may occur throughout lifetime due to negative environmental stimuli. It is recognized that harmful early life experiences, such as inadequate familial relations, increase the risk for a depressive episode (Willner et al. 2013; Slavich and Irwin 2014). Also, it is consensually accepted that there is a familial predisposition to inherit this disorder through "stress-provoking" genes passing on across generations and providing vulnerability to develop MDD (Slavich and Irwin 2014). However, such genetic transmission pattern, which does not follow the Mendelian laws, is highly complex, and even with the evolution of technical means and the human genome sequencing, it has not been clarified yet. These scientific difficulties to understand the mechanisms underlying depression, can be explained by the multiple neurological systems that are likely involved in the etiopathogenesis of depression (Marsden 2013). Due to the lack of knowledge in this scientific field, there are still many unmet medical needs to address.

Looking for a way to successfully revert and treat major depression, several hypotheses have been proposed to clarify the neurobiological mechanisms underlying the onset, maintenance and recovery from this psychiatric disorder: the neurochemical and neurotrophin hypotheses; involvement of cytoquines and inflammatory agents; glutamate excitotoxicity; altered HPA axis; the phase-shift; and the neurogenic hypothesis (Bessa et al. 2009a; Hasler 2010). Depression has a great impact on the central nervous system (CNS) inducing structural and neuroplasticity alterations in brain regions such as the prefrontal cortex (PFC), the amygdala, the ventral striatum (including the nucleus accumbens), and the hippocampus (Pittenger and Duman 2008). Indeed, in the past three decades, a vast number of studies have revealed that during a depressive episode, it is possible to observe cell loss and neuronal atrophy in the hippocampus, which is a brain area with relevant roles both in adult brain neuroplasticity and behavioral control (Pittenger and Duman 2008; Serafini 2012; Mateus-Pinheiro et al. 2013). Several mechanisms were proposed to explain this cell loss and neuronal atrophy, among which we could find the glucocorticoid and glutamate toxicity for both glia and neurons (Duman 2009; Kudryashova 2015), the decreased neurotrophic factors expression (Castren et al. 2007) and also the reduced neuronal plasticity (dendritic arborization atrophy and neurogenesis reduction in the hippocampal neurogenic niche) (Bessa et al. 2009a) in animal models of depression. The potential link between adult neurogenesis and MDD has drawn some attention in the past decades (WuiMan et al. 2013). Although being a controversial topic, the so called neurogenic hypothesis of depression, has brought different relevant questions that challenge the classical conceptions regarding depression, and addresses the neurogenic process as a key pathological player and therapeutical target in stress-related disorders (Eisch and Petrik 2012).

Assuring the implication of adult neurogenesis in depression will support the need to better understand the adult hippocampal neurogenic niche not only in physiological but also pathological conditions. Such knowledge will possibly lead to additional therapeutical approaches by artificially regulating the endogenous neural progenitors pool, in order to sustain hippocampal neurogenesis, and counteract the inhibitory effects induced by depression. This goal could be achievable through genetic or epigenetic regulation of the adult hippocampal neurogenesis process. For this, research should focus in finding key transcriptional factors and epigenetic modulatory molecules that regulate hippocampal neurogenesis, and thus present strong therapeutical potential. Altogether, it may be relevant to bring new insights on the transcriptional network and the epigenetic mechanisms underlying adult neurogenesis in the healthy and “diseased” brain, to fully understand this highly complex neurobiological process, and its potential role as a therapeutical solution for depression.

### 1.1.2. Modeling depression in animal models

Modeling of human neuropsychiatric disorders, such as depression, in animals is extremely challenging given the subjective nature of many key symptoms, the lack of objective tests, and the poor knowledge regarding the onset, maintenance and recovery from such diseases (Nestler and Hyman 2010). Nonetheless, a lot of effort has been made in order to construct animal models and protocols to understand the pathophysiology of depression and develop different modulatory drugs with therapeutic actions.

Knowledge of the etiopathogenesis of depression has progressed substantially in the last years, in part due to studies in animals models (Patricio et al. 2013). The validity of an animal model for formulation of hypotheses and for the development of novel therapeutic strategies encompasses: the use of known etiological factors (etiological validity), it must mimic the behavioral and neurological symptoms observed in human disease (face validity) and importantly, it must respond to clinically effective treatments (predicted validity) (Berton et al. 2012; Patricio et al. 2013). Although selected depressive symptoms may be irreproducible in animals, such as suicidal ideation, a number of models exhibit considerable construct validity when targeting other clinical phenotypes of depression. One of the most important advances in understanding psychiatric disorders, like depression, has been the development of mice with altered expression of specific targets, being it a receptor, transporter, enzyme or signal transduction molecule (Tecott and Wehner 2001; Cryan and Mombereau 2004). These new tools have the potential to verify novel targets for antidepressant activity for which few established pharmacological tools exist. Moreover, these genetically altered mice will enable better testing of the validity of current molecular theories of depression (Cryan and Mombereau 2004). Although there are a large number of mice strains that have been generated with a phenotype that has been interpreted as being related to depression or antidepressant action, there are three most recommend mice strains to use in a depression study: C57Bl/6J, SV/129 and BALB/c mice (Bergner et al. 2016).

There are several models of depression described in the literature: chronic unpredictable stress (CUS), unpredictable chronic mild stress (uCMS), social stress, early life stress, learned helplessness, fear conditioning and olfactory bulbectomy (Duman 2010; Patricio et al. 2013). Despite none of these models can fully recapitulate the complexity and heterogeneity of the human disease, they are considered robust approaches to study depression. However, the uCMS protocol (Willner et al. 2013), based in the principles of the CMS and CUS protocols was proven to be a more robust approach to model the human depression at the lab. In this model, after exposure to chronic mild stressors implemented in an unpredictable way, stressed animals present depressive-like symptoms such as anhedonia, anxiety and

cognitive deficits, showing in this way impairments in all three behavioral dimensions known to be affected in humans with depression (Bessa et al. 2009a; Mateus-Pinheiro et al. 2013). Moreover, these stressed animals show impaired neuroplasticity, and compromised regulation of the corticosterone levels, another well-known molecular phenotypes of depression and other stress-related disorders (Mateus-Pinheiro et al. 2013; Patricio et al. 2015). Although the uCMS was first described using a rat animal model, this model of depression induction has also been validated in mice (Surget and Belzung 2009), maintaining its translational value as it induces some core alterations that are similar to those observed in depressed patients (Sibille et al. 2009; Nollet et al. 2013).

In sum, although the currently available rodent models have significant limitations, ranging from weak validation to poor predictive power for drug efficacy in human disease, they have been a powerful tool to investigate the pathophysiology of depression. Further understanding the mechanisms underlying the pathophysiology of depression is of the upmost importance to allow improvement of the experimental animal models and lead to more complete and targeted depressive studies.

## 1.2. Adult neurogenesis in the mammalian brain: genetic and epigenetic modulation of the hippocampal neurogenic process

### 1.2.1. Neurogenesis in the adult mammalian brain

The discovery of neurogenesis in the adult mammalian brain, overturned the long-held dogma that the adult central nervous system (CNS) was immutable, and had no capacity for generating new cells (Altman and Das 1965; Deng et al. 2010). Although the emergence of adult neurogenesis as a research field in neuroscience has brought much excitement, there was a lot of reluctance manifested towards the first reports in this area (Egeland et al. 2015). Despite the initial skepticism, it is now well established that new neurons are continuously generated, differentiated and integrated in the preexisting brain neuronal networks (Doetsch et al. 1999; Gage 2002; Deng et al. 2010). Adult neurogenesis is not a single isolated event, is thus a complex process, involving a wide range of highly regulated steps, starting with the proliferation of neural stem cells (NSCs) that will then divide to give rise to transient amplifying progenitors (TAPs) which will be responsible for the rapid expansion of the multipotent progenitor cells pool. TAPs will then differentiate in immature cells, committed to a neuronal phenotype (neuroblasts), that will undergo morphological and physiological maturation with acquisition of neuronal characteristics, and will finally become functionally integrated in the pre-existing network (Figure 1) (Balu and Lucki 2009).

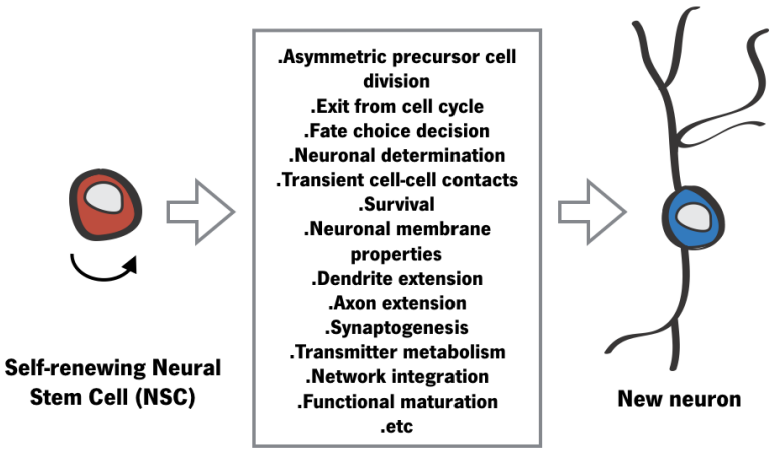



**Figure 1. Neurogenesis is a process not an isolated event.**

Adult neurogenesis is regulated at many different stages of cell development. Here the term neurogenesis comprises all necessary steps, starting with the division of a NSC and resulting in the existence of a functionally fully integrated newborn neuron. Interestingly, a high percentage of the newborn neurons die before becoming fully integrated in the network. Gliogenesis also happen at a lower percentage in the adult neurogenic niches. Newborn glial cells are thought to be generated from the same progenitor cells that give rise to neurons. Adapted from (Kempermann, 2011).

In the adult brain there are specific areas where neurogenesis persists throughout life, known as neurogenic niches (Urban and Guillemot 2014). Such spatially defined brain regions where neurogenesis occurs display the presence of immature NSCs from which new neurons can develop, and a permissive microenvironment rich in cell-extrinsic factors needed to favor the generation of new cells (Urban and Guillemot 2014).

Although being a controversial topic, there are two consensual neurogenic brain regions broadly recognized in the adult mammal brain: the subependymal zone (SEZ) lining the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Figure 2) (Zhao et al. 2008; Balu and Lucki 2009; Urban and Guillemot 2014). These two neurogenic niches are largely responsible for the formation of distinct types of neurons. In the SEZ the precursor cells are mostly found in the temporal walls of the lateral ventricles. Here, newly-born precursor cells generate neuroblasts that migrate along the rostral migratory stream (RMS), reaching the olfactory bulb (OB). At the OB, neuroblasts differentiate and mature, becoming largely GABAergic granule, and periglomerular inhibitory interneurons (Chumley et al. 2007; Belenguer et al. 2016). In the DG, the precursor cell population resides throughout the SGZ with specific gradients (Silva et al. 2006). After being formed in the SGZ, the newly-born neuronal cells – neuroblasts, become committed to a neuronal lineage and migrate into the granular cell layer (GCL), where they fully differentiate into excitatory glutamatergic granule neurons (Brill et al. 2009). In addition to these two consensually recognized neurogenic niches,

some research groups have presented evidence that neurogenesis can occur in other brain regions like the striatum (Luzzati et al. 2006; Inta et al. 2016), the cortex (Kodama et al. 2004; Ohira et al. 2010), the amygdala (Goncalves et al. 2008) and the hypothalamus (Fowler et al. 2002; Kokoeva et al. 2005). However, these results are quite controversial and further studies are needed to ashore that these neurogenic niches have indeed NSCs and a permissive microenvironment to allow the formation of new functional neurons.

Despite the increasingly intense research, a great number of questions regarding the adult neurogenesis process remain to be answered and understood. It is unquestionably recognized that in the healthy adult mammalian brain new neurons can be generated, but its functional relevance remains to be fully comprehended. While this singularity is confined to a few privileged brain regions, the generation of new neurons in the post-natal brain represents a new dimension of plasticity, impacting both directly and indirectly on neuronal remodeling and repair. This promising therapeutical target, for a wide range of neuropathological contexts, is one of the main reasons why this field is so interesting.

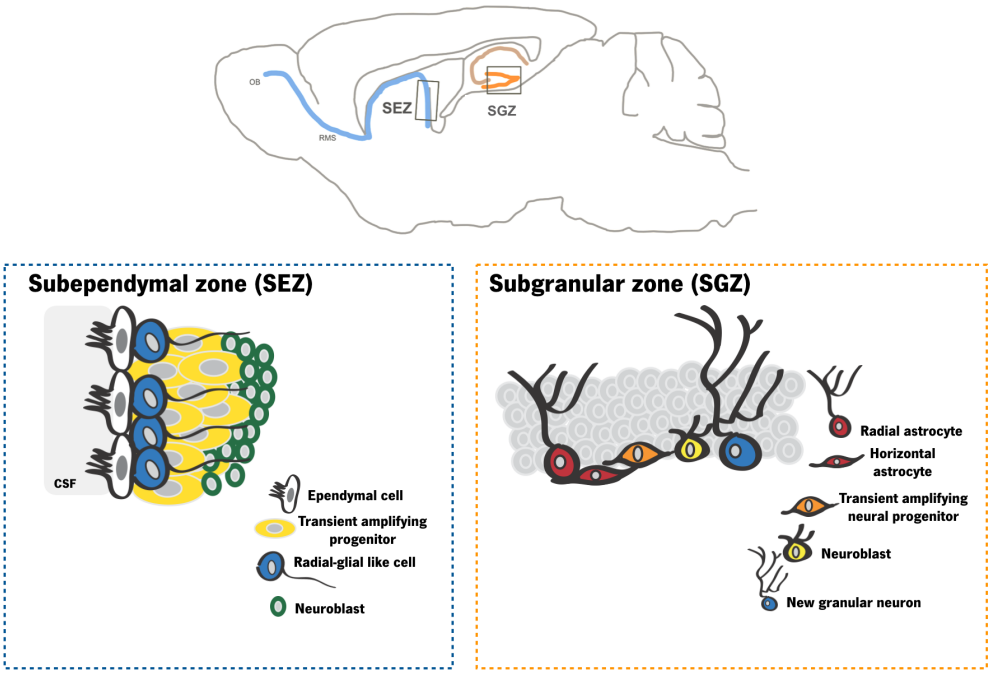



**Figure 2. The two neurogenic niches in the adult mammalian brain.**

Two regions of the adult mammalian brain are broadly recognized to be neurogenic under physiological conditions: (SEZ) Precursor cells residing in the walls of the lateral ventricles give rise to interneurons that integrate into the olfactory bulb. (SGZ) Neurogenesis in the adult hippocampal dentate gyrus generates new excitatory granule cells throughout life. These two processes of adult neurogenesis originate from different precursor cell populations, are independently regulated, and serve entirely different regions (Kempermann 2011). Abbreviations: CSF - Cerebral spinal fluid.

In the healthy brain, the functional importance of adult neurogenesis has been differently associated between the two most commonly accepted neurogenic niches. While the SEZ has already been associated with olfactory discrimination (Moreno et al. 2009), hippocampal neurogenesis has been related with memory, learning, pattern separation and even emotional behavior (Deng et al. 2010). Somehow the functional importance of adult neurogenesis in the SEZ has not yet been highly associated with a wide range of neuropathological conditions. However, there are already some studies reporting impairments in this specific neurogenic niche in neurodegenerative conditions, such as Alzheimer's disease (AD) (Curtis et al. 2007). Abnormal alterations in the hippocampal neurogenesis have been associated to a variety of pathologies, including neuropsychiatric disorders (Chambers 2013; Schoenfeld and Cameron 2015; Kang et al. 2016). Numerous research groups are trying to unveil the biological mechanisms underlying these disorders, also allowing us to better understand the process of hippocampal neurogenesis.

### 1.2.2. The hippocampal neurogenic niche: an overview of the adult hippocampal neurogenic process

In the adult mammalian brain, hippocampal neurogenesis produces new excitatory granule cells in the DG, in a highly regulated and complex process, that can be divided into four major steps: (1) the precursor cell phase, that comprises the proliferation of NSCs and the expansion of the precursor cell pool; (2) the early survival phase, during which the majority of the newborn cells are eliminated even before they make synaptic contacts with reach their target regions; (3) the post-mitotic maturation phase where it is possible to observe the functional integration of newly-born neurons into pre-established neural networks; and (4) the late survival phase, in which the establishment of new synapses with pre-existing surrounding cells is completed and a final selection occurs based on the newborn neurons functionality (Kempermann et al. 2004; Balu and Lucki 2009; Nicola et al. 2015) (Figure 3). Interestingly, this post-natal neurogenesis mimics the embryonic neurogenic process, but differing in the fact that, in the adult brain, the newborn neurons are generated in an already mature microenvironment, and as such they have to integrate the pre-existing neuronal circuits.

The SGZ of the hippocampal DG, contains a heterogeneous progenitor cell population with distinct degrees of stemness, that can be identified by a specific group of molecules, expressed by each cell type. These different molecules (presented in Figure 2) are strongly associated with the different phases of the adult hippocampal neurogenesis process. The type-1 progenitor cells, also known as quiescent neural progenitor (QNPs) cells and NSCs, are believed to be multipotent stem cells with

unlimited self-renewal capacities. These cells have astroglial and radial glia-like properties that can be further distinguishable into two classes: horizontal astrocytes (hA) and radial astrocytes (rA). The asymmetrical division of type-1 progenitor cells give rise to two consecutive stages of transient amplifying neural progenitor cells (ANPs): type-2a progenitor cells, followed by the type-2b progenitor cells. The main differences between these ANPs are their proliferative potential and its increasing stage of differentiation. It is in this phase of the neurogenic process that emerges a neuronal or non-neuronal lineage commitment, being for this reason, a decisive checkpoint in the determination of the neural progenitors' cell-fate. Different reports have demonstrated that these ANPs are highly mitotic cells with symmetric divisions (Doetsch et al. 1999; Encinas et al. 2006). But at some point they exit the cell cycle and enter into a postmitotic stage in which they give rise to neuroblasts (also known as type-3 progenitor cells) and establish network connections with the pre-existing neural circuits (Kempermann et al. 2004). These last cells are intermediate progenitors in the formation of new granule neurons, expressing the microtubule associated protein doublecortin (DCX) that will be crucial, to the maturation and migration of the newly-born cells into its final location in the GCL (Balu and Lucki 2009; Nicola et al. 2015). Here, they fully mature and integrate the pre-existing neural-circuits, elongating their axons and establishing new functional connections. It is currently assumed that the interval that takes to a newly-born cell to become a fully maturated and integrated granular neuron is typically referred to be approximately 4 to 5 weeks (Zhao et al. 2006; Zhao et al. 2008). Nevertheless, there are some authors who claim that the complete period of adult neurogenesis can take as much as 7 weeks, as this is the time needed by the new neurons to be electrophysiologically indistinguishable from the remaining pre-existing neuronal cells (Ambrogini et al. 2004).

Breaking down adult hippocampal neurogenesis in these few accessible phases opens up a new view on how neuronal development occurs under the condition of the adult hippocampus (Kempermann et al. 2004). The hippocampal neurogenic niche turns out to be a fined tuned complex process with many developmental steps sensitive to different regulatory influences. These regulatory mechanisms are still to be fully understood, but in the past years, several efforts have been made to comprehend the complex transcriptional and epigenetic orchestration of adult hippocampal neurogenesis.

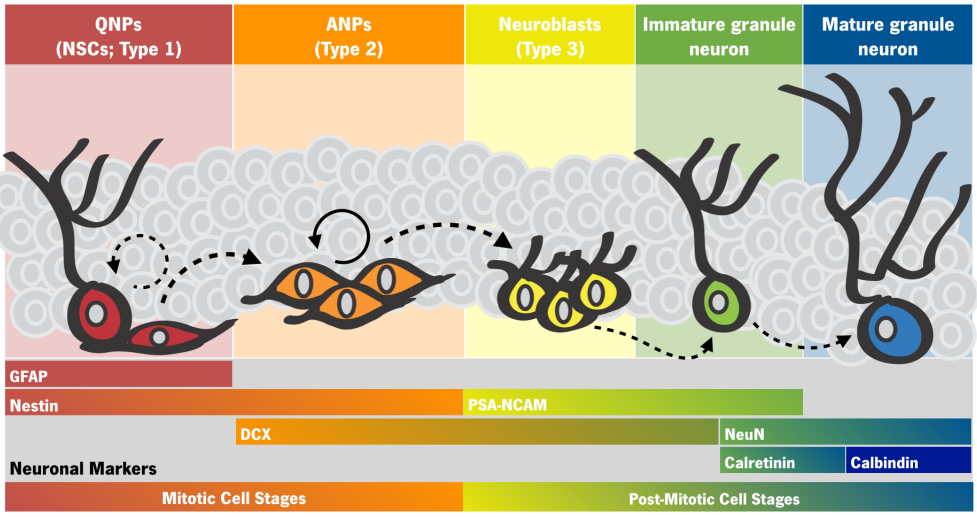



**Figure 3. Developmental stages in the adult hippocampal neurogenic process.**

Neuronal development in the adult DG encompasses several highly regulated steps. This process begins with the division of neural stem cells (NSCs), also known as quiescent neural progenitors (QNPs or type 1 progenitors), giving rise to amplifying neural progenitors (ANPs or type 2 progenitors). ANPs start to exhibit the first signs of cell-lineage commitment and eventually exit the mitotic phase to become neuroblasts (type 3 progenitors). Then, the neuroblasts will differentiate and migrate towards its final destination where they will integrate and maturate into fully mature granular neurons, establishing synapses within the pre-existing circuits. Each cell type can be distinctively identified by cellular and neuronal markers, some of which are indicated in the figure. It is currently accepted that the entire process of adult glutamatergic neurogenesis takes around 4-7 weeks. Abbreviations: GFAP - Glial fibrillary acidic protein; DCX - Doublecortin; PSA-NCAM - Polysialylated-neural cell adhesion molecule; NeuN - Neuronal Nuclei.

### 1.2.3. Transcriptional network underlying hippocampal neurogenesis: a focus on the transcription factor activating protein 2 gamma (AP2γ)

In the past years, there has been a great effort in the field to understand the transcriptional regulators involved in the hippocampal glutamatergic neurogenesis process, both in early developmental stages and also during adulthood. Complementing the current knowledge regarding the transcriptional network responsible for post-natal neurogenesis is highly relevant, so that new regulatory molecules could be used in repair and therapeutical strategies for neurological diseases. During cortical development, regulation of glutamatergic neurogenesis is controlled by a set of transcriptional factors including Pax6, Ngn2, Tbr2, NeuroD and Tbr1 (Englund et al. 2005). Interestingly, it was found that during post-natal glutamatergic neurogenesis, interneurons recapitulate this transcriptional sequence (Sox2→Pax6→Ngn2→Tbr2→NeuroD→Tbr1) that hallmarks the embryonic glutamatergic neurogenic process in the developing cerebral cortex (Brill et al. 2009).

Transcription factors such as Sox2, Pax6, Tbr2, Ngn2 and NeuroD have already been proved not only to participate in this transcriptional sequence, but to have key modulatory actions on the

glutamatergic neurogenic process. Sox2 transcription factor is also essential for pluripotency of the epiblast, embryonic stem cells (ESCs) and reprogrammed induced pluripotent stem cells. Moreover, Sox2 is expressed at early stages of CNS development and during post-natal neurogenesis by type 1 and type 2a cells. Previous studies have already reported that mutations and targeted ablation of this transcription factor leads to a reduced number of type 1 cells and decreased proliferation and glutamatergic neurogenesis in the SGZ of the hippocampal DG (Favaro et al. 2009). The same output was provided by animals with targeted deficiencies in Pax6, which has key modulatory actions in brain development and post-natal neurogenesis, being involved in the control of cell proliferation and neuronal fate determination (Maekawa et al. 2005). Furthermore, reduced expression of the transcription factor Tbr2 in the SGZ of the DG have already been showed to cause impairments in glutamatergic neurogenesis, since its highly involved in the coordination and regulation of the TAPs (Hodge et al. 2008). Likewise, studies with deletion and overexpression of Ngn2 and NeuroD, which are involved in granule neuroblasts production and neuronal fate specification, respectively, revealed the key actions of these transcripts in the modulation of the glutamatergic neurogenic process (Gao et al. 2009; Roybon et al. 2009).

Activating protein 2 gamma (AP2γ) is a transcription factor that integrates the transcriptional network regulating the glutamatergic neurogenic process, acting as a downstream target for Pax6, and being involved in the regulation of basal progenitors determinants, such as, Tbr2 and NeuroD (Figure 4). Importantly, AP2γ is critical for the specification of glutamatergic neocortical neurons and their progenitors (Pinto et al. 2009).

The AP2γ gene is part of the AP2 transcription factor family, which in mammals, comprises 5 members, AP2α, AP2β, AP2γ, AP2δ and AP2ε, all sharing common structural and functional features (Bosher et al. 1996; Oulad-Abdelghani et al. 1996; Moser et al. 1997; Zhao et al. 2001; Eckert et al. 2005). AP2 proteins have a conserved transcriptional activator domain at the amino-terminal end, acting as homo- or heterodimers, and their dimerization-binding mechanisms are mediated by a basic helix-span-helix motif (Pinto 2008). Furthermore, both dimerization as well as the basic domain are essential for DNA-binding. These family of transcription factors was also identified in chicken, Xenopus and bony fish (Eckert et al. 2005). The poor similarity between these homologs, their paralogs in *Drosophila* and *Caenorhabditis,* and the inexistence of AP2 transcription factors in yeast, is suggestive of a late emergence of these transcription factors in evolution and its predominance in vertebrate species (Eckert et al. 2005).

Generally, these AP2 proteins are recognized to be involved in various systems and biological processes (such as, cell proliferation, cell adhesion, developmental morphogenesis, tumor progression and cell fate determination), through the regulation of a large number of target genes with different biological functions (Batsche et al. 1998; Ebert et al. 1998; Maconochie et al. 1999). The different functions of these proteins seems to be largely dependent on their interaction partners in the spatially and locally defined system where they act. Many proteins are known to physically interact with this family of proteins, and therefore are influenced with their presence.

Throughout the developmental phases, AP2 family of transcription factors are often co-expressed, and their proteins seem to have, at least, partially redundant functions. However, different phenotypes are obtained with the deletion of a specific AP2 gene, and there is no resembles between the mutant of another member of this family (Pinto 2008). For instance, selective loss of AP2$\alpha$ leads to severe malfunctions in craniofacial features (both skeletal and epidermal tissue), first appearing at embryonic day (E) 9.5, which was supposed to be due to a significant increase in apoptosis of migratory neural crest cells at E9 (Schorle et al. 1996). In the knockout (KO) mice for AP2$\beta$ evident kidney abnormalities are found, during the embryonic development. At E16.5 the tubuli and collecting ducts undergo cystic transformation due to cell-autonomous apoptosis of renal epithelia (Moser et al. 1997). Both of these different phenotypes, resulting from the deletion of AP2$\alpha$ and AP2$\beta$, are lethal.

In mouse, AP2$\gamma$ (or Tcfap2c or Tfap2c according to the mouse genome informatics database) is expressed during developmental stages, both in central and peripheral nervous system, as well in the adult mouse forebrain (Pinto et al. 2009). In the developing mouse embryo, expression of AP2$\gamma$ was early detected in all trophoblast cells at day 3.5 (E3.5), and its expression is maintained in all trophoblast cell lineages, with higher expression levels laterally and rostrally, following the gradient of neurogenesis (Werling and Schorle 2002; Eckert et al. 2005). Expression levels further increase to mid-neurogenesis (E14) in the progenitor layer, declining from then on (Eckert et al. 2005). AP2$\gamma$ protein is also expressed in a subset of apical ventricular zone progenitors including the population that starts to express Tbr2 (Pinto et al. 2009). This protein is present in numerous regions of the adult mouse brain, specifically in the GCL of the adult cerebellum and in the white matter of the forebrain (Pinto et al. 2009). AP2$\gamma$ mRNA is highly expressed in both of the referred neurogenic niches (SGZ of the DG and SEZ of the lateral ventricles), and also highly expressed in the RMS, in the GCL, glomerular layer and mitral cell layer of the OB (Pinto, 2008; Mateus-Pinheiro et al. 2016). In the SEZ, AP2$\gamma$ is expressed in a group of bromodeoxyuridine (BrdU)-retaining stem cells, suggesting that the involvement of this

transcription factor might not be restricted to primordial developmental stages, and that can be involved in adult glutamatergic neurogenesis (Pinto, 2008).

All of the above observations strongly suggest that AP2γ functional role is not restricted to primordial developmental stages, being also involved in the modulation of the adult glutamatergic neurogenesis. Therefore, characterizing this transcription factor is crucial to understand its functional relevance in the regulation of the adult hippocampal neurogenic process. Recently published findings from our group, showed AP2γ transcription factor as a positive regulator of adult neurogenesis in the hippocampal DG, as its overexpression increments the generation of new neurons in this region, and its deletion, both *in vitro* and *in vivo*, results in a marked reduction of the neuroblasts population (Mateus-Pinheiro et al. 2016). Mechanistically, AP2γ acts as an effector of Sox2 and Pax6 in the promotion of Tbr2 expression in hippocampal progenitor cells. AP2γ expression produces a net effect in Tbr2 protein levels within the hippocampal DG (decreasing significantly when deleting AP2γ), suggesting that AP2γ regulates post-natal glutamatergic neurogenesis by mobilizing TAPs, rather than interfering with the NSCs pool. The presence of an alternative regulatory pathway using AP2γ as an intermediate transcription regulator, in parallel with direct regulation of Tbr2 by Pax6, suggest that AP2γ function may allow a fine-tuning of the neurogenic process, by either rapidly expanding or restricting the TAPs pool.

In summary, AP2γ is an important modulator of the adult hippocampal neurogenic process, and as such this transcription factor may be a promising target to use for novel therapeutical tools in pathological conditions in which neurogenesis is affected.

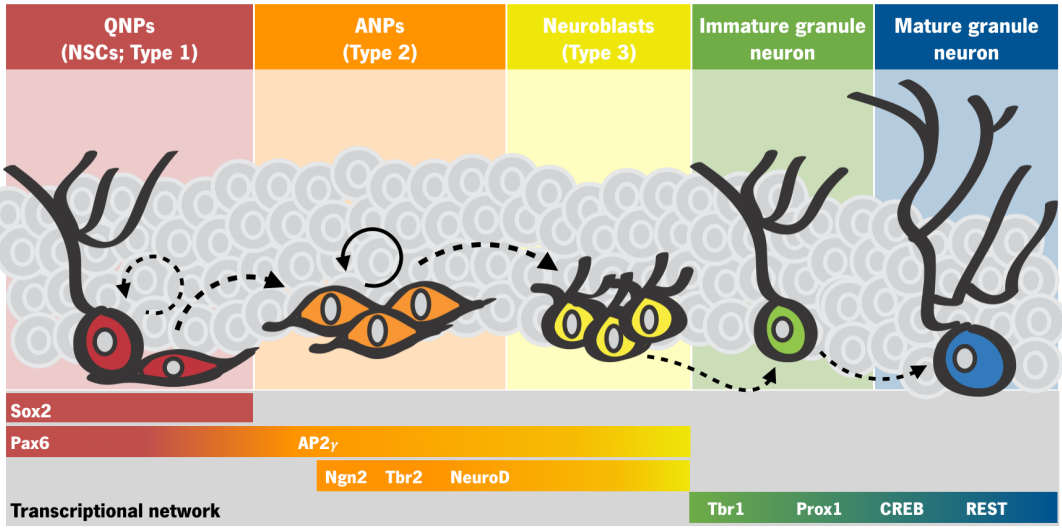



**Figure 4. Transcriptional factors involved in the regulation of the adult hippocampal neurogenic process**

The different neuronal developmental steps in the adult hippocampal neurogenic process are largely associated with the expression of different transcriptional factors. Sox2 is a transcription factor that controls the development of the nervous system from its earliest stages, being highly expressed by NSCs. Pax6 is highly involved in the brain development being highly expressed by type 1 and type 2a cells. AP2γ transcription factor, besides having key roles in developmental stages, integrates this transcriptional network, acting as a downstream target of Pax6 and having key modulatory actions upon Trb2 and NeuroD. Ngn2 transcription factor is expressed by type 2a cells, whereas Tbr2 and NeuroD are expressed by type 2b and type 3 cells, having key modulatory actions upon the neurogenic process. Tbr1 is expressed by immature neurons and granule cells, displaying important roles also in the cortical formation. Prox1 is also expressed in immature granular cells with specific roles in cell development. CREB and REST transcription factors are expressed by both immature and mature granule neurons, being involved in neuronal survival, fate choice and differentiation.

### 1.2.4. Epigenetic regulation of adult hippocampal neurogenesis: DNA methylation and DNA demethylation as epigenetic choreographers

The concept of epigenetics was first introduced almost a century ago to describe the molecular events that are involved in early embryonic development (Yao et al. 2016). Epigenetics is now widely accepted as the interface between genes and the environment, using for this, cellular processes that do not change the genomic sequence, but have the ability to elicit relatively persistent biological effects (Ma et al. 2010). Several mechanisms have been hardly associated with changes in gene expression, that do not arise from alterations in DNA sequence, among which we can find: DNA methylation, DNA demethylation, histone modifications, chromatin remodeling and regulation mediated by non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (Yao et al. 2016). Despite being a relatively recent concept in the neuroscience field, different epigenetic mechanisms have been linked with pivotal roles in different stages of neurogenesis. The participation of these epigenetic mechanisms in the regulation of NSCs proliferation, fate specification and differentiation, is now

becoming to be recognized as fundamental for the balanced production of new neuronal and glial cells, needed for the homeostatic brain function (Mateus-Pinheiro et al. 2011). Cumulative evidence now suggests that epigenetic dysregulation also plays an important part in neurodegenerative disorders, and more interestingly, in psychiatric disorders (Yao et al. 2016).

Epigenetic mechanisms are becoming gradually accepted to play dynamic roles in adult neurogenesis. Notably, the intracellular epigenetic program regulating adult neurogenesis is suggested to be quite similar to the epigenetic modulation occurring during the embryonic developmental neurogenesis, but is also determined by new extrinsic physiological and environmental stimulus that allow the alignment of neurogenesis with the external needs (Ninkovic and Gotz 2007). Even though epigenetics in the field of adult neurogenesis is still in its nascent stage, a global picture of the epigenetic involvement on this field begins to emerge (Figure 5).

Although there are different epigenetic mechanisms playing important and specific roles in adult hippocampal neurogenesis, in this work we mainly focused in two: DNA methylation and DNA demethylation.

#### 1.2.4.1. DNA methylation

DNA methylation involves the chemical covalent addition of a methyl group to the fifth carbon in the cytosine pyrimidine ring: that is, the production of 5-methylcytosine (5mC). Usually, studies of DNA methylation have focused on regions that enclose a high frequency of CG dinucleotides, which are commonly known as CpG islands (Montalban-Loro et al. 2015; Yao et al. 2016). In most mammalian, CpG islands are hypomethylated, which ensures genomic stability, imprinted gene silencing and X-inactivation. Interestingly, it was found that the majority of the dynamic DNA methylation in neurons does not occur at CpG islands and instead takes place in regions low in CpG densities (Yao et al. 2016). After the DNA methylation marks are established, a group of methyl-CpG-binding proteins behave as readers to interpret 5mC signal and mediate its function. Methyl-CpG-binding domain protein 1 (MBD1) occupies and protects the methylation of the promoter for basic fibroblast growth factor 2 (FGF2), which generates growth factors essential for the neural development (Yao et al. 2016). The depletion of MBD1 impairs adult hippocampal neurogenesis and genomic stability, due to a hypomethylation and depression of FGF2 in NSCs, resulting in this way in the failure of these cells to differentiate (Zhao et al. 2003; Li et al. 2009). Also involved in DNA methylation reading are many transcription factors with specific binding to methylated and unmethylated DNA motifs of distinct sequences (Hu et al. 2013). Thus, in contrast to the prevalent idea that 5mC nucleotides diminishes

transcription factor binding, DNA methylation increases the variety of binding sites for transcription factors highly known to be involved in the regulation of neurogenesis (Yao et al. 2016). However, there is the need to understand these binding sites specificities and their effect on gene expression during neurogenesis.

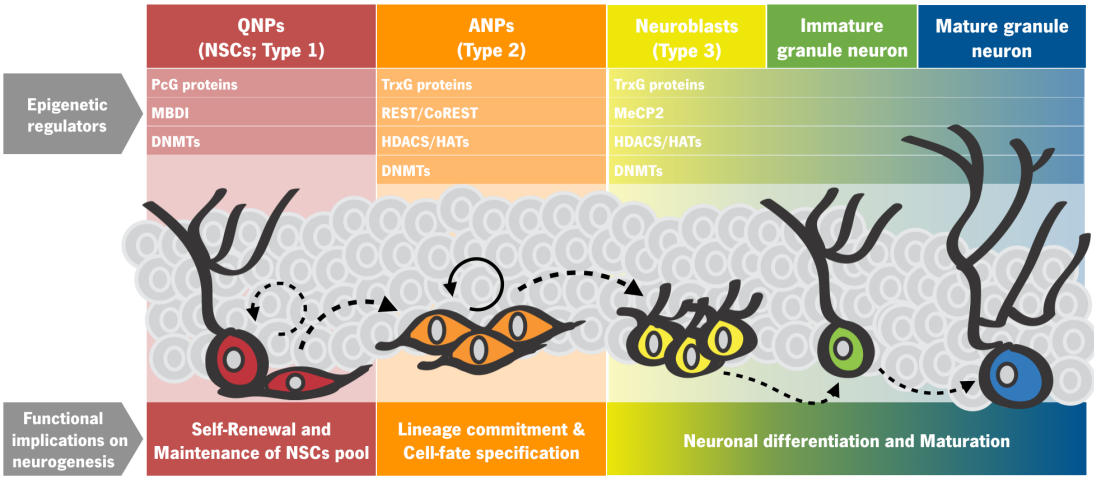



**Figure 5. Epigenetic regulators of the adult hippocampal neurogenic process.**

The adult hippocampal neurogenic process is exposed to a complex epigenetic regulation, with important functional implications. Distinct types of regulators have been identified and associated with different epigenetic mechanisms. Epigenetic regulators such as PcG protein and MDB1 are involved in the regulation of the initial steps of neurogenesis, contributing to NSCs self-renewal and maintenance. The transcriptional activation of specific genes by TrxG proteins, together with the action of chromatin remodeling complexes such as REST/CoREST complex and its molecular partners will allow the progenitor cells to exit the proliferation cycle and become committed to a neural cell lineage. The action of regulators like MeCP2, will contribute to post-mitotic neuronal differentiation and maturation. Some epigenetic regulators like HDACs, Hats and DNMTs are involved in several regulatory mechanisms of the adult neurogenic process, integrating several regulatory complexes involved in the transcriptional activation of pro-neurogenic genes.

DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs) that are responsible for maintaining or producing 5mCs on the genome. There are two types of methylation reactions, both mediated by DNMTs. The *de novo* methylation, which is catalyzed by DNMT3a and DNMT3b, is highly important for embryogenesis, neural development and the establishment of methylation patterns (Montalban-Loro et al. 2015). The other methylation reaction is promoted by the action of DNMT1, and is responsible to copy the existing methylation patterns during DNA replication for inheritance. DNMT1 is abundantly expressed in the embryonic, perinatal, and adult CNS in both dividing NSCs and mature neurons, where it maintains DNA methylation state, whereas, DNMT3a and DNMT3b are highly expressed in postnatal NSCs and are required for neurogenesis and neuronal maturation (Montalban-Loro et al. 2015; Yao et al. 2016). A mutation in any of the three major DNMTs genes in mice leads to severe developmental abnormalities and embryonic, or early postnatal lethality

(Li et al. 1992; Okano et al. 1999). In mice, a deficiency for DNMT1 leads to deficits of neuronal function and lethality in neural progenitors at embryonic stages (Montalban-Loro et al. 2015). *In vitro*, it was possible to see that the depletion of DNMT3a leads to gene silencing, and loss of DNMT3b promotes a deficient NSCs differentiation instead of proliferation (Martins-Taylor et al. 2012). Although at this point, there are some evidences regarding the functions of DNMTs in the neurogenic process, further studies are required to comprehend their genomic targets and their context-dependent roles.

#### 1.2.4.2. DNA demethylation

DNA methylation marks are reversible through both passive replication dependent demethylation and active demethylation. Involved in the active demethylation process are the ten-eleven translocation (TET) family of methylcytosine oxygenases, that in mammals comprise 3 members: TET1, TET2 and TET3. These enzymes promote an active DNA demethylation through the oxidation of 5mC into the recently characterized epigenetic marker 5-hydroxymethylcytosine (5hmC) (Tahiliani et al. 2009). In further studies it was revealed that the TET enzymes could further oxidize 5hmC to 5-formylcytosine (5fC) and then to 5-carboxylcytosine (5caC) (Yao et al. 2016).

The notion of DNA demethylation as a neurogenic choreographer emerged by the finding that growth arrest and DNA-damage-inducible protein 45β (GADD45β) promotes adult hippocampal neurogenesis (Ma et al. 2009; Yao et al. 2016). The GADD45 family members are highly associated with active DNA demethylation in different systems (Rai et al. 2008; Yao et al. 2016). In the embryonic and adult brain, GADD45β protein enhances promoter DNA demethylation and the expression of several genes, such as brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 1 (Fgf1), in glutamatergic neurons, which in turn promotes the proliferation of NSCs and generation of new neurons in the hippocampal DG (Yao et al. 2016). Genome-wide profiling revealed that 5hmC is relatively abundant in the mouse ESCs, in the early developing embryo and also in the adult brain (Montalban-Loro et al. 2015). During embryonic neurogenesis, 5hmC accumulates, as NSCs give rise to mature neurons, and its overall level continues to increase during ageing. By contrast, the differentiation of ESCs caused a reduction of 5hmC expression. Interestingly, the acquisition of 5hmC in several developmentally activated genes does not coincide with the demethylation of 5mC, confirming that 5hmC itself can serve as an epigenetic marker (Yao et al. 2016).

Until this moment, studies regarding the function of TET proteins in the brain are mostly focused on TET1, but it is already known that the different isoforms have preferences for distinct genomic sites to demethylate, suggesting in this way, that these proteins have independent but

interactive roles in neurogenesis (Santiago et al. 2014; Yao et al. 2016). TET1 was considered a key regulator for the progenitor cell pool in the hippocampal DG, since the KO mice for this gene exhibit a decreased number of NSCs in the adult SGZ, and these progenitor cells showed decreased proliferation capacities when isolated and grown as neurospheres (Huang et al. 2014). Regarding the function of both TET2 and TET3 in neurogenesis and in neuronal differentiation little is know, apart from some evidences showing that neuronal differentiation is accompanied by an upregulation of these proteins (Hahn et al. 2013). However, some insights regarding the role of TET3 in neurogenesis have emerged lately, through the depletion of this protein in *Xenopus laevis* embryos in which it was possible to see a repression of many key developmental genes also involved in neurogenesis, such as Pax6, Ngn2 and Sox2 (Yao et al. 2016).

Although there is some progress in the elucidation of the DNA demethylation role in the neurogenic process, there are still a lot of blank spaces. Importantly, we still need to understand how this epigenetic mechanism is influencing the adult neurogenic process, and its interactions with other epigenetic intervenients regulating adult hippocampal neurogenesis.

## 1.3. Implications of adult hippocampal neurogenesis deregulation in the etiopathogenesis of depression

### 1.3.1. Adult hippocampal neuroplasticity on the pathophysiology of depression

Adult hippocampal neurogenesis represents a crucial form of neuroplasticity in the hippocampal formation, which is a brain structure deeply involved in various neuropsychiatric disorders (Kang et al. 2016). Deregulation of adult hippocampal neuroplasticity is currently accepted to be involved in the pathophysiology of several neuropsychiatric diseases (Balu and Lucki 2009). As highlighted in the first part of this thesis, possibly one of the most striking findings in this scientific field was the discovery of adult neurogenesis imbalances involvement in depression, leading to the so called "neurogenic hypothesis of major depression" (Kempermann et al. 2008). This was the first theory connecting adult neurogenesis imbalances to this psychiatric disorder, and even more important, the first cellular hypothesis of depression (Kempermann et al. 2008). It postulates that impairments on the production of new neurons and reduced neuroplasticity may be related to depressive-like behaviors, based primarily on findings that stress inhibits adult hippocampal neurogenesis, and causes dendritic atrophy (Schoenfeld and Cameron 2015). This hypothesis is being highly supported by different evidences linking reduced neurogenesis to depressive-behavior, and by the observed pro-neurogenic

action of different antidepressant drugs (ADs) (Bessa et al. 2009a; Kang et al. 2016). However, some precautions have to be taken in consideration when we describe the association between the neurogenic process and depression. For example, different studies used specific methods to ablate hippocampal neurogenesis, like irradiation, and depressive-like phenotype was not induced in animals. More so, there are descriptions in the literature of ADs with both neurogenic-dependent and neurogenic-independent actions (Kang et al. 2016).

A third link between hippocampal neurogenesis and depression lies in the functional importance of this specific neurogenic process in behavioral domains commonly affected in depressive patients, like mood, anxiety and cognition (Figure 6) (Dupret et al. 2008; Bessa et al. 2009a; Clelland et al. 2009). The relationship between adult hippocampal neurogenesis and these 3 dimensions affected in depression is rather complex and not yet fully understood. However, enhancement of hippocampal neurogenesis via exercise, pharmacological, or genetic manipulations, was shown to induce anxiolytic and antidepressant-like effect in animals, suggesting that increased neurogenesis is sufficient to modulate these behaviors (Wu and Hen 2014; Hill et al. 2015; Kang et al. 2016).

In sum, hippocampal neurogenesis has a great impact in the pathophysiology of depression, both by being directly involved in the behavioral dimensions affected in depression or by indirectly mediating ADs efficacy in animals or patients suffering from this psychiatric disorder. As such, further insights in the mechanistic modulation of hippocampal neurogenesis in depression are needed, to more precisely evaluate the scientific validity of the neurogenic hypothesis of depression and possibly open up a new array of therapeutic targets to treat depression.

### 1.3.2. Transcriptional and epigenetic deregulation of adult hippocampal neurogenesis as a possible precipitator of depression

Given the crucial role of adult neurogenesis in several aspects of brain function, such as cognitive, emotional and mood regulation it is not surprising that dysregulation of this process may contribute to various brain disorders. During the last decade, cumulative evidences have emerged for the participation of genetic and epigenetic regulatory mechanisms in adult hippocampal neurogenesis. Thus, dysfunctions in these regulatory mechanisms might be a key mediator of the neurogenic imbalances observed in some neuropsychiatric disorders, such as depression.

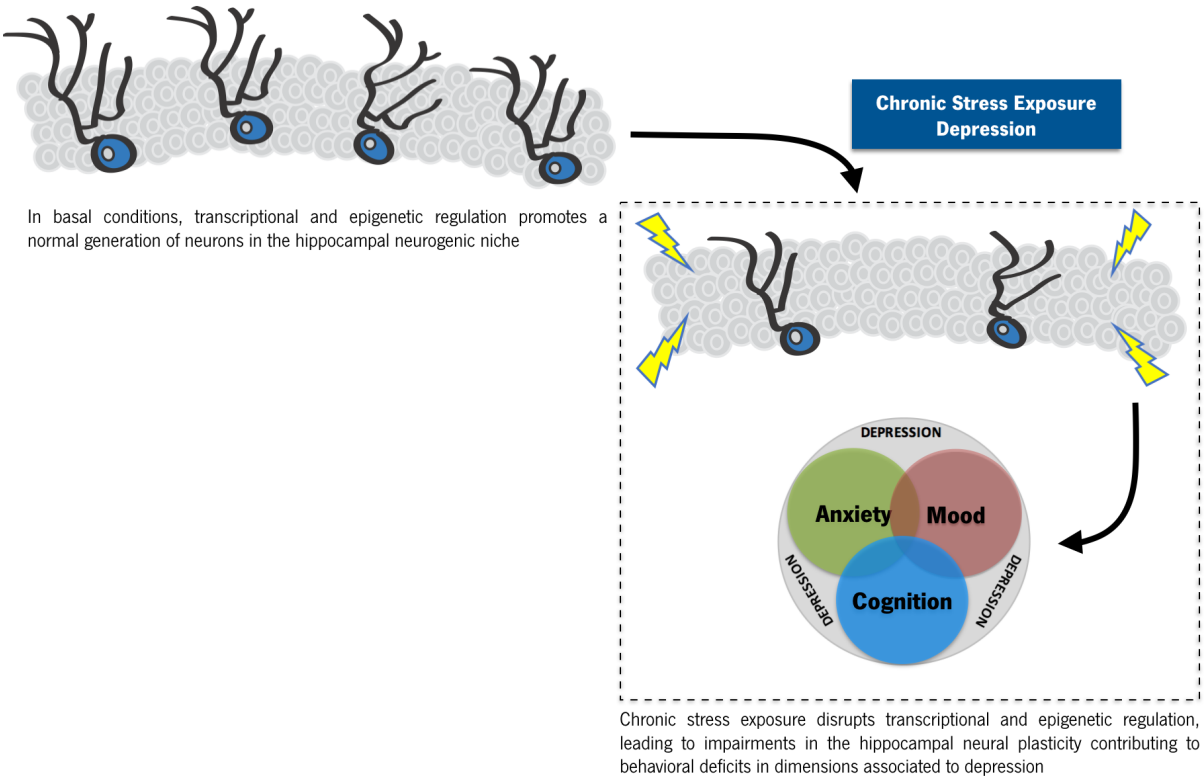



**Figure 6. Impact of hippocampal glutamatergic neurogenesis in depression.**

Chronic stress exposure leads to hippocampal neuroplastic impairments causing dendritic atrophy on pre-existing granular neurons and compromising the generation of new neurons. Impairments in the hippocampal neurogenic process can be attributed to dysfunctions in transcriptional and epigenetic regulators, possibly leading to the multidimensional behavioral deficits associated with depression.

Several studies aimed to identify genes deregulated in depressive and other stress-related disorders. In one such study, human hippocampal progenitor cells *in vitro* were treated with high levels of cortisol during the proliferation phase, to mimic the effects of chronic stress exposure (Anacker et al. 2013). Signaling pathway analysis of gene transcription revealed different pathways affected by the glucocorticoids treatment, including three well known to regulate adult neurogenesis, namely, the forkhead box protein O3 (FOXO3A) pathway, which was activated, the transforming growth factor-β (TGFβ)-SMAD2-SMAD3 and the Hedgehog pathway, which were inactivated (Egeland et al. 2015). Interestingly, gene transcription and subsequent analyses of hippocampal tissue from rodents exposed to prenatal stress showed similar results regarding the inactivation of Hedgehog and TGFβ-SMAD2-SMAD3 pathways. Despite the fact that *in vitro* and *in vivo* comparisons should be carefully made, the consistency of results between the human neuronal cell line and the rodent hippocampus suggests that these signaling pathways are likely to be very important in the regulation of post-natal neurogenesis by stress exposure (Egeland et al. 2015). Another study using a rodent model revealed several changes in

gene expression in the hippocampal DG after chronic stress exposure. In this study, exposure to a chronic stress protocol induced a decrease in the level of cAMP response element binding protein (CREB), impairing in this way this pathway and disabling the expression of genes involved in the adult glutamatergic neurogenic process (Datson et al. 2012). CREB is a transcription factor that controls adult neurogenesis, as its phosphorylation allows its binding to the brain derived neurotrophic factor (BDNF) promoter, thus modulating BDNF function (Kempermann 2011). BDNF has highly relevant roles in the adult glutamatergic neurogenesis process, being greatly involved in dendritic growth and spine maturation of hippocampal neurons. Moreover, in pathological conditions BDNF deficits leads to impairments in the adult neurogenic process, not only causing deficiencies in neuroplasticity but also impairing neuronal survival, differentiation and maturation (Kempermann 2011). Another indicator of the importance of BDNF in depressive conditions comes from the pharmacological action of ADs. One of the well known targets of ADs is indeed BDNF, which has an increased expression upon ADs chronic administration, leading to the reversal of neuronal atrophy and cell loss, further improving the behavioral output in anxiety, motivation and cognition (Martinowich et al. 2007).

Moreover, phosphorylated CREB highly co-localizes with polysialylated form of neural cell adhesion molecule (PSA-NCAM) in the hippocampal DG (Nakagawa et al. 2002). PSA-NCAM is a well known cell-adhesion molecule known to be involved in many facets of neural plasticity, playing important roles in synaptic plasticity and mediating effects of neurotrophic factors like BDNF (Wainwright and Galea 2013). This molecule is reduced in depressed patients and in animal models of depression while chronic treatment with ADs increases its expression. Importantly, in pathological conditions decreased expression of PSA-NCAM, leads to impairments in adult neurogenesis, in neurites outgrowth and in the maturation of the synapse sites of mature neurons (Wainwright and Galea 2013).

Although being still a controversial topic, several studies showed that impairments in epigenetic regulatory mechanisms of adult hippocampal neurogenesis are also affected in animal models of depression. This deregulation may contribute to aberrant expression of risk-associated genes causing negative impact on neurogenesis, and contributing in this way, to the pathophysiology of depression. A case supporting the epigenetic deregulation of adult neurogenesis in depression is the already mentioned methyl binding protein MBD1. In previous studies, by Allan and colleagues, showed that MBD1-deficient mice, besides having decreased NSCs proliferation, also displayed significant deficits in different behavioral dimensions affected by depression. These animals showed increased anxious phenotype both in elevated-plus maze (EPM) and light-dark box, cognitive deficits demonstrated during the execution of Morris water maze (MWM) spatial learning tasks and behavioral despair detected

through the forced swimming test (Allan et al. 2008). These different findings suggested that MBD1 binding protein impacts on adult neurogenesis epigenetic modulation, and consequently in the pathophysiology of depression.

Moreover, epigenetic regulators involved directly in post-mitotic neuronal maturation and differentiation, have also been related to different behavioral and cognitive impairments observed in various neuropsychiatric disorders, including depression. One well-known example is the methyl-CpG-binding protein 2 (MeCP2), reported to be involved in adult hippocampal neurogenesis and in the pathophysiology of major depression. This well characterized epigenetic regulator is highly responsible for the expression of BDNF. Mechanistically, MeCP2 specifically recognizes methylated DNA at a promoter of the BDNF and further recruits the transcription repressors histone deacetylases (HDACs) and SIN3A (Yao et al. 2016). Furthermore, it has been shown, using a MeCP2 KO mice, that deficiency in this binding protein causes reduction of hippocampal neurogenesis, severe deficiencies in the maturation of newborn neurons in the SGZ, including delayed differentiation and diminished dendritic spine density (Smrt et al. 2007). As such, MeCP2 may interfere in neurological pathways that mediate adult neurogenesis, and thus can be involved in its deregulation in a depressive situation. The DNMTs family of enzymes was also shown to be involved in epigenetic deregulation in depression, specifically causing impairments in memory formation (Miller and Sweatt 2007; Miller et al. 2008). Interestingly, an enzyme that belongs to this family, DNMT3b, has increased expression in depressive suicide completers (Poulter et al. 2008).

Another indicator of the involvement of epigenetic mechanisms deregulation involvement in the modulation of the neurogenic process and precipitation of depression can be endorsed by the pharmacological action of ADs in depression. The action of the antidepressant imipramine, a tricyclic agent with a well described pro-neurogenic action (Bessa et al. 2009a) and behavioral improving features in a socially defeated mice model, induces the downregulation of HDAC in the hippocampal region (Tsankova et al. 2006). Moreover, overexpression of specific HDACs, such as HDAC5, counteracts the positive behavioral effects of chronic treatment with imipramine in animal models. This deacetylase is involved in the regulation of adult hippocampal neurogenesis, controlling both newborn neurons maturation and also its survival (Mateus-Pinheiro et al. 2011). The clinical effectiveness as mood stabilizer of another used drug in depression, valproic acid (VPA), has been associated to its neurogenic improvement effect (Yu et al. 2009). Additional studies, proved that VPA alone, or in conjugation sodium butyrate or when administered with the antidepressant fluoxetine ameliorates performance in animal models of behavioral despair. Taking in consideration that VPA is an HDAC

inhibitor (HDACi), and that HDAC inhibition is a known target of adult hippocampal neurogenesis, these studies reinforced the importance of the pharmacological modulation of epigenetic regulators to the efficacy of some ADs (Mateus-Pinheiro et al. 2011).

Epigenetic deregulation is also involved in the vulnerability to stress, enhancing the susceptibility to stress-related disorders, such as depression. The role of epigenetics in stress is a quite recent concept, but it has been shown that in rats exposed to stress early in life, have an increased expression in a specific DNA binding protein REST (repression element 1 silencing transcription factor) – REST4. These animals showed higher susceptibility to high-levels of anxiety-like behavior and stress-induced impairments in some cognitive domains, like spatial reference memory and attention (Uchida et al. 2010). In a different study, using a mice model with ablation of a crucial component of a repressive chromatin complex, KAP1, this epigenetic regulator was shown to impact on anxiety, since these KO animals showed high-levels of anxiety-like behavior and other significant stress-induced impairments (Bredy et al. 2010). These referred epigenetic mechanisms are not directly involved in the modulation of adult neurogenesis, but are a great example of the importance that deregulation of epigenetic factors may have in increasing susceptibility to depression (Mateus-Pinheiro et al. 2011).

In summary, there are mounting evidences strongly suggesting the crucial role of different genetic and epigenetic regulators in the modulation of adult hippocampal neurogenesis in some neuropsychiatric disorders. Therefore, it is important to study its deregulation in pathological situations, in order to truly understand the mechanism underlying its function and comprehend if it is suitable to use in therapeutical approaches. This will open a new range of therapeutical possibilities, since it will facilitate the design of new molecule-directed drugs to counteract the neuropathological effects of major depression. Also, characterizing and understanding the functional roles of these neurogenic modulators can lead to gene-directed therapies aiming to have a neuroprotective role in emerging psychiatric disorders.

CHAPTER 2

# RESEARCH OBJECTIVES

# 2. RESEARCH OBJECTIVES

Previous studies from the group unveiled an important function of the transcription factor AP2γ in the modulation of adult glutamatergic neurogenesis and behavior. The central objective of this master thesis is to further explore the impact of this transcription factor in brain neurophysiology and behavior during development and at adult stages, dissecting also its underlying mechanisms both in basal and depressive states. For this, the work was divided into 2 major parts:

PART 1

In the first part of this thesis we characterized the constitutive AP2γ KO animal model, to discriminate if the molecular and behavioral deficits seen in the adult mice are indeed an adult response or if there is already an impact of AP2γ reduction on post-natal developmental stages and on juvenile stages. For this purpose, three objectives were designed:

1) Explore the impact of AP2γ on post-natal developmental;

2) Understand if reduction of AP2γ has impact on behavior of juvenile mice;

3) Elucidate the mechanism of action of AP2γ on the modulation of post-natal glutamatergic neurogenesis.

PART 2

In the second part of this thesis we aimed to bring some insights regarding the impact of AP2γ on depressive-like behavior. Therefore, we used both constitutive and conditional AP2γ KO animal models, and designed two main objectives:

1) Investigate the impact of AP2γ on behavior of depressive-like animals;

2) Study if AP2γ deficiency modulates adult hippocampal neurogenesis in depressive-like animals.

CHAPTER 3

# MATERIALS AND METHODS

# 3. MATERIALS AND METHODS

## 3.1. Animals

The experimental procedures performed in this master thesis were conducted in accordance with the Portuguese national authority for animal experimentation, Direcção-Geral de Alimentação e Veterinária (ID: DGAV4542). Animals were kept and handle in accordance with the guidelines for the care and handling of laboratory animals in the directive 2010/63/EU of the European Parliament and of the Council. Efforts were made to minimize the number of animals used and their suffering.

To all animals was given the standard diet (4RF25 during gestation and postnatal periods, and 4RF21 after weaning; Mucedola SRL, Settimo Milanese, Italy) and water *ad libitum*, with exception to the privation periods that occurred whenever the experiment required so. After weaning, animals were group-housed (3-5 per cage) and maintained under standard laboratory conditions on a 12/12h light/dark cycles (lights on at 8 a.m.) with an ambient temperature at 22°C, and relative humidity of 55%.

In order to address the proposed objectives two animal models were used. To fulfill the first part of this thesis the constitutive AP2γ KO animal model was used, and for the second part both constitutive and conditional AP2γ KO animal models were used.

Constitutive AP2γ KO animal model:

AP2γ heterozygous KO ($AP2\gamma^{+/-}$) mice (kindly provided by Dr. Hubert Schorle, Institute for Pathology, University of Bonn Medical School, Bonn, Germany) were maintained in a SV/129 background and identified the genotypes by PCR of genomic DNA. For the post-natal evaluation of the developmental milestones, male animals were divided according to its genotype: WT (n=9) and $AP2\gamma^{+/-}$ (n=9) animals. Due to the strong side effects of the developmental milestones protocol a different set of male animals was used for the juvenile behavior and histological evaluation, and once again the animals were divided according to its genotype: WT (n=12) and $AP2\gamma^{+/-}$ (n=10) animals. Adult male animals with 2 months of age were divided into four experimental groups: WT control mice (n=8), $AP2\gamma^{+/-}$ control mice (n=7), WT mice exposed to uCMS (n=8), and $AP2\gamma^{+/-}$ exposed to uCMS (n=7).

Conditional AP2γ KO animal model:

$AP2\gamma^{loxP/loxP}$ ($AP2\gamma^{fl/fl}$) conditional KO mice (being loxP a locus of crossing over of bacteriophage) and Glast:$CreER^{T2}$ mice were maintained on a SV/129 and C57Bl/6J background and the genotypes were identified by PCR of genomic DNA. loxP sites flanked exon 5, whose deletion caused a loss of the

helix-span-helix domain near the protein carboxyl terminus. AP2γ$^{fl/fl}$ mice were crossed with Glast:CreER$^{T2}$. Both male WT (AP2γ$^{+/+}$//Glast:CreER$^{T2+/-}$, henceforth referred to as cAP2γ$^{+/+}$) animals and KO (AP2γ$^{fl/fl}$//Glast:CreER$^{T2+/-}$, henceforth referred to as cAP2γ$^{-/-}$) mice with two months of age were divided into four experimental groups: cAP2γ$^{+/+}$ control mice (n=12), cAP2γ$^{-/-}$ control mice (n=5), cAP2γ$^{+/+}$ mice exposed to uCMS (n=12) and cAP2γ$^{-/-}$ mice exposed to uCMS (n=8).

## 3.2. Genotyping

After collecting tail tips, the samples were transferred into 300 μL of lysis solution (Citogene, Brazil) with 1.5 μL of Proteinase K (Thermo Scientific, USA) and incubated overnight at 55°C. In the following day, 100 μL of protein precipitation solution was added (Citogene), and after tissue lysis was completed, bones and hairs were removed through centrifugation [5 min at 13000 rotations per minute (rpm)]. The supernatant was transferred to 300 μL of cold isopropanol tubes that were inverted several times until the DNA precipitation was completed. Afterwards, the tubes were centrifuged for 5 min at 13000 rpm, the supernatant was rejected and the DNA pellet was washed with 300 μL of ethanol 70%. To remove the ethanol, a centrifugation was performed for 1 min at 13000 rpm, and the supernatant was carefully discarded, leaving the pellet to dry at room temperature (RT) for 1 h. The DNA was then dissolved in DNase and RNase free water (Sigma, USA) at 65°C for 1 h. DNA concentrations were accessed through the use of NanoDrop (ND-100 Spectrophotometer, Alfagene, Portugal) apparatus, and adjusted to a final concentration of 50 ng/μL.

To access the genotype of mice with one null allele of the AP2γ gene, it was used 2 μL of DNA (extracted like it was described) as a template for a multiplex polymerase chain reaction (PCR) using the primers presented in Table 1.

**Table 1. List of PCR primers sequence needed to genotype AP2γ gene and respective melting temperature (TM)**

| Primer abbreviation | Primer database code | Sequence | TM (°C) |
|---|---|---|---|
| **P1** | Ap2γ_In4down | 5'-aacaggttatcatttggttgggatt- 3' | 61 |
| **P2** | Ap2γ_Ex5up | 5'-caattttgtccaacttctccctcaa-3' | 62,5 |
| **P3** | Ap2γ_Ex6up | 5'-aatagtcagccaccgctttactagg-3' | 65 |

The primers P1 and P2 are used to identify the WT allele (343 bp), through an amplification of this allele, while the P3 primer allows us to identify the null allele (700 bp) of the AP2γ gene (Figure 7).

To genotype GLAST: CreER$^{T2}$ mice it was used 2 µL of DNA as a template for a multiplex PCR using the primers presented in Table 2.

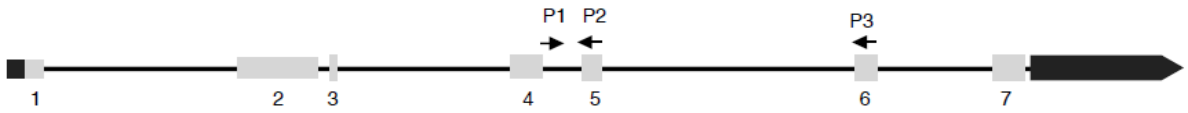



**Figure 7. Representation of the 6-kb genomic fragment of AP2γ.**

The exons are numbered and identified in grey. Primers P1 and P2 were used to amplify the WT allele, while the P3 was used to distinguish the null allele. Adapted from (Mateus-Pinheiro 2011).

**Table 2. List of PCR primers sequence needed to genotype GLAST: CreER$^{T2}$ mice and respective melting temperature (TM)**

| Primer abbreviation | Primer database code | Sequence | TM (°C) |
|---|---|---|---|
| **P4** | GLAST_F8 | 5'-gaggcacttggctaggctctgagga- 3' | 71 |
| **P5** | GLAST_R3 | 5'-caggagatcctgacccgatcagttgg -3' | 69 |
| **P6** | CER1 (CreER$^{T2}$ specific primer) | 5'-ggtgtacggtcagtaaattggacat-3' | 64 |

The primers P4 and P5 were used to amplify the WT allele (700 bp), while the P6 primer allowed to identify the recombinant allele (400 bp).

To perform the PCR, a thermal cycler (MWG AG Biotech Primus 96 plus, Cole-Parmer, USA) was used. The PCR tubes (Sarstedt, Germany), with a total volume of 20 µL of the master mix and reaction conditions, are presented in Table 3.

**Table 3. Primer mix composition and reaction conditions**

| Reagents | Master mix AP2γ genotyping | Master mix GLAST:CreER$^{T2}$ Genotyping | DNA template | AP2γ PCR conditions | GLAST:CreEr$^{T2}$ PCR conditions |
|---|---|---|---|---|---|
| **P1 (10µM)** | 1 µL | - | 3 µL (50ng/ µL) | 95°C 120'' | 94°C 120'' |
| **P2 (10µM)** | 1 µL | - | | **40 cycles of:** | **40 cycles of:** |
| **P3 (10µM)** | 1 µL | - | | 95°C 45'' | 94°C 20'' |
| **P4 (10µM)** | - | 1 µL | | 55°C 35'' | 55°C 20'' |
| **P5 (10µM)** | - | 1 µL | | 72°C 60'' | 72°C 30'' |
| **P6 (10µM)** | - | 1 µL | | 72°C 180'' | 72°C 300'' |
| **Taq Polymerase**[a] | 0.4 µL | 0.4 µL | | 4°C ∞ | 4°C ∞ |
| **Taq Buffer**[b] | 10 µL | 5 µL | | | |
| **Q solution (Quiagen, Germany)** | - | 6 µL | | | |
| **$H_2O$ (Sigma)** | 3.6 µL | 12.6 µL | | | |

[a] Nzytech Taq DNA Polymerase (recombinant) (5u.µL$^{-1}$)

[b] Nzytech Taq Buffer with 1 mM DTT; 20mM Tris-HCl (pH 7.5 at 25°C); 0.1M EDTA; 50% (v/v) glycerol

PCR products were separated in a 3% agarose gel stained with Greensafe premium (Nzytech, Portugal). The gel was obtained by mixing 100 mL of TAE (Tris base, Acetic acid and EDTA) buffer with 3 g of agarose (GeneON, UK), and then, 3 μL of *Greensafe premium* was added. After the gel polymerization was completed, 3 μL of 6xDNA loading dye (Fermentas, USA) was added to each sample, and 10 μL of the referred samples were charged in different wells. To guarantee a correct interpretation of the size bands, to each row of the gel 4 μL of 1kb DNA ladder (0.5μg/μL Generuler™ Fermentas) was loaded in 2 wells. The electrophoresis occurred at 100 V for a period of 30 min. After completion of the run, the gel was visualized on a cassette of the Geldoc™ IZ imager (Biorad, USA), and the image obtained was cut and cleaned using Image Lab™ Software (Biorad).

## 3.3. *In vivo* tamoxifen injections

To promote inducible excision of AP2γ, all AP2γ conditional KO animals groups (both cAP2γ$^{+/+}$ and cAP2γ$^{-/-}$) were injected with tamoxifen (T-5648, Sigma, USA). Tamoxifen was dissolved in corn oil (Sigma; C-8267) at 20 mg/mL and 1 mg was injected intraperitoneally (i.p.) twice a day for 5 consecutive days (Mori et al. 2006; Feil et al. 2009). After a 7 days' interval period, this process was repeated once more.

## 3.4. Unpredictable chronic mild stress protocol

Both animal models (AP2γ constitutive KO model and the AP2γ conditional KO model) were exposed to a unpredictable chronic mild stress (uCMS) protocol, which is described and employed in rats (Bessa et al. 2009b), but in previous studies from the group, the employment of this protocol with some adaptions induced core depressive-like symptoms in mice. Briefly, the uCMS protocol encompasses several mild stressors: confinement to a restricted space for 1 h; housing in an overcrowded cage for 1 h; housing in shaking cages for 2 h; overnight housing in titled cages; overnight damp bedding; exposure to stroboscopic lights during 4 h and noise exposure for 4 h. Animals were random exposed to these stressors during 6 weeks. Control animals were handled every week throughout the protocol. All behavioral analyses were conducted after the uCMS period (weeks 7 and 8) during the diurnal phase, between 09:00 am and 18:00 pm.

## 3.5. Serum corticosterone measurements

At the end of the uCMS protocol, blood samples were collected in order to assess the level of corticosteroids on both adult AP2γ KO animal models (AP2γ constitutive KO model: $n_{Control\ WT}$=8; $n_{Control\ AP2\gamma^{+/-}}$=8; $n_{uCMS\ WT}$=7; $n_{uCMSI\ AP2\gamma^{+/-}}$=7; AP2γ conditional KO model: $n_{Control\ cAP2\gamma^{+/+}}$=12 ;$n_{Control\ cAP2\gamma^{-/-}}$=5; $n_{uCMS\ cAP2\gamma^{+/+}}$=12 ;$n_{uCMSI\ cAP2\gamma^{-/-}}$=8). This was performed to evaluate if indeed the uCMS protocol induced core molecular alterations in the animals subjected to this stress protocol, since corticosterone is a major indicator of stress (stress increases the production of corticosteroids). Blood sampling (tail venipuncture) was performed during the diurnal nadir (N, 08.00-09:00) and nocturnal zenith (Z, 20:00-21:00), with an interval of 24 h in between. Serum was obtained by centrifugation (13000 rpm for 10 min) and stored at -20°C until further analysis. Subsequent serum corticosteroid levels were measured by corticosterone enzyme-linked immunossorbant assay (ELISA) kit, following the instructions provided by the manufacture (Enzo Life Sciences, Switzerland)

## 3.6. Behavioral tests

### 3.6.1. Developmental milestones

The assessment of the neurobehavioral neonatal development included the performance of a diverse and well-described range of tests (Lim et al. 2008). These tests were used to evaluate different neurologic parameters such as motor coordination, spatial and auditory reflexes, and strength development. This procedure was designed to allow a fast analysis so that several animals could be examined daily in a relatively short period of time, reducing in this way the stress induced in the mothers and litter (Lim et al. 2008).

The day of birth was considered as postnatal day (PND) 0, and after some experience with the protocol we decided to not perform any kind of test in this day due to be too traumatic for the mothers. From this day on, mice were daily inspected for the acquisition of developmental milestones and weight gain until PND21, the weaning day. To allow a fast identification of each mouse, pups were numbered and only after the weaning day, tail tips were cut to genotyping purposes.

On the day of testing, the home cage was moved into the testing room and left to habituate for at least 15 min. During the experimental performance, the pups were left in the same room as the mother and the separation time was minimized as possible. The experimenter was always the same

and the execution of each test was random, as well as the animal's order. The experiment procedures were conducted blind to genotype.

This developmental milestones protocol encompasses several tests that have to be performed in a specific a range of time during the 21 days (Table 4). The results of the tests have in consideration the time to accurately perform, or reply to a stimulus or posture. Thus, the latency of time (in seconds, s) that the animal took to perform the test was registered and later converted to dichotomic scores, as indicated in Table 5, allowing in this way a quantitative measurement of the behavior. The animal is considered to display the mature response on a specific test when the highest score is attributed for three consecutive days.

This developmental milestones protocol can be divided into two major categories of tests: test that assess somatic parameters and tests that evaluate neurobiological reflexes (Table 6).

**Table 4. Data sheet for developmental milestones**

Neonatal mouse pups were examined daily from PND 0 to 21 for performance on a wide range of developmental tests evaluating strength, motor coordination and the appearance of reflexes. The shaded areas identify the different tests performed on each day.

| Milestones tests | Postnatal day | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| Weight | | | | | | | | | | | | | | | | | | | | | | |
| Anogenital distance | | | | | | | | | | | | | | | | | | | | | | |
| Surface righting | | | | | | | | | | | | | | | | | | | | | | |
| Negative geotaxis | | | | | | | | | | | | | | | | | | | | | | |
| Cliff aversion | | | | | | | | | | | | | | | | | | | | | | |
| Rooting | | | | | | | | | | | | | | | | | | | | | | |
| Postural reflex | | | | | | | | | | | | | | | | | | | | | | |
| Wire suspension | | | | | | | | | | | | | | | | | | | | | | |
| Walking | | | | | | | | | | | | | | | | | | | | | | |
| Grasping | | | | | | | | | | | | | | | | | | | | | | |
| Auditory startle | | | | | | | | | | | | | | | | | | | | | | |
| Ear twitch | | | | | | | | | | | | | | | | | | | | | | |
| Open field | | | | | | | | | | | | | | | | | | | | | | |
| Air righting | | | | | | | | | | | | | | | | | | | | | | |
| Eye opening | | | | | | | | | | | | | | | | | | | | | | |
| Homing | | | | | | | | | | | | | | | | | | | | | | |

Somatic Parameters

As a measure for morphological development animals were daily weighed and the anogenital distance (which refers to the distance between the anus and the opening of the genitalia) was also measured. The opening of both eyes was inspected everyday and scored according to Table 5.

Neurobiological reflexes

**Surface righting,** PND1-13 (Labyrinthine reflex and body righting mechanism, strength and coordination). The mouse pup was held gently (in the four limbs) on its back and released. The time for the pup to flip over with all four paws touching the surface of the soft screen was registered, and later converted into dichotomic scores. If the animal did no responded within 30 s, the test was ended. When the pups were able to right themselves in less than 1 s for three consecutive days, the mature response was attributed.

**Negative geotaxis,** PND1-14 (Labyrinthine reflex and body righting mechanism, strength and coordination). The animal was placed head down on a square of a scratchy screen set at an angle of 45°. The latency of time for the pup to turn 180° to the "head up" position was recorded and converted to a dichotomic score. If the pup lost footing and slipped on the screen, the test was repeated once more. If the animal did not respond within 30 s, the test was stopped. The test was daily repeated and the mature response was attributed once the animal performed correctly the movement in less than 30 s for three consecutive days.

**Cliff aversion,** PND1-14 (Labyrinthine reflex and body righting mechanism, strength and coordination). The animal was placed on the edge of a small box, with the digits of the forepaws and the snout hanging over the box. The time that it took the animal to turn and begin crawling away from the edge was registered and later converted to a dichotomic score. If the pup lost footing and slipped off the box, the test was repeated once more. If the animal did not respond within 30 s, the test was stopped. The test was daily repeated and the mature response was attributed once the animal performed correctly the movement in less than 30 s for three consecutive days.

**Air righting,** PND8-21 (Labyrinthine reflex and body righting mechanism, strength and coordination). In this test the pup was held upside down and released from a height of approximately

30 cm, while its landing position was observed. The mature response was recognized when the animals landed on all four paws for three consecutive days.

**Table 5. Adaptation of time latency registered for each test into dichotomic scores**

[adapted from (Hill et al. 2008)]

| Milestones tests | Score | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| **Surface righting** | No response within 30 s | Rights itself but slowly (10 - 25 s) | Rights itself but it takes around 10 s to do it | Rights itself immediately |
| **Negative geotaxis** | No response within 30 s | Turns its body 180° to the "head up" position in less than 30 s | | |
| **Cliff aversion** | Freezes/does not respond within 30 s | Turns very slowly back to the surface (10 - 25 s) | Avoids the cliff, but it still takes some time to turn (up to 10 s) | Turn back in less than 3 s |
| **Rooting** | Does not move the head towards the filament | Moves the head towards the filament in one way | Moves the head towards the filament in two ways | |
| **Postural reflex** | Not present | Present | | |
| **Wire suspension** | Falls immediately | Grasps the bar with all four limbs (maximum time set 30 s) | | |
| **Walking** | No locomotion | Pivoting – moves around with the help of and forelimbs, but it does not uses the hind limbs | Crawling – Moves around with all four limbs, but it still drags the belly over the surface | Walking – Mature locomotion with body fully supported by the four limbs |
| **Grasping** | No grasping | Places its paws on the thin wire, but it does not hold on firmly | Places its paw on the thin wire, but when the stick is pulled, it can not hold it | Grasps the thin wire very firmly |
| **Auditory startle** | No response | Reacts to the sound | | |
| **Ear twitch** | Not present | Present | | |
| **Open field** | No response within 30 s | Moves out of the arena in less than 30 s | | |
| **Air righting** | Lands on its back | Lands on the surface with all four paws | | |
| **Eye opening** | Both closed | One eye open | Both eyes open | |

**Rooting,** PND1-12 (Tactile reflex and motor coordination). To perform this test a fine filament of a cotton bud was used. The filament was gently and slowly rubbed from front to back alongside of the animal's head. It was considered a successful test if the pup moved it head towards the filament. The test was repeated on the other side of the head to evaluate the appearance of this neurobiological reflexes on both sides. If the animal did not respond to the filament the test was repeated once. The mature response was recognized when the animals responded on both sides to the cotton filament for 3 consecutive days.

**Grasping,** PND5-21 (Freeing reflex). The mouse palm forelimb was stimulated with a thin wire. This reflex disappears with the development of the nervous system, and because of that the mature response was assumed to appear as soon as the animal grasped firmly the thin wire immediately.

**Postural reflex,** PND5-21 (Reflex). The pups were placed in a small plastic box and gently shaken up and down and left and right. When the animals were able to maintain its original position in the box by extending all four paws, it was assumed that the animals acquired the mature response.

**Auditory startle,** PND7-18 (Auditory reflex). In this test the animals were placed on a laboratory bench and had to react to a handclap at a distance of 10 cm. When the pups responded correctly with a quick involuntary jump for three consecutive days it was presumed that the animals developed the mature response.

**Ear twitch,** PND7-15 (Tactile reflex). To execute the test a fine filament twisted from a cotton swab was used to gently brush the ear of the pups three times. The animal was considered to accomplish a mature response when it responded by flattening the ear against the side of the head for three consecutive days.

**Wire Suspension,** PND4-14 (Strength). The animal, hold with its forepaws in a 3-mm diameter metal wire suspended at 5 cm above a soft surface, was released and the interval of time that the pup maintained grasping the thin wire was acquired. If the pup immediately failed off, the test was repeated once more. The mature response was achieved when the animal was able to grasp the bar holding it with all four paws.

**Open field,** PND8-21 (Locomotive coordination). To execute this test, the animal was placed in a small and adapted arena (a circle with 13 cm in diameter), and the interval of time taken to move was recorded. If the pup was not able to move, the test was ended. When the mouse was able to leave the circle in less than 30 s in three consecutive days, it was considered that the animal presented a mature response.

**Walking,** PND5-21 (Locomotive coordination and muscular strength). In this test, the animals were able to freely move around on the laboratory bench for 60 s. The mature response was achieved when the animals were able to move with the body completely supported by the four limbs.

**Homing,** PND12 (Locomotive coordination and olfactory capabilities). Individual animals were placed in the center of a standard cage containing 1/3 of bedding from the home cage and the remaining of the cage with new clean bedding. In this test it was assessed the latency to turn and move onto the home cage bedding in three separate trials. Each trial lasted a maximum of 120 s with an inter-trial interval of 10 s. The mouse was considered to recognize the home cage bedding when all four paws had crossed onto the home bedding and the pup remained there for at least 10 s.

**Table 6. Summary of each test analyzed and its evaluated dimension**

| Milestones tests | Somatic | Reflexes | Coordination | Strength | Vestibular | Olfactory |
|---|---|---|---|---|---|---|
| Weight | | | | | | |
| Anogenital distance | | | | | | |
| Eye opening | | | | | | |
| Surface righting | | | | | | |
| Negative geotaxis | | | | | | |
| Cliff aversion | | | | | | |
| Air righting | | | | | | |
| Rooting | | | | | | |
| Wire suspension | | | | | | |
| Walking | | | | | | |
| Open field | | | | | | |
| Grasping | | | | | | |
| Postural reflex | | | | | | |
| Auditory startle | | | | | | |
| Ear twitch | | | | | | |
| Homing | | | | | | |

### 3.6.2. Elevated-plus maze

The elevated-plus maze (EPM) is a widely used behavioral assay for rodents and it has been validated to access mechanisms underlying anxiety-related behavior (Walf and Frye 2007). The assessment of anxiety-like behavior of the rodents is possible through the ratio of time spent on the open arms to the time spent on the closed arms. Unlike other behavioral tests used to evaluate anxiety responses upon the presentation of noxious stimuli, that typically produce a conditioned response, the EPM relies on rodents tendency to explore novel environments (Bailey and Crawley 2009). Despite of this tendency, mice tend to prefer dark, enclosed spaces (approach) and have an unconditioned fear of heights/open spaces (avoidance) (Walf and Frye 2007). This approach-avoidance conflict results in behaviors that have been correlated with increases in physiological stress indicators.

A black propylene-made EPM apparatus (ENV-560; MedAssociates, USA) was used, and it basically consists in two opposite open arms (50.8x10.2 cm) in perpendicular orientation towards two additional opposite closed arms (50.8x10.2x40.6 cm), elevated 72.4 cm above the floor. The central area connecting both arms measured 10x10cm. Each animal was individually positioned in the center of the maze, facing an edge of a closed-arm and was allowed 5 min of exploration. Behavior parameters were recorded using an infrared photobeam system. Videos were analyzed using the video tracking software Ethovision XT (Noldus, Netherlands).

### 3.6.3. Open Field test

The open field (OF) is a commonly used test to evaluate anxious-like behavior and measure exploratory behavior and general activity in rodents. In order to do that, different parameters have to be measured: for instance, to evaluate the movement it is necessary to take in consideration distance traveled and time spending moving. Anxiety-like behavior can be assessed by measuring the activity in the center of the arena and comparing it with the activity that the animal has in the peripheries of the OF (Bailey and Crawley 2009). Normally decreased time and ratio central/total locomotion or latency to enter in the central part of the arena is an indication of anxiety-like behavior (Prut and Belzung 2003).

The OF apparatus consisted of a highly illuminated square arena of 43.2x43.2 cm, closed by a 30.5 cm high wall. Mice were placed individually in the center of the OF arena and their movement was traced for 20 min (in juvenile animals) and 5 min (adult mice), using a 16-beam infrared system. The resulting data was analyzed using the Activity Monitor software (MedAssociates).

### 3.6.4. Forced Swimming test

Learned-helplessness was evaluated through the forced swimming test (FST) for the assessment of depressive-like behaviors. The experimental parameters employed in the FST were conducted using a slightly modified protocol described by Porsolt and colleagues 1977 (Porsolt et al. 1977). Briefly, each animal was individually placed in glass cylinders filled with water (23°C; depth 30 cm) for 5 min. Test sessions were video-recorded and the immobility time was measured using the video tracking software Ethovision XT (Noldus). Through this software it was possible to define different parameters to ashore that indeed the animal is immobile. A mouse was judged immobile when it ceased all active behaviors (struggling, swimming and jumping) and remained passively floating or making minimal movements need to maintain the nostrils above water. To assess learned-helplessness, the first 3 min of the single session trial was considered as a habituation period and the last 2 min as the test period. Therefore, only the last 2 min of the trial were considered for behavioral analysis.

### 3.6.5. Tail suspension test

The tail suspension test (TST) is also a commonly used behavioral test to assess depressive-like features in mice. The principle of this behavioral paradigm is highly similar to the FST, evaluating also the learned-helplessness of the animals. In this test, mice were suspended by the tail to the edge of a laboratory bench 80 cm above the floor (using adhesive tape), during 6 min (both for juvenile and adult mice). Trials were video-recorded, and immobility and climbing time was measured through the use of video tracking software Ethovision XT (Noldus). To assess learned-helplessness the first 3 min of the single session trial was considered as a habituation period and the last 3 min as the test period. Therefore, only the last 3 min of the trial were considered for behavioral analysis.

### 3.6.6. Sucrose splash test

The sucrose splash test, henceforth referred as splash test, as described by Yalcin and colleagues (Yalcin et al. 2008) consists in spraying a 10% sucrose solution on the dorsal coat of mice in their home cage. Because of its viscosity, the sucrose solution dirties the coat and induces grooming behavior. After applying the sucrose solution, the time spent grooming was video-recorded for a period of 5 min as an index of self-care and motivational behavior, and then manually analyzed using the behavioral scoring program Observador v.0.2.7 (Department of Pharmacology, University of Athens). Due to the simplicity of this paradigm, this behavioral test was only applied in the juvenile AP2γ constitutive KO animal model to analyze if there were any differences between WT and AP2$\gamma^{+/-}$ animals.

### 3.6.7. Cognitive function assessment

For the assessment of the cognitive function in both adult AP2γ KO animal models two well described behavioral paradigms were used, the Morris water maze (MWM), and the contextual fear conditioning (CFC) tests.

#### 3.6.7.1. Water Maze Tasks

The water maze tests were used to assess the performance in spatial reference and behavioral flexibility tasks as described by Bessa and colleagues (Bessa et al. 2009b). These tests were conducted in a circular white pool (170 cm diameter) filled with water at 22°C to a depth of 34 cm in a room with extrinsic clues (triangle, square, cross and horizontal stripes) and a dim light. The water tank was divided in four quadrants by imaginary lines and a clear acrylic platform (12 cm diameter; 30 cm high), was placed in one of the quadrants. Trials were video captured by a video tracking system (Viewpoint, Champahne au mont d'or, France).

Working memory task:

The working memory task (Cerqueira et al. 2007) was used to estimate the cognitive domain that relies on the interplay between the hippocampal and pre-frontal cortex (PFC) functions. The goal of this task, that results from adaptations on the original spatial reference memory test (Morris 1984), is to evaluate the capacity of animals to learn the position of the clear and hidden platform and to retain this information for four consecutives trials. The platform was placed in one of the quadrants, and it was maintained in the same position during the four daily trials. This task was evaluated during a period of 4 days, and in each day the platform was repositioned in a new quadrant in a clockwise-fashion. In each of the daily trials animals were positioned in a different starting point (north, east, west, and south) and a trial was considered terminated when the platform was reached within the time limit of 120 s. If the animals did not reach the platform during the trial time, they were guided to the platform and allowed to stay in it for 30 s. The time of escape latency and the path described to reach the platform (distance swam) were recorded for each trial.

Reference memory task:

Through this water maze task, it was possible to assess the hippocampal dependent cognitive function. Briefly, in order to determine this task, the platform remained for 4 days in the same quadrant and animals were tested in 4 daily trials according to the same procedure previously described in the

working memory task. The time of escape latency and the path described to reach the platform (distance swam) were recorded for each trial.

Reversal learning task:

After conducting tests for 4 days, maintaining the platform in the same quadrant, the reversal learning performance of animals (which is a PFC dependent function) was tested by positioning the platform in a new (opposite) quadrant. Animals were tested in 4 trials according to the same procedure previously described. The time of escape latency and the path described to reach the platform (distance swam) were recorded for each trial.

#### 3.6.7.2. Contextual fear conditioning (CFC)

The CFC test was conducted in white acrylic chambers with internal dimensions of 20 cm wide, 16 cm deep and 20.5 cm high (MedAssociates), with a fixed light bulb mounted directly above the chamber to provide illumination. Each chamber contained a stainless steel shock grid floor inside a clear acrylic cylinder, where animals were placed. Animal were exposed to two probes, a context probe and a cue (light) probe, as previously described by Gu and colleagues (Gu et al. 2012). Mouse freezing behavior was monitored via video cameras and manually scored with the help of the behavioral scoring program Observador v.0.2.7 (University of Athens). The CFC procedures were conducted over 3 days.

Day 1: Mice were placed in the conditioning white chamber (Context A) and received 3 pairings between a light (20 s) and a co-terminating shock (1 s, ≈ 0.5 mA). The interval between pairings was set as 180 s, and the first tone presentation commended 180 s after the mouse was placed into the chamber. At the end of the three pairings, mice remained in the chamber for a further 30 s before being returned to their home cage. The chambers were cleaned with a 10% ethanol solution between each trial.

Day 2: for the context probe, animals were placed in the white chamber (Context A), where they were originally shocked, 24 h after the light-shock pairings. Freezing behavior was measured during 3 min. Two hours later, animals were put in a modified version of the chamber (Context B) that was sheeted with a black plasticized cover that was previously sprayed with vanilla scent, in order to alter both spatial and odor references; the ventilation was not operated, and the experimenter wore a different style of gloves and lab coat. Again, freezing behavior was measured for 3 min. The freezing behavior state was defined as the complete privation of motion, for a minimum of 1 s. The chambers were cleaned between each trial with a 10% ethanol solution.

Day 3: For the cue probe, test setting for Context B were maintained and animals were placed inside the chamber 24 h after the context probe. After 3 min, the light bulb was turned on during 20 s and freezing behavior was subsequently measured during 1 min.

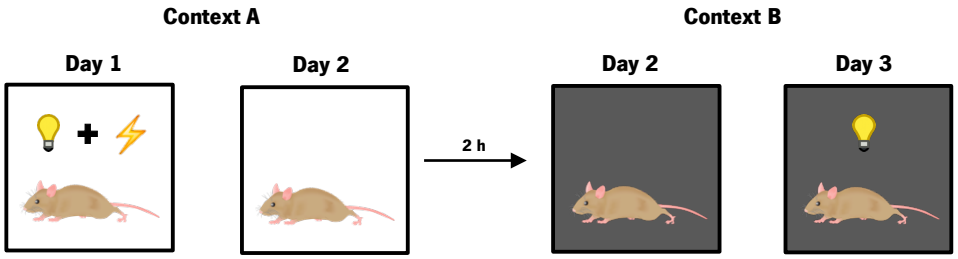



**Figure 8. Schematic representation of the CFC protocol.**

## 3.7. BrdU labeling *in vivo*

To assess the effect of AP2γ on the proliferation of fast-dividing progenitor cells, and comprehend its influence in the hippocampal neurogenic process of depressive-like animals, all animal groups were injected intraperitoneally with a thymidine analogous that is incorporated in the DNA during the S-phase (Taupin 2007), the bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU; 50 mg/kg) at the end of all behavioral test, and sacrificed 24 h later.

## 3.8. Fixation of mice brains and tissue processing

At the moment of sacrifice all animals were deeply anesthetized with a ketamine (75 mg/kg; Imalgene, USA) and medetomidine (1mg/kg; Syba, USA) solution, and then, different steps were performed depending on the purpose that we had for the sacrificed animal.

For vibratome sectioning: After anesthetized animals were transcardially perfused with cold 4% paraformaldehyde (PFA) in 0.1M PBS 1x solution. Brains were carefully removed from the skull and post fixed in 4% PFA for 12 h at 4°C, rinsed in phosphate buffered saline (PBS) and stored in a 30% sucrose/PBS solution with 0.1% azide at 4°C. Brains were coronal processed at the vibrotome (Leica VT 1000S, Germany) with a thickness of 40 - 50 µm, extending over the entire length of the hippocampal formation.

For macrodissection: Animals that were destined to different tests like western-blots and quantitative real-time PCR were only perfused transcardially with a 0.9% saline solution, and brains were carefully removed, after decapitation, and different brain regions were macrodissected: PFC,

cortex, dorsal and ventral hippocampi, dorsal and ventral DG, SEZ, amygdala and cerebellum. The different brain regions were immediately stored at -80°C until need for testing.

## 3.9. Immunostainings

Initially, the coronal vibratome processed sections were rinsed in PBS buffer prior to the staining procedure. Eventual pretreatment of the sections is mentioned in the list of used antibodies Table 7. The coronal sections were then washed in PBS with 0.5% of Triton-X100 (Sigma; 3 times for 15 min), since the antibodies used are intracellular markers. Afterwards, the blockage of non-specific binding was performed with PBS containing 10% fetal bovine serum (FBS, Biochrom, Germany). Then sections were incubated overnight at 4°C with primary antibodies (specified in Table 7) diluted in PBS-Triton with 10% FBS. The sections were washed in PBS-Triton several times. Secondary antibodies (Table 8), dissolved in PBS-Triton, were added and incubated for 2 h at RT. All coronal sections were stained with 4,6-diamidino-2-phenylindole (DAPI, 1:100, Invitrogen, USA) and mounted with immune-mounting medium (Immu-Mount, Thermo Scientific). Finally, images were obtained at the confocal microscope (Olympus FluoViewTM FV1000, Germany) for further analyses. Solutions composition is described in Table 9.

**Table 7. List of primary antibodies**

| Primary Antibody - Specie | Working dilution | Pretreatment | Company |
|---|---|---|---|
| **DCX – Rabbit** | 1:100 | 30 min HCl (2M) + 15 min in pre-heated Cytrate buffer | Abcam (UK) |
| **BrdU – Rat** | 1:50 | | |

**Table 8. List of secondary antibodies**

| Secondary Antibody - Antigenicity | Working dilution | Company |
|---|---|---|
| **Alexa Fluor 568 – Anti-rabbit** | 1:1000 | Invitrogen |
| **Alexa Fluor 488 – Anti-rat** | 1:1000 | Invitrogen |

**Table 9. Solutions compositions**

| Solution | Composition |
|---|---|
| **PBS** | 1,3 M NaCl, 27 mM KCl, 15 mM $KH_2PO_4$, 83 mM $NA_2HPO_4$, pH 7.6 |
| **PBS-T** | PBS 0.1%, Triton 0.5% |
| **PFA 4%** | PBS, 4%, pH 7.4 |

## 3.10. Western blot

DG of both adult constitutive and conditional AP2γ KO animal models were carefully macrodissected after decapitation. Tissue was weighted and homogenized in RIPA buffer [containing 50 mM Tris HCl, 2 mM EDTA, 250 mM NaCl, 10 % glycerol, 1 mM PMSF protease inhibitors (Roche, Switzerland) and then sonicated (Sonics & Materials, USA) for 2 min. The samples were centrifuged for 25 min at 10.000 rpm at 4°C. The protein concentration of the supernatant was determined using Bradford assay. Samples with equal amounts of protein, 30 μg, were analyzed using the following primary antibodies: beta-actin (Life Technologies, mouse, 1:200), AP2γ (Abcam, goat, 1:250), Pax6 (Millipore, rabbit, 1:2000), Sox2 (Millipore, rabbit, 1:500) and Tbr2 (Abcam, rabbit, 1:500). Secondary antibodies were purchased from BioRad (anti-mouse, 1:10.000; anti-rabbit, 1:10.000) and Santa-Cruz Biotechnologies (anti-goat, 1:5000). Membranes were developed using ECL Clarity reagent (BioRad) and developed in ChemiDoc XRS System from BioRad. After developing, images were quantified using ImageLabTM Software (BioRad).

## 3.11. Quantitative real-time PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) is a method used to amplify complementary DNA (cDNA) copies of RNA using a reverse transcriptase. Sensitive and versatile, RT-PCR is used to clone the 5' and 3' termini of mRNAs and to generate large cDNA libraries from very small amounts of mRNA. Quantitative real-time PCR (qRT-PCR) combine conventional PCR methodologies with a detection mechanism and quantification by fluorescence, allowing to monitor the progress of the PCR as it occurs (i.e., in real time). Using sequence-specific primers, the number of copies of a particular RNA sequence can be determined. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. The amount of cDNA is measured via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules generated. In the present study, these techniques were used to analyze the expression levels of specific genes involved in epigenetic mechanisms.

### 3.11.1. RNA extraction

Dorsal and ventral DG of adult constitutive AP2γ KO animal model were carefully dissected after decapitation. The RNA from theses tissues was then separately extracted by the Direct-zol RNA mini prep kit following the instructions provided by the manufacture (Direct-zol RNA mini prep, Zymo Research, USA).

### 3.11.2. cDNA transformation and quantitative Real-time PCR

The cDNA synthesis was performed using iScript™ cDNA Synthesis Kit (BioRad, USA). 1μg of purified RNA was used as template and volumes normalized with nuclease-free water. The complete reaction mix (RNA sample, 5x iScript reaction mix and iScript reverse transcriptase, Table 10) was incubated for 5 min at 25°C followed by 30 min at 42°C and for final 5 min at 85°C, in a thermal cycler (Applied Biosystems, USA).

**Table 10. cDNA mix composition**

| Components | Volume per Reaction |
|---|---|
| **5x iScript reaction mix** | 4 μL |
| **iScript reverse transcriptase** | 1 μL |
| **Nuclease-free water** | x μL |
| **RNA sample** | x μL |
| **Total volume** | 20 μL |

Differences in gene expression were assessed by qRT-PCR. For quantitative gene expression analysis, the cDNA was subject to PCR amplification using Eva Green technology (Ssofast Evagreen supermix, BioRad) on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA) in order to obtain the real-time detection of PCR products. Reaction solution was obtained with 5 μL of Ssofas Evagreen supermix, plus 10 μM from each primer, forward and reverse (Table 11). As template 1 μL of cDNA was used. The cycling conditions applying in this procedure were 30 s at 95°C for the enzyme activation, followed by 40 cycles of 5 s at 95°C for denaturation, 5 s at 60°C for annealing and 5 s at 72°C for extension step (Table 12). As reference gene Hspcb was used. The fold increase was determined using the $2^{-\Delta\Delta_{Ct}}$ (Livak) relative quantification method (Livak and Schmittgen 2001; Cardozo et al. 2012).

**Table 11. List of PCR primers used to amplify genes associated with epigenetic mechanisms**

| Gene | Primer sequence | Product size (bp) |
|---|---|---|
| **HSPCB** | Forward 5'-GCTGGCTGAGGACAAGGAGA -3'<br>Reverse 5'-CGTCGGTTAGTGGAATCTTCATG-3' | 93 |
| **DNMT3a** | Forward 5'- CCTGCAATGACCTCTCCATT -3'<br>Reverse 5'- CAGGAGGCGGTAGAACTCAA-3' | 89 |
| **DNMT3b** | Forward 5'- TGGTGATTGGTGGAAGCC -3'<br>Reverse 5'- AATGGACGGTTGTCGCC -3' | 145 |
| **TET1** | Forward 5'- CCATTCTCACAAGGACATTCACA -3'<br>Reverse 5'- GCAGGACGTGGAGTTGTTCA -3' | 116 |
| **TET2** | Forward 5'- GCCATTCTCAGGAGTCACTGC -3'<br>Reverse 5'- ACTTCTCGATTGTCTTCTCTATTGAGG -3' | 120 |
| **TET3** | Forward 5'- GGTCACAGCCTGCATGGACT-3'<br>Reverse 5'- AGCGATTGTCTTCCTTGGTCAG -3' | 104 |

**Table 12. PCR mix composition and reaction conditions**

| Mix Components | Volume (μL) | cDNA template | PCR conditions |
|---|---|---|---|
| **Evagreen Supermix** | 5 | 1 μL | 95°C, 30 s<br>95°C, 5 s<br>60°C, 5 s<br>72°C, 5 s |
| **Primer Forward** | 0.5 | | |
| **Primer Reverse** | 0.5 | | |
| **Nuclease-Free Water** | 3 | | |

## 3.12. Data analysis

Statistical analysis was performed using IBM SPSS Statistics v.22 (IBM Com, USA) and graph's representation using GraphPad Prism v.6 (GraphPad Software, La Jolla, USA).

In order to compare the mean values for two groups, a two-tailed independent-sample t-test was applied. If an evaluation along time was required, a repeated measures ANOVA was performed. On the other hand, statistical evaluation for animal behavior tests and histological analysis for the adult mice, a two-way ANOVA was used to compare the interaction between two variables. Moreover, when we analyzed the corticosterone levels (nadir and zenith) presented in the animals' serum was used a Paired t-test.

Normality was measured using the Kolmogorov-Smirnov and Shapiro-Wilk statistical tests and taking into account the respective histograms and measures of skewness and kurtosis. Equality of variances and Sphericity were measured using the Levene's and Mauchly's tests, respectively, and was assumed when $p>0.05$. Multiple comparisons between groups were accomplished through the Bonferroni statistical test.

Values were accepted as significant if the p-value was higher than 0.05 and all results were expressed as group mean ± SEM (standard error of the mean). Effect size was calculated using the Cohen's d or $\eta^2_{partial}$.

## 3.13. Experimental design

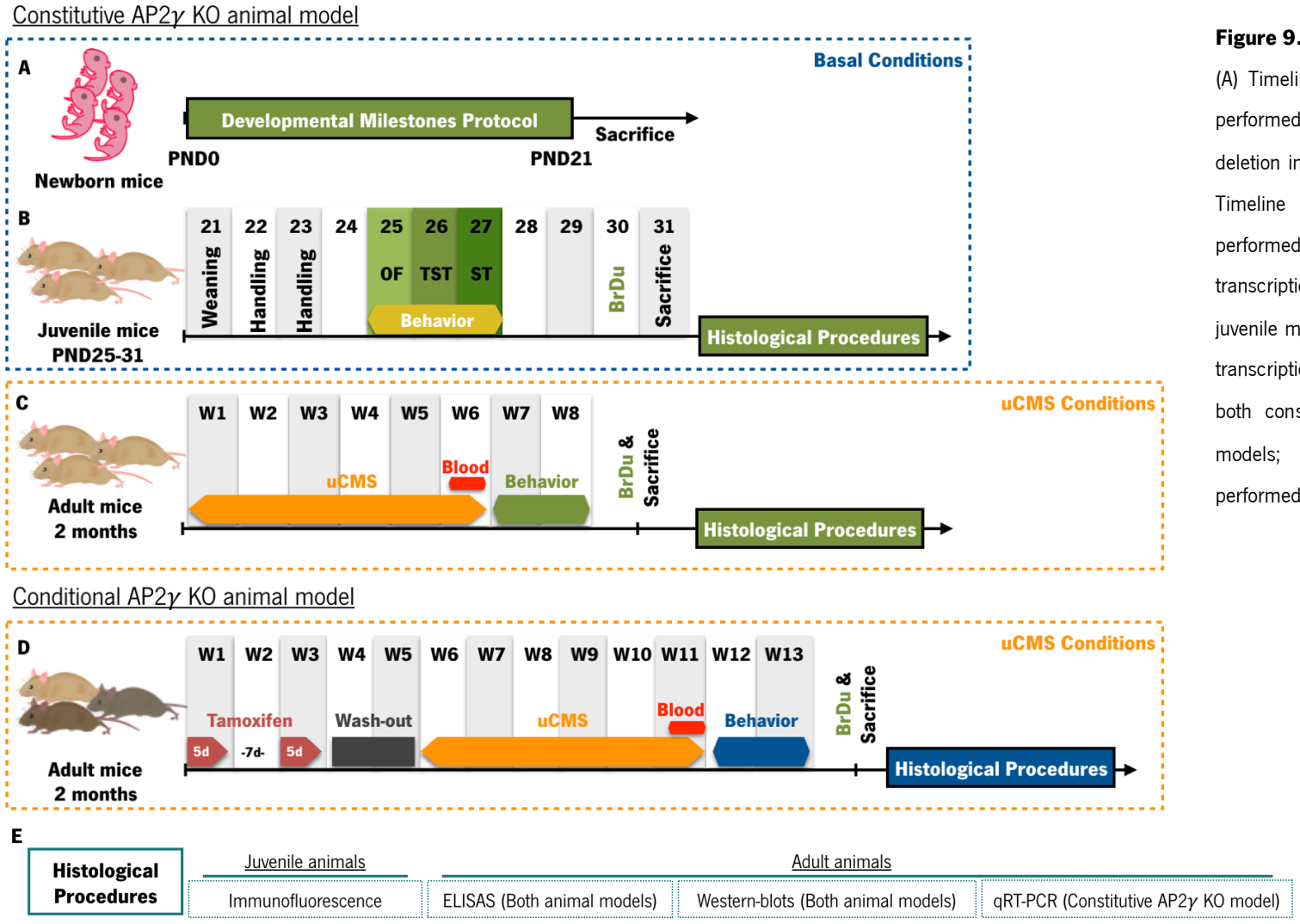



**Figure 9. Experimental design**

(A) Timeline of the experimental procedures performed to assess the impact of AP2γ deletion in the developmental milestones; (B) Timeline of the experimental procedures performed to study the impact of the transcription factor AP2γ on the behavior of juvenile mice; (C, D) Functional impact of the transcription factor AP2γ in Depression, in both constitutive and conditional AP2γ KO models; (E) Histological procedures performed in this study.

CHAPTER 4

# RESULTS

## 4.1. Functional impact of the transcription factor AP2γ in the neurodevelopment

### 4.1.1. Developmental milestones assessment

The results of the different behavioral parameters analyzed are presented as the average of three independent experimental sets and summarized in Figure 10 and Table 13-14 (presented as the average of day of mature response within the 21 days of the milestones protocol).

Somatic Parameters

When evaluating the general body weight and anogenital distance throughout the 21 days of the milestones protocol no differences where found between genotypes (Figure 10A-B and Table 13). Another hallmark for somatic development is the day of eye opening and for that we had constitutively registered the state of eyes opening until both eyes were found opened. We found that AP2γ$^{+/-}$ animals opened both of their eyes around one day earlier than the WT group (AP2γ$^{+/-}$ animals group opened both of their eyes around PND11 and the WT group around PND12) (Figure 10C and Table 13).

**Table 13. Summary of body weight and anogenital distance throughout the 21 days of the milestones protocol and eye opening day**

Data presented as mean ± SEM. In grey shading is the somatic parameter where differences between groups were observed.

| Milestones test | | WT | AP2γ$^{+/-}$ | Statistical test, significance, effect size |
|---|---|---|---|---|
| Weight | **Day 1** | 1.62 ± 0.07 | 1.57 ± 0.09 | $F_{(1,16)}$=0.32, p=0.58, $\eta^2_{partial}$=0.019 |
| | **Day 2** | 1.83 ± 0,10 | 1,97 ± 0.17 | |
| | **Day 3** | 2.16 ± 0.14 | 2,37 ± 0.14 | |
| | **Day 4** | 2.59 ± 0.19 | 2,79 ± 0.16 | |
| | **Day 5** | 3.07 ± 0.25 | 3,20 ± 0.15 | |
| | **Day 6** | 3.56 ± 0.27 | 7,60 ± 0.14 | |
| | **Day 7** | 4,03 ± 0.45 | 4,36 ± 0.19 | |
| | **Day 8** | 4,58 ± 0.47 | 5,00 ± 0.14 | |
| | **Day 9** | 5,16 ± 0,46 | 5,43 ± 0.14 | |
| | **Day 10** | 5,81 ± 0,38 | 5,97 ± 0.13 | |
| | **Day 11** | 6,43 ± 0.36 | 6,59 ± 0.11 | |
| | **Day 12** | 7,00 ± 0.42 | 7,20 ± 0.12 | |
| | **Day 13** | 7,41 ± 0.42 | 7,66 ± 0.14 | |
| | **Day 14** | 8,02 ± 0.35 | 8,08 ± 0.15 | |
| | **Day 15** | 8,35 ± 0.32 | 8,41 ± 0.03 | |
| | **Day 16** | 8,52 ± 0.36 | 8,58 ± 0.15 | |
| | **Day 17** | 8,62 ± 0.40 | 8,58 ± 0.14 | |
| | **Day 18** | 8,60 ± 0.46 | 8,73 ± 0.13 | |
| | **Day 19** | 8,60 ± 0.50 | 8,89 ± 0.12 | |
| | **Day 20** | 8,68 ± 0.50 | 9,09 ± 0.15 | |
| | **Day 21** | 8,99 ± 0.53 | 9,22 ± 0.16 | |

| | | | | |
|---|---|---|---|---|
| **Normalized Anogenital Distance (AD/Weight)** | **Day 3** | 0.75 ± 0.07 | 0.81 ± 0.08 | $F_{(1,16)}$=0.87, p=0.37, $\eta^2_{partial}$=0.051 |
| | **Day 4** | 0.80 ± 0.07 | 0.80 ± 0.1 | |
| | **Day 5** | 0.72 ± 0.05 | 0.82 ± 0.05 | |
| | **Day 6** | 0.71 ± 0.06 | 0.73 ± 0.04 | |
| | **Day 7** | 0.76 ± 0.11 | 0.73 ± 0.03 | |
| | **Day 8** | 0.76 ± 0.09 | 0.73 ± 0.25 | |
| | **Day 9** | 0.72 ± 0.08 | 0.73 ± 0.02 | |
| | **Day 10** | 0.70 ± 0.03 | 0.76 ± 0.02 | |
| | **Day 11** | 0.70 ± 0.22 | 0.74 ± 0.15 | |
| | **Day 12** | 0.65 ± 0.02 | 0.67 ± 0.01 | |
| | **Day 13** | 0.65 ± 0.02 | 0.67 ± 0.01 | |
| | **Day 14** | 0.64 ± 0.02 | 0.65 ± 0.01 | |
| | **Day 15** | 0.64 ± 0.02 | 0.68 ± 0.01 | |
| | **Day 16** | 0.64 ± 0.02 | 0.68 ± 0.01 | |
| | **Day 17** | 0.64 ± 0.02 | 0.70 ± 0.01 | |
| | **Day 18** | 0.67 ± 0.03 | 0.71 ± 0.01 | |
| | **Day 19** | 0.69 ± 0.03 | 0.71 ± 0.01 | |
| | **Day 20** | 0.72 ± 0.02 | 0.72 ± 0.01 | |
| | **Day 21** | 0.72 ± 0.02 | 0.72 ± 0.01 | |
| **Eye opening** | | 12.38 ± 0.18 | 11.33 ± 0.17 | $t_{(15)}$= 4.22, p= 0.0007, Cohen's d= 2.06 |

Neurobiological reflexes

In the parameters examined to evaluate the physical maturation of motor reflexes, coordination and strength needed for motor skills no major differences were observed in surface righting, cliff aversion and air righting tests (Figure 10D, F-G and Table 14). However, in the negative geotaxis test the AP2$\gamma^{+/-}$ group seems to acquire the mature response later than the WT group, with the mature response of the first group around 1.3 days later than the WT (Figure 10E and Table 14).

Regarding the evaluation of postural reflex, meaning the posture that animals present when they are gently shacked, no impairments were found between groups, with all animals exhibiting the mature response around PND9 (Figure 10H and Table 14). The same results were observed for the more specific tests used to assess reflexes, namely rooting, ear twitch, auditory startle and grasping where all animals regardless of genotype achieved a mature response around PND7, PND16, PND9 and PND8 respectively (Figure 10I-L and Table 14).

In the wire suspension test, which is used for strength measurements, we found that AP2$\gamma^{+/-}$ animals acquire the mature response earlier than the WT group, appearing it around 2.3 days later than the WT group (Figure 10M and Table 14). As for the tests specifically used for motor coordination assessment, namely walking and open field, no differences were found between genotypes, with an average of maturation day around PND10 for walking and PND8 for open field (Figure 10N-O and Table 14).

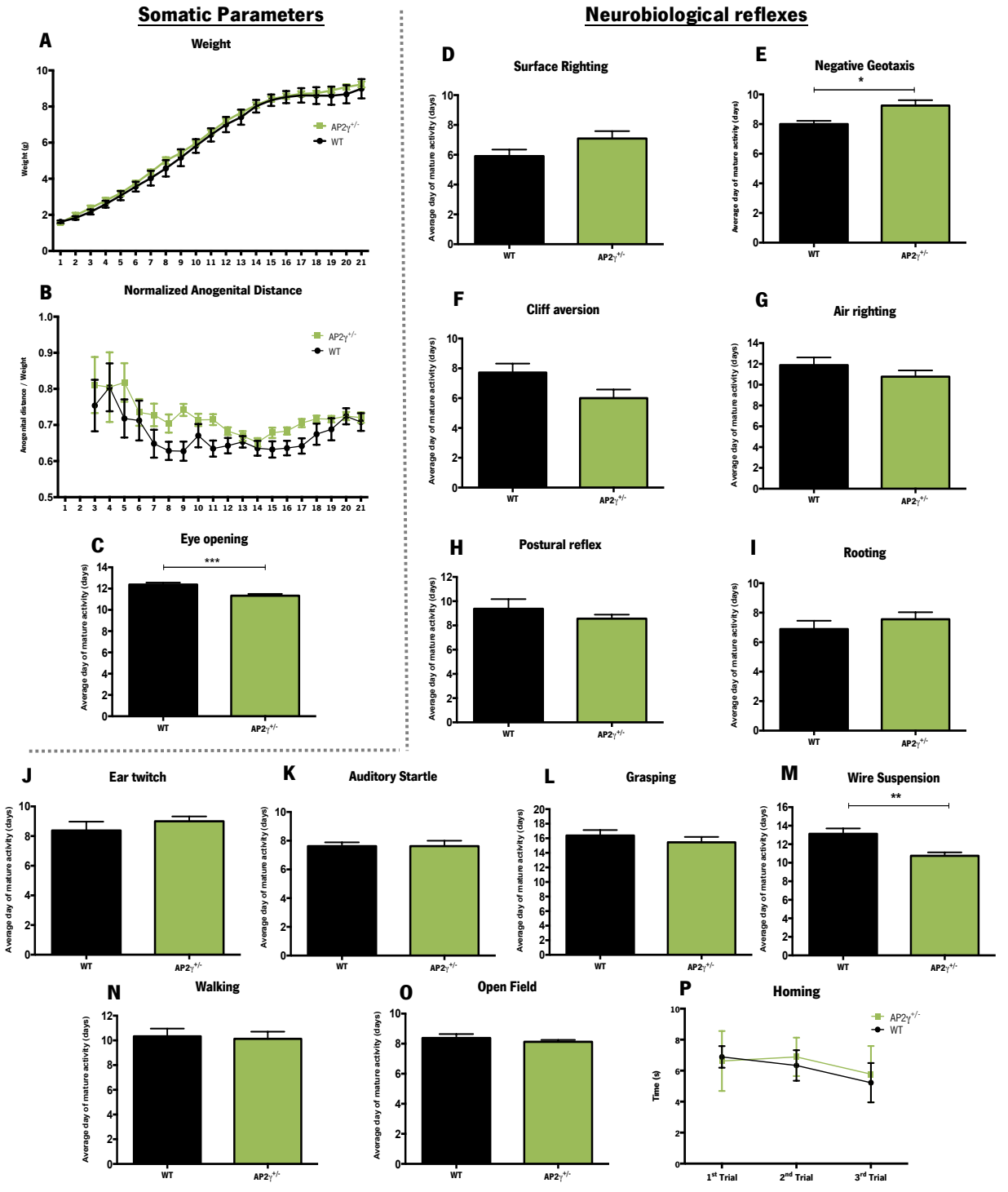



**Figure 10. The constitutive deletion of one allele of AP2γ impact on the milestones development and maturity.**

Using a set of established tests used to evaluate both somatic and neurologic parameters acquisition, from PND0 until PND21, AP2γ$^{+/-}$ animals were found to have an anticipated eye opening (C) day, and also an anticipated mature response in the strength test wire suspension (M). Through the milestones protocol it was also possible to unveil a delayed mature response of the AP2γ$^{+/-}$ group in the negative geotaxis test (E). All tests are presented as the average day of mature activity ($n_{WT}$= 9; $n_{AP2\gamma^{+/-}}$=9. Data presented as mean ± SEM, *<0.05; **<0.01; ***p<0.001

Through the homing test (Figure 10P and Table 14), which was performed at PND12, it was not possible to observe any major impairment between groups, suggesting that there were no olfactory and motor deficits in these animals, in both genotypes.

**Table 14. Summary of the average days of mature response obtained in each genotype during the neurobiological reflexes milestones assessment.**

Data presented as mean ± SEM. In grey shading are the tests where it was observable differences between groups.

| Milestones test | | WT | AP2γ$^{+/-}$ | Statistical test, significance, effect size |
|---|---|---|---|---|
| **Surface righting** | | 5.9 ± 0.44 | 7.0 ± 0.49 | $t_{(20)}$= 1.79, p=0.09, Cohen's d= 0.76 |
| **Negative geotaxis** | | 8.0 ± 0.22 | 9.3 ± 0.37 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 1.49 |
| **Cliff aversion** | | 7.7 ± 0.61 | 6.0 ± 0.58 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 1.09 |
| **Air righting** | | 11.9 ± 0.73 | 10.8 ± 0.60 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 0.55 |
| **Postural reflex** | | 9.4 ± 0.80 | 8.6 ± 0.34 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 0.47 |
| **Rooting** | | 6.9 ± 0.56 | 7.6 ± 0.47 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 0.43 |
| **Ear twitch** | | 8.4 ± 0.60 | 9.0 ± 0.33 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 0.46 |
| **Auditory startle** | | 7.6 ± 0.26 | 7.6 ± 0.38 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 0 |
| **Grasping** | | 16.4 ± 0.75 | 15.4 ± 0.75 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 0.43 |
| **Wire suspension** | | 13.1 ± 0.58 | 10.8 ± 0.37 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 1.73 |
| **Walking** | | 10.3 ± 0.62 | 10.1 ± 0.58 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 0.11 |
| **Open field** | | 8.4 ± 0.26 | 8.1 ± 0.13 | $t_{(13)}$= 2.83, p=0.01, Cohen's d= 0.43 |
| **Homing** | **Trial 1** | 6.89 ± 0.44 | 6.89 ± 1.32 | $F_{(1,16)}$=0.11, p=0.74, $\eta^2_{partial}$= 0.007 |
| | **Trial 2** | 6.33 ± 1.12 | 6.89 ± 1.12 | |
| | **Trial 3** | 5.22 ± 1.57 | 5.78 ± 1.57 | |

### 4.1.2. The transcription factor AP2γ impact on behavior of juvenile mice

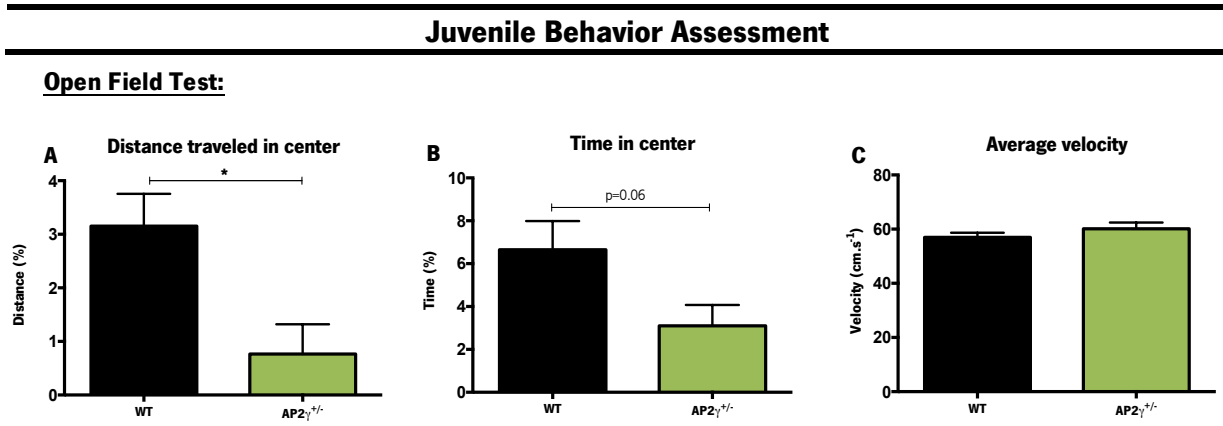
To understand if the transcription factor AP2γ could have an impact on the juvenile behavior, we used a set of established behavioral tests in animals with age between PND25 and PND31. We started the juvenile behavior characterization by evaluating if AP2γ transcription factor has an impact on anxious-like behavior through the OF test. Although it is not described as a classical test for anxiety, the OF test can also be used as an indicator of possible anxious-like behavioral states of rodents, by evaluating the ratio of time spent and distanced traveled in the center versus the periphery of the arena (Prut and Belzung 2003). With this test, it was possible to observe that the AP2γ$^{+/-}$ mice traveled lower distances (Figure 11A and Table 15) and spent less time in the center of the arena (Figure 11B and Table 15) suggesting higher anxiety levels when compared to the WT group. Furthermore, it was possible to evaluate locomotor activity, measured as the average velocity of mice during the execution of the test, which did not differ between WT and AP2γ$^{+/-}$ mice (Figure 11C and Table 15), thus validating subsequent behavioral testing that relay on locomotor activity. Also through the OF test, we observed

that the AP2γ$^{+/-}$ mice were less willing to explore the arena as they showed higher percentage of time in resting state (Figure 11D and Table 15) and presented a lower total distance traveled (Figure 11E and Table 15). Then we assessed if in basal conditions there are any signs of susceptibility to behavioral despair, through the TST. We did not found any differences between the two groups in the percentage of time that mice stay immobile during the test (Figure 11F and Table 15), suggesting the absence of depressive-like symptoms in AP2γ$^{+/-}$ juvenile mice. Also in the splash test we did not found differences between groups in the time that the animals spent grooming (Figure 11G and Table 15) after applying a sucrose solution to their coat, indicating that AP2γ$^{+/-}$ juvenile mice do not present core alterations regarding motivational self-care.

**Table 15. Statistical analysis of the juvenile behavior tests.**

Data presented as mean ± SEM. In grey shading are the behavioral tests where it was possible to observe differences between groups.

| | Behavioral Test | WT | AP2γ$^{+/-}$ | Statistical test, significance, effect size |
|---|---|---|---|---|
| OF | Distance traveled center (%) | 3.152 ± 0.60 | 0.77 ± 0.55 | t(19)= 2.29, p=0.03, Cohen's d= 1.35 |
| | Time in center (%) | 6.651 ± 1.34 | 3.01 ± 0.98 | t(19)= 2.06, p=0.06, Cohen's d= 1.26 |
| | Average velocity (cm.s-1) | 56.95 ± 1.73 | 60.10 ± 2.34 | t(19)= 1.01, p=0.28, Cohen's d= 0.82 |
| | Time in resting state (%) | 82.87 ± 0.94 | 86.28 ± 1.04 | t(19)= 0.18, p=0.03, Cohen's d= 1.13 |
| | Total distance (cm) | 3799 ± 514.3 | 1645 ± 589.7 | t(19)= 2.73, p=0.01, Cohen's d= 1.19 |
| TST | Immobility time (%) | 51.01 ± 5.88 | 49.70 ± 4.53 | t(19)= 0.18, p=0.86, Cohen's d= 0.066 |
| Splash test | Time grooming (%) | 76.86 ± 3.19 | 78.00 ± 5.67 | t(10)= 0.19, p=0.85, Cohen's d= 0.106 |



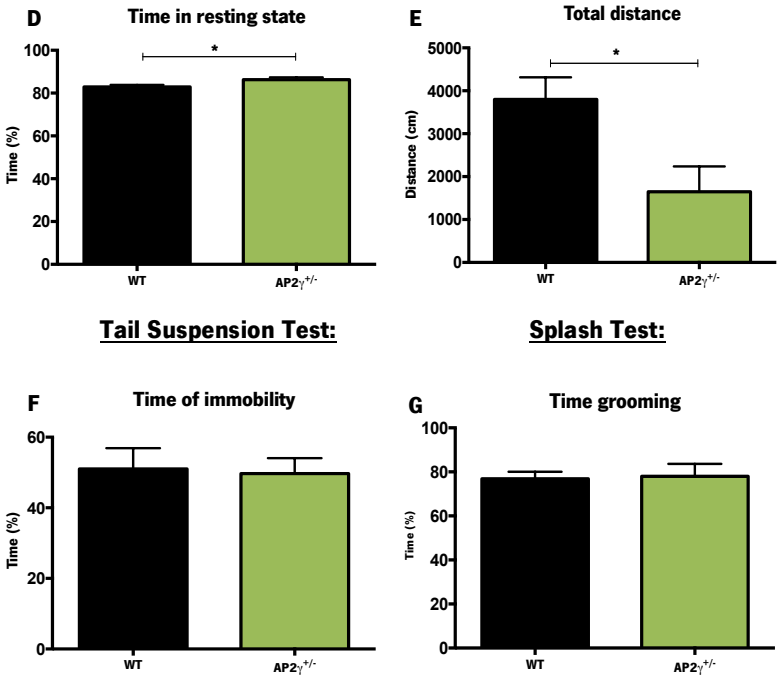



**Figure 11. The impact of the constitutive deletion of one allele of AP2γ in the emotional and mood behavioral dimension of juvenile mice.**

Using a set of established behavioral tests, it was possible to assess anxiety, mood, and motivational care in juvenile mice from PND25 to PND31. Through the OF test we observed that AP2γ$^{+/-}$ showed a higher anxiety level, since they traveled lower distances and spent less time in the center of the arena (A-B). No locomotor deficits were found between groups (C), but the AP2γ$^{+/-}$ mice were less willing to explore the arena since they spent an higher period in resting state (D) and had a lower distance traveled (E). In the TST (F) and in the Splash test (G) no differences were found between groups suggesting that in basal state AP2γ$^{+/-}$ mice do not display depressive-like symptoms. (OF and TST: $n_{WT}$= 12 ; $n_{AP2\gamma^{+/-}}$= 9 ; Splash test: $n_{WT}$= 7; $n_{AP2\gamma^{+/-}}$= 5. Data presented as mean ± SEM, *p<0.05).

### 4.1.3. The transcription factor AP2γ impact on hippocampal glutamatergic neurogenesis in juvenile mice

In previous studies from the group hippocampal dentate gyrus (DG) cell proliferation and glutamatergic neurogenesis in the brain of AP2γ$^{+/-}$ adult mice was already characterized. However, it was not analyzed if these effects in the adult mice promoted by deletion of AP2γ are indeed specifically observed at adult stages, or if during the developmental and maturity stages there is already an effect of the constitutive deletion of one allele of the AP2γ gene in the hippocampal neurogenesis.

To comprehend if the reduced expression of AP2γ could have already an impact on hippocampal glutamatergic neurogenesis during development and maturity stages, we injected juvenile animals with BrdU and sacrificed them 24h later (at PND31), allowing us, through immunostaining, to assess the proliferation of progenitor cells and newborn neurons (neuroblasts expressing doublecortin – DCX). Analysis of total cell proliferation in the DG of WT and AP2γ$^{+/-}$ animals revealed no significant

differences between groups, although the proliferation values of AP2γ$^{+/-}$ animals were slightly reduced (Figure 12B, Table 16).

In addition, we analyzed proliferation of neuroblasts with double staining for BrdU and DCX, revealing a significant difference between WT and AP2γ$^{+/-}$ animals. In AP2γ$^{+/-}$ mice, a decreased proliferation of neuroblasts was observed as shown by the reduced number of double-positive BrdU/DCX cells, when compared to the WT control group (Figure 12C, Table 16).

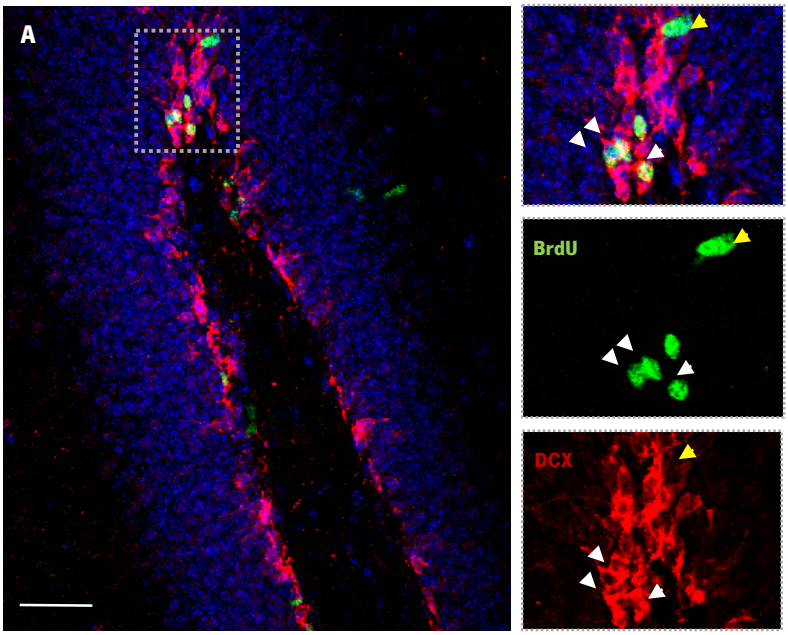
These differences in neuronal proliferation observed in juvenile animals with constitutive deletion of one allele of AP2γ in comparison to the WT animals confirm the involvement of this transcription factor in the modulation of post-natal glutamatergic neurogenesis (in the healthy brain) already during development and maturity of these animals.

**Table 16. Statistical analysis of the post-natal glutamatergic neurogenesis of juvenile animals.**

Data presented as mean ± SEM. In grey shading is the cell-count where it was possible to observe differences between groups.

| Markers | WT | AP2γ | Statistical test, significance, effect size |
|---|---|---|---|
| **BrdU single-positive cells** | 194.3 ± 15.42 | 123.1 ± 22.27 | $t_{(8)}$= 2.63, p=0.12, Cohen's d= 2.63 |
| **BrdU/DCX double positive cells** | 123.6 ± 15.83 | 49.27 ± 6.94 | $t_{(8)}$= 4.30, p=0.05, Cohen's d= 4.30 |

**Hippocampal glutamatergic neurogenesis in juvenile mice**



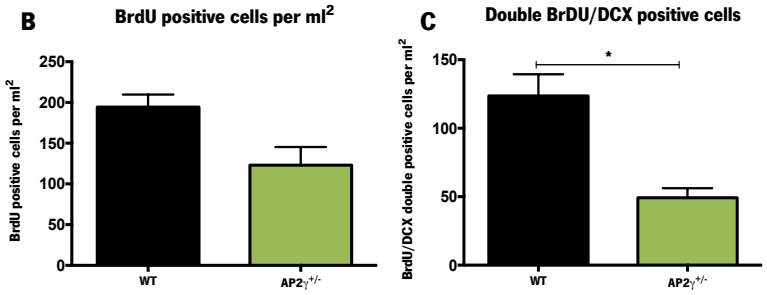



**Figure 12. Impact of the constitutive deletion of one allele of AP2γ in the post-natal hippocampal glutamatergic neurogenesis of juvenile animals.**

Hippocampal sections were immunostained for BrdU and DCX (A). Immature neurons (neuroblasts) are identified as BrdU and DCX double-positive cells (white arrows), whereas the remaining proliferative cells are single-BrdU positive cells (yellow arrow). Global proliferation (total BrdU positive cells/per area of DG) have been determined (B) as well as the proliferation of neuroblasts (total double positive BrdU/DCX cells/per area of DG) in the complete DG (C). ($n_{WT}$ = 3; $n_{AP2\gamma^{+/-}}$ = 3; Data presented as mean ± SEM, *p<0.05).

## 4.2. Functional impact of the transcription factor AP2γ in Depression

### 4.2.1. Validation of the uCMS model of depression

In order to validate the uCMS protocol implemented in this study we used two major measures to control its efficacy. Throughout the six weeks of the uCMS protocol we controlled the animals' weight weekly to perceive if the animals from control and uCMS groups were showing different body mass gains. It was possible to observe in both constitutive (Figure 13A and Table 17) and conditional (Figure 13A and Table 18) KO animal models that the uCMS protocol was producing different effects when comparing the control and uCMS groups, in which the first group was gaining and maintaining the gained weight, and the second one was first loosing weight and maintaining the weight lost throughout the six weeks of the stress protocol. The genotype variable did not impact the weight gain or loss in both animal models (Supplementary figures 1 and 2). Since this gain/loss weight measure is not sufficient to fully understand and validate if the uCMS protocol was efficient in inducing core symptoms of depression, we proceed to molecular analysis.

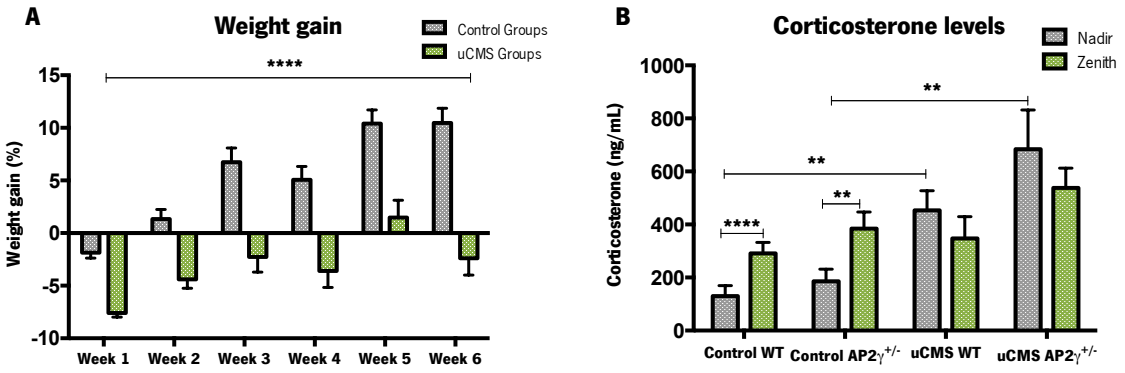



**Figure 13. Validation of the uCMS model of depression in the constitutive AP2γ KO animal model.**

Using the percentage of weight gain (A) throughout the six weeks of the uCMS protocol, and the corticosterone (B) levels measured in the animals' serum between 8:00 and 9:00 (basal levels; nadir) and between 20:00 and 21:00 (peak levels; zenith) at the 6th week of the uCMS protocol it was possible to confirm its efficacy. (Weight gain: $n_{Controls}$=15, $n_{uCMS}$=15; Corticosterone levels: $n_{WT}$=5, $n_{AP2\gamma^{+/-}}$=4, $n_{WT}$=5, $n_{AP2\gamma^{+/-}}$=4; Data presented as mean ± SEM; **p<0.01; ****p<0.0001).

uCMS exposure is known to disrupt the hypothalamic-pituitary-adrenal (HPA) axis (Ottenweller et al. 1994), and therefore the measurement of corticosterone (produced by the adrenals) levels in blood provides a correlate of the stress-level in the tested animals (Sousa et al. 1998; Ventura-Silva et al. 2013; Patricio et al. 2015). Knowing that control animals display a corticosterone peak at diurnal zenith (night time, peak levels) (D'Agostino et al. 1982), which is affected in animals exposed to chronic stress, we measured the levels of corticosterone at the end of the uCMS protocol (6th week) both at nadir (day time, basal) and zenith. It was possible to notice that both constitutive (Figure 13B and Table 17) and conditional (Figure 14B and Table 18) control groups maintained a normal circadian rhythm of corticosterone secretion, since there is a significantly peak of corticosterone at the zenith time-point. The uCMS exposed-groups showed a disruption of the HPA axis by presenting a significantly higher nadir levels of corticosterone than the control groups, and no circadian regulation, as there are no differences between nadir and zenith in both constitutive (Figure 13B and Table 17) and conditional (Figure 14B and Table 18) uCMS groups.

## Validation of the uCMS model of depression in the conditional AP2γ KO animal model

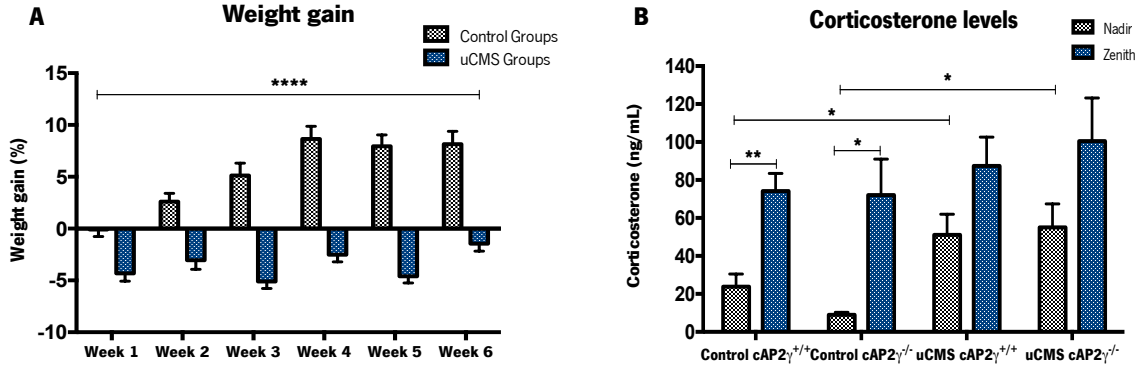



**Figure 14. Validation of the uCMS model of depression in the conditional AP2γ KO animal model.**

Using the percentage of weight gain (A) throughout the six weeks of the uCMS protocol, and the corticosterone (B) levels measured in the animals' serum between 8:00 and 9:00 (basal levels; nadir) and between 20:00 and 21:00 (peak levels; zenith) it was possible to confirm the efficacy of the uCMS protocol. [Weigh gain: $n_{Controls}$=17, $n_{uCMS}$=18; Corticosterone levels: Controls ($n_{cAP2\gamma^{+/+}}$=8, $n_{cAP2\gamma^{-/-}}$=3), uCMS ($n_{cAP2\gamma^{+/+}}$=9, $n_{AP2\gamma^{-/-}}$=4); Data presented as mean ± SEM; *p<0.05; **p<0.01; ****p<0.0001].

**Table 17. Statistical analysis of the parameters used to validate the uCMS protocol in the constitutive KO animal model.**

Data presented as mean ± SEM. In grey shading are the tests were it was observable differences between the variables. Abbreviations: Week(W).

| Test | | Control | | uCMS | | Statistical test, significance, effect size | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | WT | AP2γ$^{+/-}$ | WT | AP2γ$^{+/-}$ | | | | | | |
| Weight | W1 | -1.87 ± 0.51 | | -7.6 ± 0.39 | | $F_{(1,28)}$=2.15, p< 0.0001, $\eta^2_{partial}$= 0.528 | | | | | |
| | W2 | 1.33 ± 0.91 | | -4.4 ± 0.84 | | | | | | | |
| | W3 | 6.73 ± 1.35 | | -2.27 ± 1.44 | | | | | | | |
| | W4 | 5.07 ± 1.27 | | -3.6 ± 1.57 | | | | | | | |
| | W5 | 10.40 ± 1.30 | | 1.47 ± 1.67 | | | | | | | |
| | W6 | 10.47 ± 1.40 | | -2.40 ± 1.59 | | | | | | | |
| Corticosterone levels | Nadir (N) | 130.9± 39.75 | 158.8 ± 45.01 | 397.3 ± 59.23 | 684 ± 147.9 | Control WT | NControl WT/ /NuCMS WT | Control AP2γ$^{+/-}$ | NControl AP2γ$^{+/-}$/ /NuCMS AP2γ$^{+/-}$ | uCMS WT | uCMS AP2γ$^{+/-}$ |
| | Zenith (Z) | 291.2± 41.23 | 437.3 ± 40.46 | 347.7 ± 81.84 | 538 ± 74.38 | $t_d$= 19.2, p<0.001 Cohen's d=1.77 | $t_r$= 3.96, p=0.005, Cohen's d=2.71 | $t_s$= 4.6, p=0.002, Cohen's d=2.91 | $t_c$=4.69 , p=0.003, Cohen's d=3.28 | $t_3$= 0.18 , p=0.87, Cohen's d=0.10 | $t_6$= 0.71, p=0.50, Cohen's d=0.58 |

**Table 18. Statistical analysis of the parameters used to validate the uCMS protocol in the conditional KO animal model.**

Data presented as mean ± SEM. In grey shading are the tests were it was observable differences between the variables. Abbreviations: Week(W).

| Test | | Control | | uCMS | | Statistical test, significance, effect size | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | cAP2γ$^{+/+}$ | cAP2γ$^{+/-}$ | cAP2γ$^{+/+}$ | cAP2γ$^{+/-}$ | | | | | | |
| Weight | W1 | 0.09 ± 0.67 | | -4.13 ± 0.70 | | $F_{(1,36)}$=2.98, p< 0.0001, $\eta^2_{partial}$= 0.63 | | | | | |
| | W2 | 2.85 ± 0.79 | | -2.64 ± 0.82 | | | | | | | |
| | W3 | 3.85 ± 0.79 | | -4.27 ± 0.66 | | | | | | | |
| | W4 | 8.67 ± 1.17 | | -1.73 ± 0.66 | | | | | | | |
| | W5 | 8.04 ± 1.06 | | -3.68 ± 0.66 | | | | | | | |
| | W6 | 8.06 ± 1.19 | | -0.52 ± 0.81 | | | | | | | |
| Corticosterone levels | Nadir (N) | 23.79± 6.72 | 9.03 ± 1.25 | 51.06 ± 10.94 | 45.69 ± 10.41 | Control cAP2γ$^{+/+}$ | NControl cAP2γ$^{+/+}$/ /NuCMS cAP2γ$^{+/+}$ | Control cAP2γ$^{+/-}$ | NControl cAP2γ$^{+/-}$/ /NuCMS cAP2γ$^{+/-}$ | uCMS cAP2γ$^{+/+}$ | uCMS cAP2γ$^{+/-}$ |
| | Zenith (Z) | 74.17± 9.31 | 72.09 ± 18.95 | 87.38 ± 18.25 | 54.81 ± 29.79 | $t_7$=3.93 p= .006 Cohen's d=2.19 | $t_{14}$=2.36 p= 0.03 Cohen's d=1.18 | $t_7$= 3.32 p= 0.03 Cohen's d=2.67 | $7_6$=2.8 p=0.03 Cohen's d=2.35 | $t_{13}$=1.99, p=0.07, Cohen's d=1.03 | $t_5$=1.84, p=0.16, Cohen's d=1.56 |

### 4.2.2. Assessment of the behavior dimensions affected by the uCMS protocol

In previous studies from the group it was already confirmed the importance of AP2γ transcription factor in the modulation of adult hippocampal glutamatergic neurogenesis, playing an important role in hippocampal proliferation and neuronal differentiation through reciprocal interactions with transcriptional regulators of glutamatergic neurogenesis (Mateus-Pinheiro et al. 2016). In this master thesis work, once again, we demonstrated the modulatory action of AP2γ on glutamatergic neurogenesis through the results in juvenile mice (Figure 12). Taking this in consideration, we aimed to assess if AP2γ could have a functional role in neurogenesis-related neuropathological contexts, namely depression. Therefore, we submitted both constitutive and conditional AP2γ KO animal models to an uCMS protocol during 6 weeks and assessed the impact of chronic stress exposure using different behavioral tests and paradigms.

uCMS exposure typically produces deficits in three behavioral dimensions that are commonly affected in depression – anxiety, mood and cognition (Bessa et al. 2009b). Thus, a multidimensional behavioral analysis was performed in this work. We started by assessing the anxiety dimension through the OF and the EPM tests. In the first behavioral test to evaluate anxiety-like traits we found similar results in both constitutive and conditional AP2γ KO animal models. It was possible to observe in both models a susceptibility of control AP2γ KO groups (AP2γ$^{+/-}$ and cAP2γ$^{+/-}$) to display anxious-like behavior

since both groups show a lower distance traveled in the center of the OF arena (Figure 15A and 16A, Table 19 and 20). Although not reaching statistical difference in the conditional KO animal model, it was possible to note also that the control KO animals have a lower percentage of time spent in the center of the arena when compared to the respective control groups (WT and cAP2$\gamma^{+/+}$). In the constitutive AP2γ KO animal model it was not possible to observe statistical differences between control and uCMS groups. However, in this model we observed a tendency of WT uCMS animals to travel smaller distances (Figure 15A, Table 19), and to spend less time in the center of the arena (Figure 15B, Table 19) when compared to the control WT group. In the conditional AP2γ KO model, statistical differences were found between the control cAP2$\gamma^{+/+}$ and the uCMS cAP2$\gamma^{+/+}$ groups in the distance traveled and in the percentage of time spent in the center of the arena (Figure 16AB, Table 20), suggesting increased anxiety levels upon stress exposure. In both models it was not possible to observe differences between the control and the uCMS AP2γ KO groups (AP2$\gamma^{+/-}$ and cAP2$\gamma^{-/-}$) (Figure 15AB and 16AB, Table 19 and 20). Furthermore, through the OF test we assessed locomotor activity, measured as the average velocity (Figure 15C and 16C, Table 19 and 20), and in both animal models no differences were found between the groups, thus validating subsequent behavioral tests that relay on locomotor activity.

In the EPM test no predisposition to an anxious-like behavior was found between the control groups, since in both animal models no differences were unveiled between the control groups (control WT/AP2$\gamma^{+/-}$ and control cAP2$\gamma^{+/+}$/cAP2$\gamma^{-/-}$) (Figure 15D and 16D, Table 19 and 20). However, WT and AP2$\gamma^{+/-}$ uCMS groups, from the constitutive model, spent a higher percentage of time in the closed arms, when compared to the respective control situations (Figure 15D, Table 19). More so, the conditional cAP2$\gamma^{+/+}$ uCMS group showed an higher percentage of time spent in the closed arms of the EPM apparatus, when compared to the cAP2$\gamma^{+/+}$ control group (Figure 16D, Table 20).

Considering the behavioral output provided together by the OF and the EPM tests, the employed uCMS protocol had impact on the anxiety levels of both mice strains analyzed. Notably, in these two behavioral tests, different trends regarding the control situations were observed, since in the OF test we could evidentiate an anxious-like behavior in the AP2γ KO groups, which was not the case in the EPM test.

## Constitutive AP2γ KO animal model – AP2γ impact in the anxiety and mood dimensions of depression

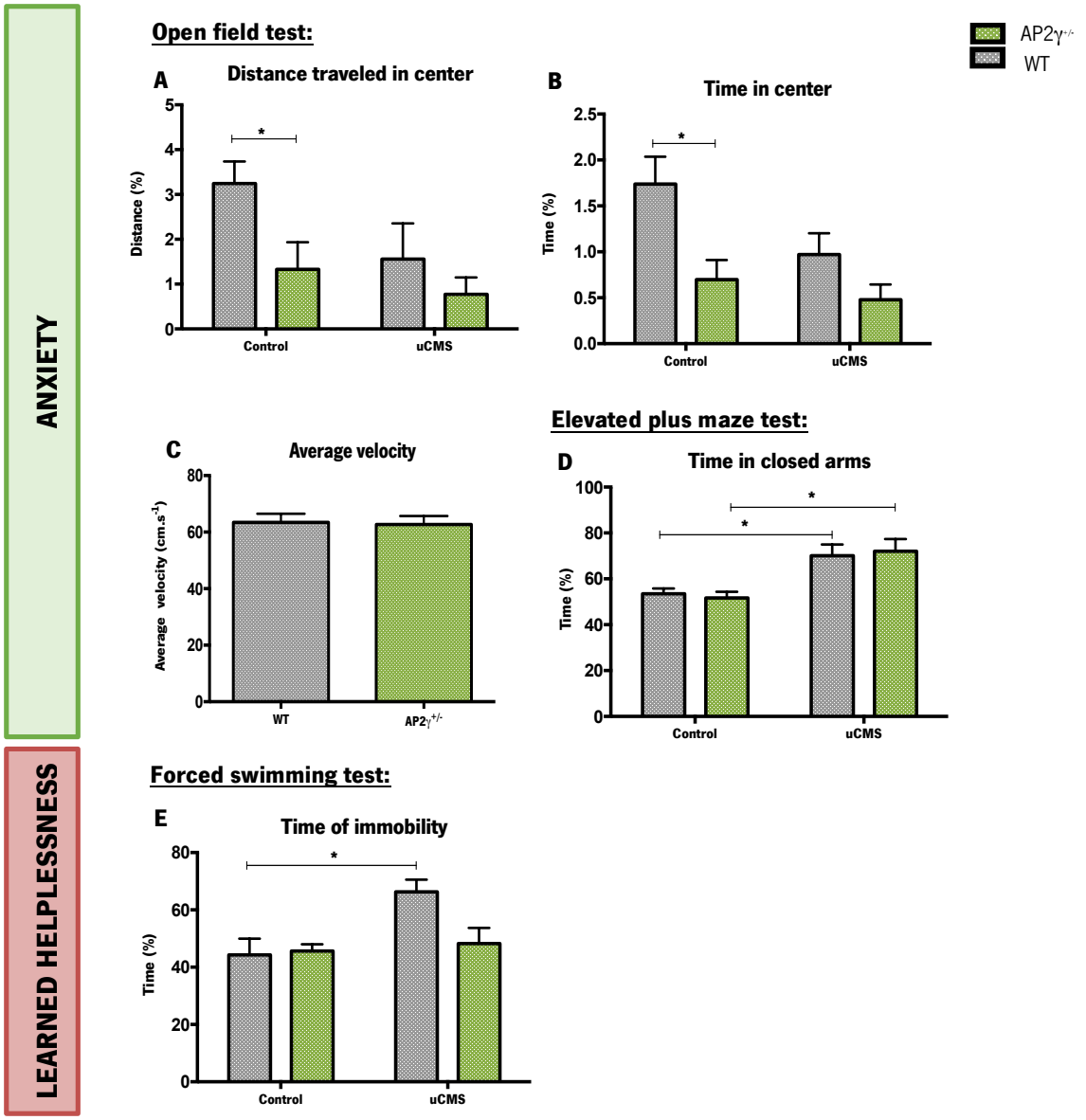



**Figure 15. Impact of the constitutive deletion of one allele of AP2γ in the anxiety and mood behavioral dimensions of depression.**

After 6 weeks of uCMS, anxiety-like signs [Distance traveled in center arena (A) and Time in center (B)] and locomotor activity were assessed in the OF test. Anxiety behavior was determined in the EPM test (D), and learned helplessness was evaluated by the FST. (Control: $n_{WT}$= 8, $n_{AP2\gamma^{+/-}}$= 7 ; uCMS: $n_{WT}$= 8, $n_{AP2\gamma^{+/-}}$=7; Data presented as mean ± SEM; *p<0.05).

**Table 19. Statistical analysis of the behavioral tests used to evaluate anxiety-like behavior and mood of the constitutive AP2γ KO animal model.**

Data presented as mean ± SEM. In grey shading are the tests were it was observable differences between the variables

| | Test | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|
| | | WT | AP2γ$^{+/-}$ | WT | AP2γ$^{+/-}$ | |
| OF | Distance traveled center | 3.24 ± 0.57 | 1.33 ±1.36 | 1.56 ± 0.83 | 0.77 ± 0.38 | Interaction Genotype vs uCMS: $F_{(1,16)}$= 0.95, p=0.34, $\eta^{2partial}$= 0.06 Interaction Control vs uCMS: $F_{(1,16)}$= 3.77, p=0.07, $\eta^{2partial}$= 0.19 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,16)}$= 5,48, p=0.03, $\eta^{2partial}$= 0.26 |
| | Time in center | 1.74 ± 0.33 | 0.69 ± 0.40 | 0.97 ± 0.31 | 0.47 ± 0.17 | Interaction Genotype vs uCMS: $F_{(1,16)}$= 1.39, p=0.26, $\eta^{2partial}$=0.08 Interaction Control vs uCMS: $F_{(1,16)}$= 4.50, p=0.04, $\eta^{2partial}$=0.22 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,16)}$= 10.85, p=0.004, $\eta^{2partial}$= 0.40 |
| | Average velocity | WT 63.45 ± 3.06 | | AP2γ$^{+/-}$ 62.70 ± 2.99 | | $t_{27}$= 0.17, p=0.68, Cohen's d= 0.25 |
| EPM | Time in closed arms | 53.49 ± 6.88 | 51.68 ± 6.70 | 70.09 ± 4,70 | 72.02 ± 5.43 | Interaction Genotype vs uCMS: $F_{(1,21)}$= 0.21, p=0.65, $\eta^{2partial}$= 0.01 Interaction Control vs uCMS: $F_{(1,21)}$= 20.10, p<0.0001, $\eta^{2partial}$= 0.49 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,21)}$= 0.0002, p=0.99, $\eta^{2partial}$= 0.001 |
| FST | Time of immobility | 44. 26 ± 3.07 | 45.63 ± 7.2 | 66.27 ± 5.71 | 48.23 ± 5.75 | Interaction Genotype vs uCMS: $F_{(1,17)}$= 4.12, p=0.06, $\eta^{2partial}$= 0.20 Interaction Control vs uCMS: $F_{(1,17)}$= 6.62, p=0.02, $\eta^{2partial}$= 0.28 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,17)}$= 3.04, p=0.09, $\eta^{2partial}$= 0.152 |

We next performed the FST and the TST to assess learned helplessness, a known depressive-like symptom. With this two tests it was possible to evaluate the depressive-like state of the animals in behavioral paradigms. Although we did not perform the TST in the constitutive AP2γ KO animal model, it was possible to see in both models similar results in the FST. Here, both animal models displayed, no kind of predisposition to depressive-like behavior in the control groups, and chronic stress exposure impacted the WT and cAP2γ$^{+/+}$ uCMS groups in the constitutive and conditional AP2γ models, since the immobility time is significantly higher in these groups, when compared to the respective control groups (Figure 15E and 16E, Table 19 and 20). However, this difference between control and uCMS was not obtained in both KO groups (AP2γ$^{+/-}$ and cAP2γ$^{-/-}$; Figure 15E and 16E, Table 19 and 20). In the TST (Figure 16F, Table 20), which was only performed in the conditional AP2γ KO animal model, we did obtain the same conclusions, since an increased immobility time between the uCMS and control cAP2γ$^{+/+}$ groups was observed. We did not observe differences between the KO groups from both constitutive and conditional animal models (AP2γ$^{+/-}$ and cAP2γ$^{-/-}$).

**Conditional AP2γ KO animal model – AP2γ impact in the anxiety and mood dimensions of depression**

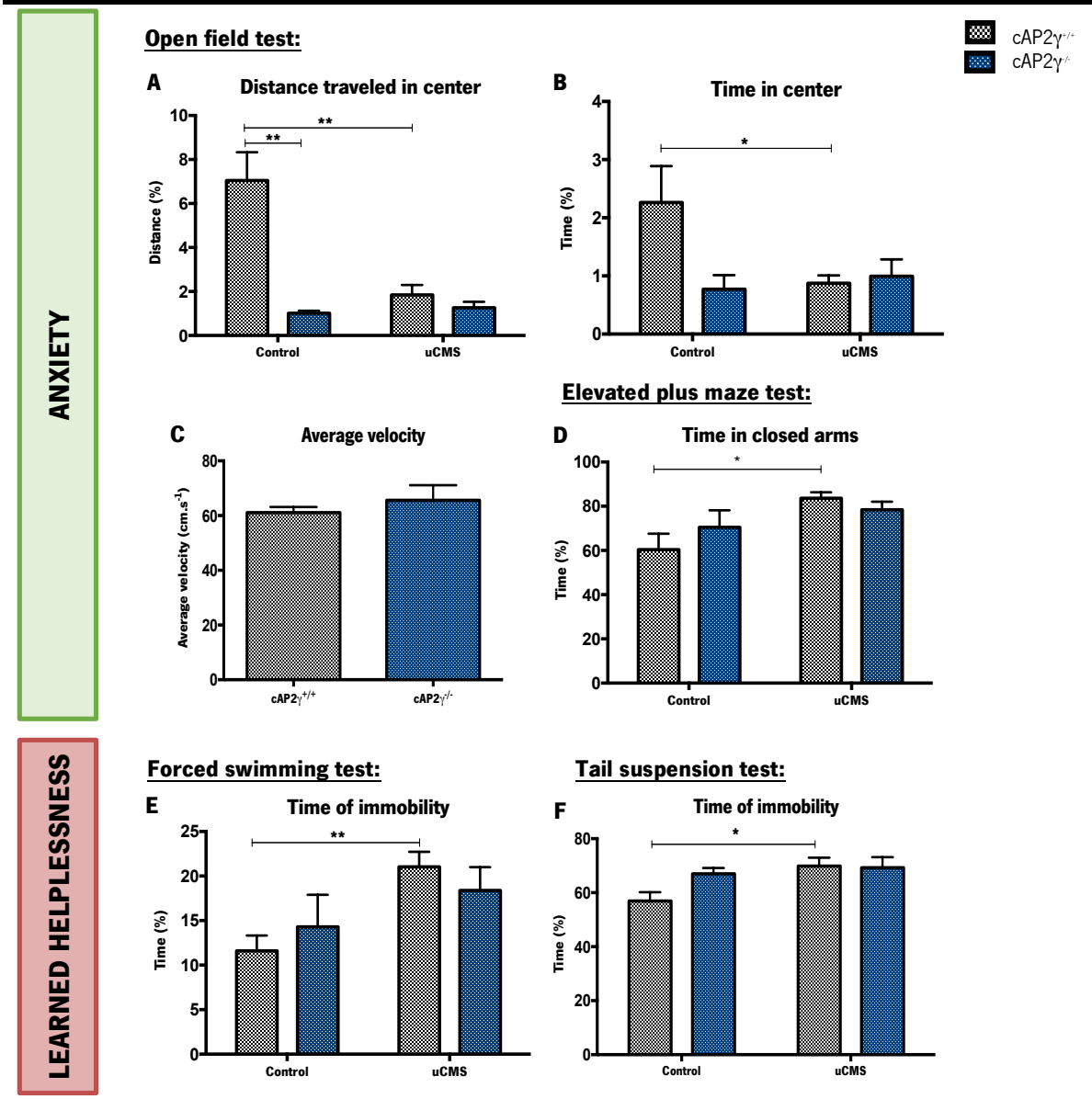



**Figure 16. Impact of the conditional deletion of one allele of AP2γ in the anxiety and mood behavioral dimensions of depression.**

After 6 weeks of uCMS, anxiety-like signs [Distance traveled in center arena (A) and Time in center (B)] and locomotor activity were assessed in the OF test. Anxiety behavior was determined in the EPM test (D), and learned helplessness was evaluated by the FST. Control: $n_{cAP2\gamma^{+/+}} = 8$, $n_{cAP2\gamma^{-/-}} = 7$ ; uCMS: $n_{cAP2\gamma^{+/+}} = 8$, $n_{cAP2\gamma^{-/-}} = 7$; Data presented as mean ± SEM; *p<0.05; **p<0.01.

**Table 20. Statistical analysis of the behavioral tests used to evaluate anxiety and mood dimension of the conditional AP2γ KO animal model.**

Data presented as mean ± SEM. In grey shading are the tests were it was observable the differences between variable

| | Test | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|
| | | cAP2γ$^{+/+}$ | cAP2γ$^{+/-}$ | cAP2γ$^{+/+}$ | cAP2γ$^{+/-}$ | |
| OF | Distance traveled center | 7.05 ± 0.97 | 1.01 ± 0.75 | 1.85 ± 0.51 | 1.26 ± 0.55 | Interaction Genotype vs uCMS: $F_{(1,36)}$= 6.1, p=0.018, $\eta^{2partial}$= 0.15 Interaction Control vs uCMS: $F_{(1,36)}$= 5.04, p=0.03, $\eta^{2partial}$= 0.12 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,36)}$= 8.98, p=0.005, $\eta^{2partial}$= 0.20 |
| | Time in center | 2.26 ± 1.06 | 0.77 ± 0.36 | 0.87 ± 0.18 | 0.99 ± 0.79 | Interaction Genotype vs uCMS: $F_{(1,36)}$= 4.40, p=0.05, $\eta^{2partial}$=0.155 Interaction Control vs uCMS: $F_{(1,36)}$= 2.29, p=0.14, $\eta^{2partial}$=0.09 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,36)}$= 3.19, p=0.08, $\eta^{2partial}$= 0.12 |
| | Average velocity | WT 61.08 ± 2.06 | | AP2γ$^{+/-}$ 65.6 ± 5.5 | | $t_{16}$= 0.96, p=0.35, Cohen's d= 0.08 |
| EPM | Time in closed arms | 60.36 ± 5.33 | 70.49 ± 13.58 | 83.63 ± 6.85 | 78.39 ± 8.19 | Interaction Genotype vs uCMS: $F_{(1,28)}$= 1.09, p=0.31, $\eta^{2partial}$= 0.042 Interaction Control vs uCMS: $F_{(1,28)}$= 4.48, p=0.04, $\eta^{2partial}$= 0.004 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,28)}$= 0.11, p=0.74, $\eta^{2partial}$= 0.001 |
| FST | Time of immobility | 11.60 ± 1.62 | 14.30 ± 1.25 | 21.02 ± 2.24 | 18.38 ± 2.91 | Interaction Genotype vs uCMS: $F_{(1,33)}$= 1.29, p=0.26, $\eta^{2partial}$= 0.04 Interaction Control vs uCMS: $F_{(1,33)}$= 8.28, p=0.007, $\eta^{2partial}$= 0.20 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,33)}$= 0.00009, p=, $\eta^{2partial}$= 0.000003 |
| TST | Time of immobility | 56.90 ± 3.01 | 67.00 ± 3.77 | 69.85 ± 3.30 | 69.23 ± 5.05 | Interaction Genotype vs uCMS: $F_{(1,33)}$= 1.59, p=0.22, $\eta^{2partial}$= 0.20 Interaction Control vs uCMS: $F_{(1,33)}$= 3.19, p=0.04, $\eta^{2partial}$= 0.28 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,33)}$= 1.24, p=0.27, $\eta^{2partial}$= 0.152 |

Next, we assessed the impact of AP2γ deletion in different cognitive domains in both constitutive and conditional animal models. We first performed different water maze paradigms, beginning with the evaluation of the working memory task, in which we observed the detrimental effects of chronic stress exposure in the uCMS WT animals, since this group showed a significant delay to reach the platform along the four trials of testing, when compared to the respective WT control group (Figure 17A, Table 21). However, it was not possible to see the same effect of chronic stress exposures on the AP2γ$^{+/-}$ animals, as both WT and AP2γ$^{+/-}$ control groups presented similar performances in this behavioral paradigm. In the conditional AP2γ model, no differences were found in the working memory task between genotypes, and stress exposure did not induce any deleterious on the conditional KO animals (Figure 18A, Table 22). In the spatial reference memory task, which relies on the integrity of hippocampal function, the control AP2γ KO groups (AP2γ$^{+/-}$ and cAP2γ$^{-/-}$) did not display reference memory deficits in comparison to the respective control WT groups (WT and cAP2γ$^{+/+}$) (Figure 17B and

18B, Table 21 and 22). Strikingly, in the constitutive animal model, upon uCMS exposure, WT animals developed significant impairment in this cognitive dimension whereas AP2γ$^{+/-}$ mice proved to be resilient to this deleterious effect of stress. In the conditional AP2γ KO model no differences were found between control and uCMS groups, suggesting that the chronic stress did not impact as much as in the constitutive KO animals, in this specific behavioral paradigm. Also through water maze paradigms, we performed the behavioral flexibility task, in which AP2γ$^{+/-}$ control animals displayed lower percentage of time spent in the new quadrant when compared to the WT control mice (Figure 17C, Table 21). Although barely failing to reach statistical difference, we could see the same tendency in the conditional KO animal model in which cAP2γ$^{-/-}$ presented lower percentage of time in the new quadrant when compared to the cAP2γ$^{+/+}$ control group (Figure 18C, Table 22). Regarding the deleterious effect of stress in the reversal learning task, no differences were found between control and uCMS groups in the constitutive model. However, in the conditional model, chronic stress induced detrimental effects on the cAP2γ$^{+/+}$ uCMS animals, and interestingly this result was not observed between the cAP2γ$^{-/-}$ groups.

After evaluating the cognitive behavioral dimension with the different water maze paradigms, we performed a cognitive test described to be sensitive to neurogenesis impairments, the CFC (Gu et al. 2012). In this test, both animal models were submitted to a context probe, aimed to test hippocampal-dependent memory, and a light-cued probe, designed to evaluate the integrity of extra-hippocampal memory circuits. In the context probe (context A), it was possible to observe in the constitutive AP2γ KO model that AP2γ$^{+/-}$ control group displayed a reduction in the percentage of time freezing when exposed to a familiar context (Figure 17D and Table 21). However, the same outcome was not observed in the cAP2γ$^{-/-}$ control, since no differences where found between this group and the cAP2γ$^{+/+}$ control group (Figure 18D and Table 22). The detrimental effects of uCMS exposure were shown by the reduced time freezing of both constitutive WT uCMS and the conditional cAP2γ$^{+/+}$ animals, when compared to the respective controls (Figure 17D and 18D, Table 21 and 22). Although not reaching statistical difference, it was also possible to see a clear tendency of cAP2γ$^{-/-}$ uCMS mice to present a reduction in the percentage of time freezing when compared to the control group.

In a second task, switching to a new environment (context B), promoted a decrease in the time freezing in all groups from both constitutive and conditional AP2γ KO animal models (Figure 17E and 18E, Table 21 and 22). In the light probe, once again, all groups presented similar responses to the light cue, in both models (Figure 17F and 18F, Table 21 and 22). Overall, these CFC results show that AP2γ KO groups (AP2γ$^{+/-}$ and cAP2γ$^{-/-}$) display specific deficits in contextual hippocampal-associated

memory which is exacerbated by the chronic stress exposure, while preserving associative non-hippocampal dependent memory in both control and uCMS situations.

**Table 21. Statistical analysis of the behavioral tests used to evaluate the cognitive dimension of the constitutive AP2γ KO animal model.**

Data presented as mean ± SEM. In grey shading are the tests were it was observable differences between variables. Abbreviations: Trial (T), Day (D).

| | Test | | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|---|
| | | | WT | AP2γ$^{+/-}$ | WT | AP2γ$^{+/-}$ | |
| CFC | Working memory | T1 | 78.79 ± 4.29 | 95.25 ± 10.56 | 112.04 ± 4.66 | 89.00 ± 10.05 | $F_{(1,17)}$=14.37, p<0.001, $\eta^2_{partial}$= 0.72 |
| | | T2 | 68.23 ± 9.64 | 83.31 ± 4.75 | 107.83 ± 2.86 | 88.40 ± 8.12 | |
| | | T3 | 58.04 ± 7.58 | 78.75 ±10.73 | 108.4 ± 10.07 | 83.50 ± 8.05 | |
| | | T4 | 60.71 ± 7.52 | 79.31 ± 13.63 | 97.83 ± 9.57 | 82.50 ± 1.71 | |
| | Spatial reference memory | D1 | 107.2 ± 7.09 | 108.75 ± 6.88 | 105.79 ± 5.20 | 68.18 ± 11.28 | $F_{(1,17)}$=12.37, p<0.001, $\eta^2_{partial}$= 0.69 |
| | | D2 | 54,00 ± 11.33 | 91.65 ± 9.06 | 117.13 ± 4.07 | 75.19 ± 17.20 | |
| | | D3 | 40.83 ± 6.33 | 55.75 ± 9.40 | 100.6 ± 15.97 | 37.82 ± 14.56 | |
| | | D4 | 28.37 ± 4.66 | 58.2 ± 9.48 | 109.0 ± 11.31 | 74.00 ± 23.23 | |
| | Behavioral flexibility | | 28.33 ± 4.08 | 14.71 ± 1.47 | 23.27 ± 2.03 | 27.32± 2.16 | Interaction Genotype vs uCMS: $F_{(1,17)}$= 9.92, p=0.007, $\eta^{2partial}$= 0.18 Interaction Control vs uCMS: $F_{(1,17)}$= 1.36, p=0.24, $\eta^{2partial}$= 0.12 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,17)}$= 3.95, p=0.05, $\eta^{2partial}$= 0.43 |
| | Context probe (context A) | | 73.56 ± 7.60 | 53.17 ± 9.25 | 57.38 ± 4.97 | 68.37 ± 2.41 | Interaction Genotype vs uCMS: $F_{(1,19)}$= 15.32, p=0.001, $\eta^{2partial}$= 0.45 Interaction Control vs uCMS: $F_{(1,19)}$= 0.02, p=0.90, $\eta^{2partial}$= 0.12 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,19)}$= 1.37, p=0.26, $\eta^{2partial}$= 0.20 |
| | Context probe (context B) | | 59.24 ± 6.59 | 36.51 ± 14.4 | 32.74 ± 6.94 | 47.86 ± 6.18 | Interaction Genotype vs uCMS: $F_{(1,19)}$= 6.4, p=0.018, $\eta^{2partial}$= 0.02 Interaction Control vs uCMS: $F_{(1,19)}$= 1.02, p=0.03, $\eta^{2partial}$= 0.32 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,19)}$= 8.98, p=0.005, $\eta^{2partial}$= 0.62 |
| | Cue probe (context B) | | 76.38 ± 6.24 | 61.52 ± 10.65 | 61.52 ± 7.56 | 90.26± 4.86 | Interaction Genotype vs uCMS: $F_{(1,19)}$= 15.51, p=0.08, $\eta^{2partial}$=0.46 Interaction Control vs uCMS: $F_{(1,19)}$= 1.57, p=0.23, $\eta^{2partial}$= 0.08 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,19)}$= 1.57, p=0.23, $\eta^{2partial}$= 0.08 |

**Constitutive AP2γ KO animal model – AP2γ transcription factor impact in the cognitive dimension of depression**

COGNITION

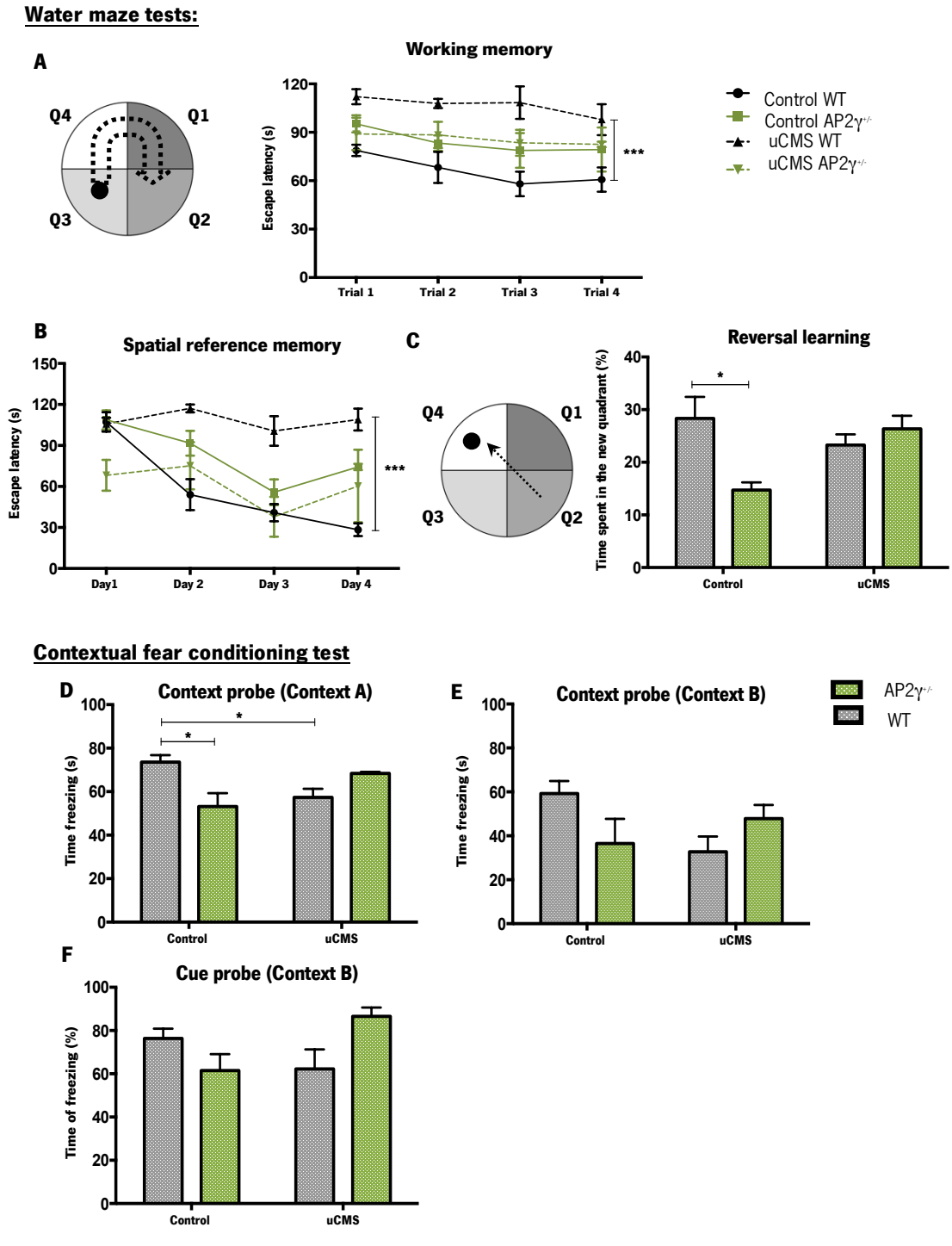



**Figure 17. Impact of the constitutive deletion of one allele of AP2γ in the cognitive behavioral dimension of depression.**

We started our cognitive dimension assessment by the water maze tests, in which we evaluated working memory (A), spatial reference (B) and reversal learning (C) tasks. In addition, animals were tested in a CFC paradigm, in the context probe (D and E) and in the cue probe (F). (Control: $n_{WT}$= 8, $n_{AP2\gamma^{+/-}}$= 7 ; uCMS: $n_{WT}$= 8, $n_{AP2\gamma^{+/-}}$=7; Data presented as mean ± SEM; *p<0.05, ***p<0.001; Data presented as mean ± SEM; p<0.05;**p<0.01.

## Conditional AP2γ KO animal model – AP2γ transcription factor impact in the cognitive dimension of depression

COGNITION

**Water maze tests:**

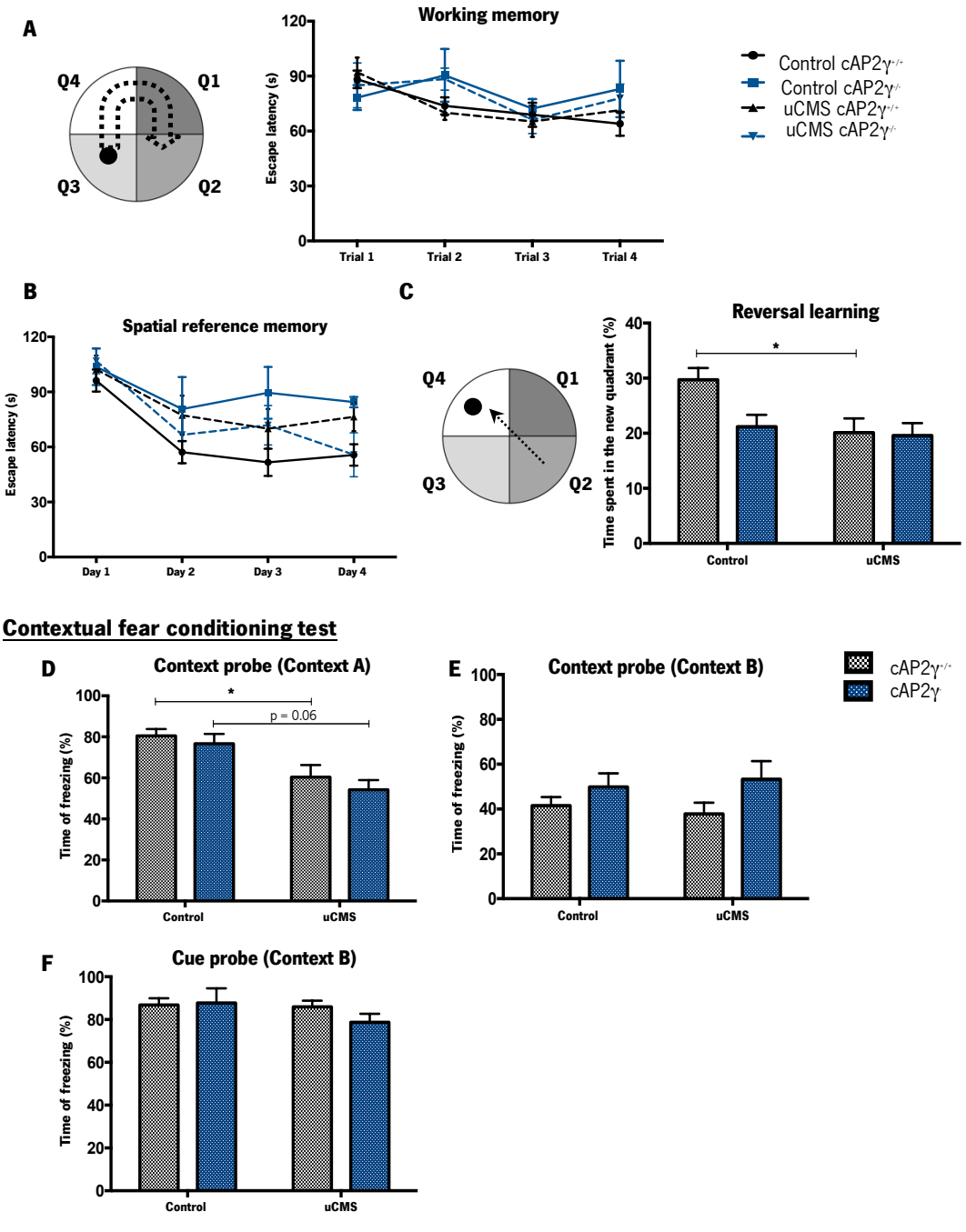



**Figure 18. Impact of the conditional deletion of AP2γ in the cognitive behavioral dimension of depression.**

We started our cognitive dimension assessment by the water maze tests, in which we evaluated working memory (A), spatial reference (B) and reversal learning (C) tasks. In addition, animals were tested in a CFC paradigm, in the context probe (D and E) and in the cue probe (F). Controls: $n_{cAP2\gamma^{+/+}}$= 12, $n_{cAP2\gamma^{-/-}}$=5; uCMS: $n_{cAP2\gamma^{+/+}}$=12, $n_{AP2\gamma^{-/-}}$=8; Data presented as mean ± SEM; *p<0.05.

**Table 22. Statistical analysis of the behavioral tests used to evaluate the cognitive dimension of the conditional AP2γ KO animal model.**

Data presented as mean ± SEM. In grey shading are the tests were it was observable differences between variables. Abbreviations: Trial (T), Day (D).

| | Test | | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|---|
| | | | cAP2γ$^{+/+}$ | cAP2γ$^{-/-}$ | cAP2γ$^{+/+}$ | cAP2γ$^{-/-}$ | |
| WMT | Working memory | T1 | 85.04 ± 5.03 | 78.31 ± 6.79 | 92.1 ± 8.02 | 86.94 ± 12.24 | $F_{(1,30)}$=0.38, p=0.77, $\eta^2_{partial}$= 0.04 |
| | | T2 | 73.00 ± 5.25 | 90.44 ± 14.39 | 70.03 ± 3.94 | 88.40 ±6.01 | |
| | | T3 | 70.72 ± 7.55 | 72.32 ± 5.19 | 65.3 ± 8.45 | 66.11 ± 7.42 | |
| | | T4 | 64.32 ± 7.23 | 83.00 ± 15.41 | 71.4 ± 7.58 | 77.89 ± 7.38 | |
| | Spatial reference memory | D1 | 96.19 ± 5.98 | 103.7 ± 24.74 | 102.3 ± 7.50 | 106.9 ± 6.97 | $F_{(1,30)}$=2.98, p=0.08, $\eta^2_{partial}$= 0.263 |
| | | D2 | 57.17 ± 6.08 | 80.63 ± 23.52 | 77.28 ± 10.66 | 66.6 ± 15.34 | |
| | | D3 | 51.60 ± 7.39 | 89.50 ± 23.45 | 69.97 ± 10.76 | 71.85 ± 10.77 | |
| | | D4 | 55.65 ± 5.82 | 84.44 ± 19.04 | 76.41 ± 7.74 | 55.75 ± 11.94 | |
| | Behavioral flexibility | | 29.71 ± 2.24 | 21.20 ± 2.15 | 20.12 ± 2.75 | 19.58 ± 1.68 | Interaction Genotype vs uCMS: $F_{(1,30)}$= 9.92, p=0.007, $\eta^{2partial}$= 0.08 Interaction Control vs uCMS: $F_{(1,30)}$= 6.96, p=0.02, $\eta^{2partial}$= 0.15 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,30)}$= 2.97, p=0.10, $\eta^{2partial}$= 0.11 |
| CFC | Context probe (context A) | | 80.52 ± 3.2 | 76.68 ± 3.3 | 60.39 ± 2.7 | 54.24 ± 3.2 | Interaction Genotype vs uCMS: $F_{(1,30)}$= 0.05, p=0.82, $\eta^{2partial}$= 0.002 Interaction Control vs uCMS: $F_{(1,30)}$= 17.8, p=0.014, $\eta^{2partial}$= 0.38 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,30)}$= 0.98, p=0.33, $\eta^{2partial}$= 0.03 |
| | Context probe (context B) | | 41.48 ± 4.44 | 49.86 ± 7.64 | 37.81 ± 6.03 | 53.31 ± 9.18 | Interaction Genotype vs uCMS: $F_{(1,30)}$= 0.33, p=0.57, $\eta^{2partial}$= 0.01 Interaction Control vs uCMS: $F_{(1,30)}$= 0.0001, p=0.99, $\eta^{2partial}$= 0.0001 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,30)}$= 3.75, p=0.08, $\eta^{2partial}$= 0.11 |
| | Cue probe (context B) | | 86.78 ± 5.35 | 87.76 ± 9.27 | 85.92 ± 5.28 | 78.75 ± 4.29 | Interaction Genotype vs uCMS: $F_{(1,30)}$= 0.93, p=0.341, $\eta^{2partial}$= 0.02 Interaction Control vs uCMS: $F_{(1,30)}$= 1.37, p=0.25, $\eta^{2partial}$= 0.04 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,30)}$= 0.54, p=0.47, $\eta^{2partial}$= 0.02 |

## 4.3. AP2γ modulatory mechanisms of adult hippocampal neurogenesis in depressive-like animals

### 4.3.1. AP2γ impact on the protein levels of different transcription factors involved in the adult hippocampal neurogenic process

To assess the functional impact of AP2γ deletion in the hippocampal glutamatergic neurogenic process, we quantified the protein expression of different transcripts involved in the transcriptional network responsible for the formation of new glutamatergic neurons in the adult hippocampal niche. To quantify these transcripts, we used western-blot (WB) analysis in brain tissue containing the total DG sample from both constitutive and conditional AP2γ KO animal models.

We started by quantifying the protein levels in the DG of constitutive AP2γ KO mice (Figure 19, Table 23) in which we confirmed a significant reduction of AP2γ protein levels in both AP2γ$^{+/-}$ control and after uCMS exposure when compared to the WT control and uCMS WT groups, respectively. Regarding the quantification of protein levels of upstream regulators of AP2γ also involved in glutamatergic neurogenesis, such as Sox2 and Pax6, no differences were observed between WT and AP2γ$^{+/-}$ control groups. However, after the noxious chronic stress stimulus we found a bolstering of Sox2 in both WT (which barely fails statistical difference) and AP2γ$^{+/-}$ uCMS groups. Interestingly, we observed that the constitutive KO group has an increase of these transcript. In the Pax6 upstream regulator of AP2γ, we observed a similar protein expression pattern, finding once again a bolstering of expression after uCMS exposure in the AP2γ$^{+/-}$ group, but no differences were seen between WT control and uCMS groups. Moreover, a significant difference among the uCMS groups was observed, with AP2γ$^{+/-}$ mice exposed to uCMS showing an increased expression of Pax6 when compared to the WT uCMS group. When we analyzed the expression of the downstream target of AP2γ, Tbr2 (expressed in intermediate progenitor cells of the adult hippocampus), the AP2γ$^{+/-}$ control group showed a lower expression of this transcript when compared to the control WT group, although not reaching statistical significance. After uCMS exposure, no differences were found among the WT groups. However, Tbr2 showed increased expression in the DG of AP2γ$^{+/-}$ mice exposed to uCMS in comparison to the control AP2γ$^{+/-}$ group.

After quantifying the protein expression of transcription factors involved in adult glutamatergic neurogenesis of the hippocampal DG in the constitutive AP2γ KO mice, we proceeded with analysis in the DG of conditional KO mice (Figure 20, Table 24). Although, in this part of the results, the WB analysis of this model is still not completed, since the uCMS groups only include 1-2 animals per group, we were already able to observe the impact of AP2γ in the protein levels of several transcription factors involved in adult glutamatergic neurogenesis. We were able to confirm a significant reduction of AP2γ protein levels in the hippocampal DG of cAP2γ$^{-/-}$ control mice. However, no differences were observed between the control and uCMS exposed animals, nor among the uCMS groups. Regarding upstream regulators of AP2γ, we measured the protein level of Pax6, which showed a significant decreased expression in the cAP2γ$^{-/-}$ group when compared to its control group. Moreover, no alterations were observed between control and uCMS groups in the quantification of Pax6 protein levels. In the DCX measurements, similarly to the Pax6 results, we observed a decreased expression in the cAP2γ$^{-/-}$ mice when compared to its control group, but also a bolstering of its expression in the uCMS cAP2γ$^{-/-}$ mice when compared to the control cAP2γ$^{-/-}$ group. We further performed protein quantification of Tbr2 in the hippocampal DG, revealing a significant decrease of its expression in the control cAP2γ$^{-/-}$ group when

compared to the control cAP2γ$^{+/+}$ mice. Furthermore, uCMS exposure induced a reduction of Tbr2 expression in the cAP2γ$^{+/+}$ mice, when compared to the respective control group.

**AP2γ impact on the protein levels of different transcripts involved in adult glutamatergic neurogenesis in the depressed-like constitutive KO animal model**

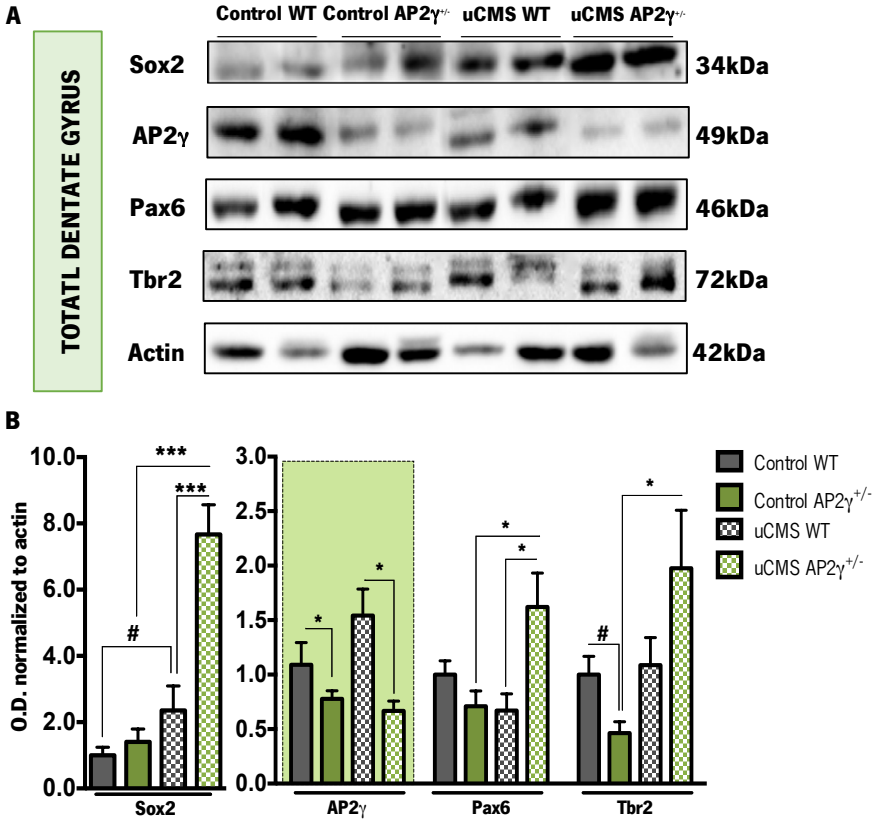



**Figure 19. Western blot analysis of Sox2, AP2γ Pax6 and Tbr2 in adult hippocampal dentate gyrus of the constitutive AP2γ KO animals.**

Representative blots for each genotype and condition (A); Protein expression levels with optical density (O.D.) normalized to actin, for each protein assessed (B). (Control: $n_{WT}$= 5, $n_{AP2\gamma^{+/-}}$= 5; uCMS: $n_{WT}$= 3, $n_{AP2\gamma^{+/-}}$= 3; Data presented as mean ± SEM; *p<0.05; ***p<0.001; #p=0.06). Abbreviations: Kilodalton (kDa).

**Table 23. Statistical analysis of the western blots performed in the hippocampal dentate gyrus of AP2γ constitutive KO animals.**

Data presented as mean ± SEM. In grey shading are the groups between each it was observable statistical difference.

| | | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|
| | | WT | AP2γ$^{+/-}$ | WT | AP2γ$^{+/-}$ | |
| **Sox 2** | **Total DG** | 1.00 ± 0.24 | 1.41 ± 0.39 | 2.35 ± 0.83 | 7.67 ± 0.89 | Interaction Genotype vs uCMS: $F_{(1,19)}$= 14.47, p=0.001, $\eta^{2partial}$= 0.45<br>Interaction Control vs uCMS: $F_{(1,19)}$= 57.39, p=0.001, $\eta^{2partial}$= 0.70<br>Interaction WT vs AP2γ$^{+/-}$: $F_{(1,19)}$= 10.63, p=0.001, $\eta^{2partial}$= 0.53 |
| **Pax6** | **Total DG** | 1.00 ± 0.13 | 0.71 ± 0.14 | 0.67 ± 0.15 | 1.62 ± 0.31 | Interaction Genotype vs uCMS: $F_{(1,20)}$= 9.81, p=0.005, $\eta^{2partial}$= 0.33<br>Interaction Control vs uCMS: $F_{(1,20)}$= 2.15, p=0.16, $\eta^{2partial}$= 0.10<br>Interaction WT vs AP2γ$^{+/-}$: $F_{(1,20)}$= 2.78, p=0.11, $\eta^{2partial}$= 0.12 |
| **AP2γ** | **Total DG** | 1.22 ± 0.16 | 0.66 ± 0.03 | 1.54 ± 0.24 | 0.67 ± 0.09 | Interaction Genotype vs uCMS: $F_{(1,14)}$= 0.89, p=0.36, $\eta^{2partial}$= 0.06<br>Interaction Control vs uCMS: $F_{(1,14)}$= 0.97, p=0.34, $\eta^{2partial}$= 0.07<br>Interaction WT vs AP2γ$^{+/-}$: $F_{(1,14)}$= 17.69, p=0.001, $\eta^{2partial}$= 0.56 |
| **Tbr2** | **Total DG** | 1.00 ± 0.17 | 0.46 ± 0.10 | 1.09 ± 0.25 | 1.98 ± 0.53 | Interaction Genotype vs uCMS: $F_{(1,26)}$= 5.88, p=0.02, $\eta^{2partial}$= 0.19<br>Interaction Control vs uCMS: $F_{(1,26)}$= 7.42, p=0.01, $\eta^{2partial}$= 0.22<br>Interaction WT vs AP2γ$^{+/-}$: $F_{(1,26)}$= 0.36, p=0.55, $\eta^{2partial}$= 0.01 |

## AP2γ impact on the protein levels of different transcripts involved in the adult glutamatergic neurogenic process in the depressed conditional KO animal model

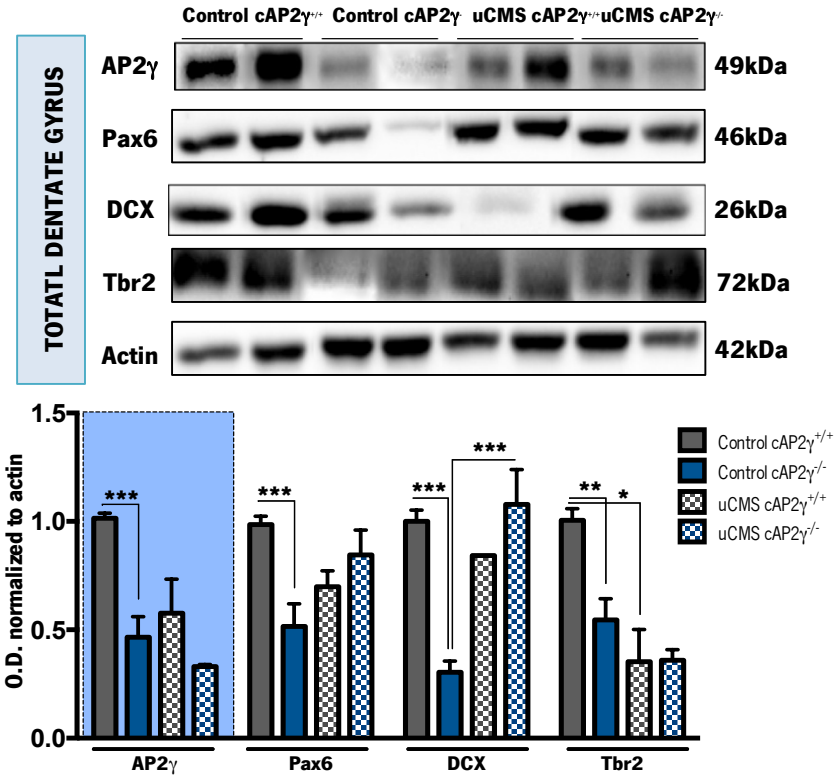



**Figure 20. Western blot analysis of AP2γ, Pax6, DCX and Tbr2 in adult hippocampal dentate gyrus of the conditional AP2γ KO animals.**

Representative blots for each genotype and condition (A); Protein expression levels with optical density (O.D.) normalized to actin, for each protein assessed (B). (Control: $n_{cAP2\gamma^{+/+}}$= 5, $n_{cAP2\gamma^{-/-}}$= 5; uCMS: $n_{cAP2\gamma^{+/+}}$= 1-2, $n_{cAP2\gamma^{-/-}}$= 2; Data presented as mean ± SEM; *p<0.05; **<0.01; ***p<0.001)

**Table 24. Statistical analysis of the western blots performed in the hippocampal dentate gyrus of AP2γ conditional KO animals.**

Data presented as mean ± SEM. In grey shading are the groups between each it was observable statistical difference.

| | | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|
| | | cAP2γ$^{+/+}$ | cAP2γ$^{-/-}$ | cAP2γ$^{+/+}$ | cAP2γ$^{-/-}$ | |
| **DCX** | **Total DG** | 1.00 ± 0.05 | 1.41 ± 0.05 | 0.84 ± 0.00 | 7.67 ± 0.16 | Interaction Genotype vs uCMS: $F_{(1,15)}$= 20.54, p=0.0004, $\eta^{2partial}$= 0.58 Interaction Control vs uCMS: $F_{(1,15)}$= 9.01, p=0.009, $\eta^{2partial}$= 0.38 Interaction WT vs AP2γ$^{-/-}$: $F_{(1,15)}$= 5.01, p=0.05, $\eta^{2partial}$= 0.53 |
| **Pax6** | **Total DG** | 0.99 ± 0.04 | 0.52 ± 0.10 | 0.70 ± 0.07 | 0.85 ± 0.11 | Interaction Genotype vs uCMS: $F_{(1,28)}$= 6.26, p=0.02, $\eta^{2partial}$= 0.18 Interaction Control vs uCMS: $F_{(1,28)}$= 0.03, p=0.86, $\eta^{2partial}$= 0.001 Interaction WT vs AP2γ$^{-/-}$: $F_{(1,28)}$= 1.72, p=0.20, $\eta^{2partial}$= 0.06 |
| **AP2γ** | **Total DG** | 1.01 ± 0.02 | 0.47 ± 0.16 | 0.49 ± 0.09 | 0.37 ± 0.01 | Interaction Genotype vs uCMS: $F_{(1,18)}$= 4.45, p=0.05, $\eta^{2partial}$= 0.20 Interaction Control vs uCMS: $F_{(1,18)}$= 10.17, p=0.06, $\eta^{2partial}$= 0.36 Interaction WT vs AP2γ$^{-/-}$: $F_{(1,18)}$= 11.3, p=0.003, $\eta^{2partial}$= 0.39 |
| **Tbr2** | **Total DG** | 1.00 ± 0.05 | 0.56 ± 0.15 | 0.35 ± 0.10 | 0.36 ± 0.05 | Interaction Genotype vs uCMS: $F_{(1,24)}$= 2.86, p=0.10, $\eta^{2partial}$= 0.10 Interaction Control vs uCMS: $F_{(1,24)}$= 9.27, p=0.006, $\eta^{2partial}$= 0.28 Interaction WT vs AP2γ$^{-/-}$: $F_{(1,24)}$= 2.70, p=0.11, $\eta^{2partial}$= 0.10 |

### 4.3.2. AP2γ transcription factor impact on epigenetic regulators of adult hippocampal glutamatergic neurogenesis

In this thesis we also started to assess epigenetic mechanisms that have been proposed to play crucial roles in adult glutamatergic neurogenesis, namely DNA methylation and demethylation mechanisms. Although until this point we do not have highly conclusive results, a broad analysis was possible to obtain.

We started an epigenetic characterization through a sensitive but general way of testing, by targeting specific known mediators of DNA methylation and demethylation in the AP2γ constitutive KO mice. DNA methylation was first evaluated through the quantification of DNMT3a and DNMT3b (enzymes involved in the chemical covalent addition of a methyl group to the fifth carbon in the cytosine pyrimidine ring) mRNA expression by quantitative real-time (qRT)-PCR in the dorsal and ventral DG samples. No differences were found in the expression of these two enzymes, both between genotypes and among control and uCMS conditions in the dorsal and ventral DG (Figure 21A-D; Table 26). However, when we assessed the DNA demethylation process through the quantification of the ten eleven translocation (TET) family of enzymes, which are largely responsible for the active DNA demethylation process, we could observe in the dorsal DG that all three enzymes (TET 1, TET 2 and

TET 3) showed a significant reduced expression in the AP2γ$^{+/-}$ group after exposure to uCMS when compared to the control AP2γ$^{+/-}$ mice (Figure 22A, C and E; Table 26). Moreover, we did observe the same tendency of TET 1 gene expression in the ventral DG (Figure 22B; Table 26). No differences were found between the WT groups in both dorsal and ventral DG, and although we could observe a tendency for the AP2γ$^{+/-}$ group to show higher expression of all three enzymes in control conditions, no statistical differences were found among genotypes.

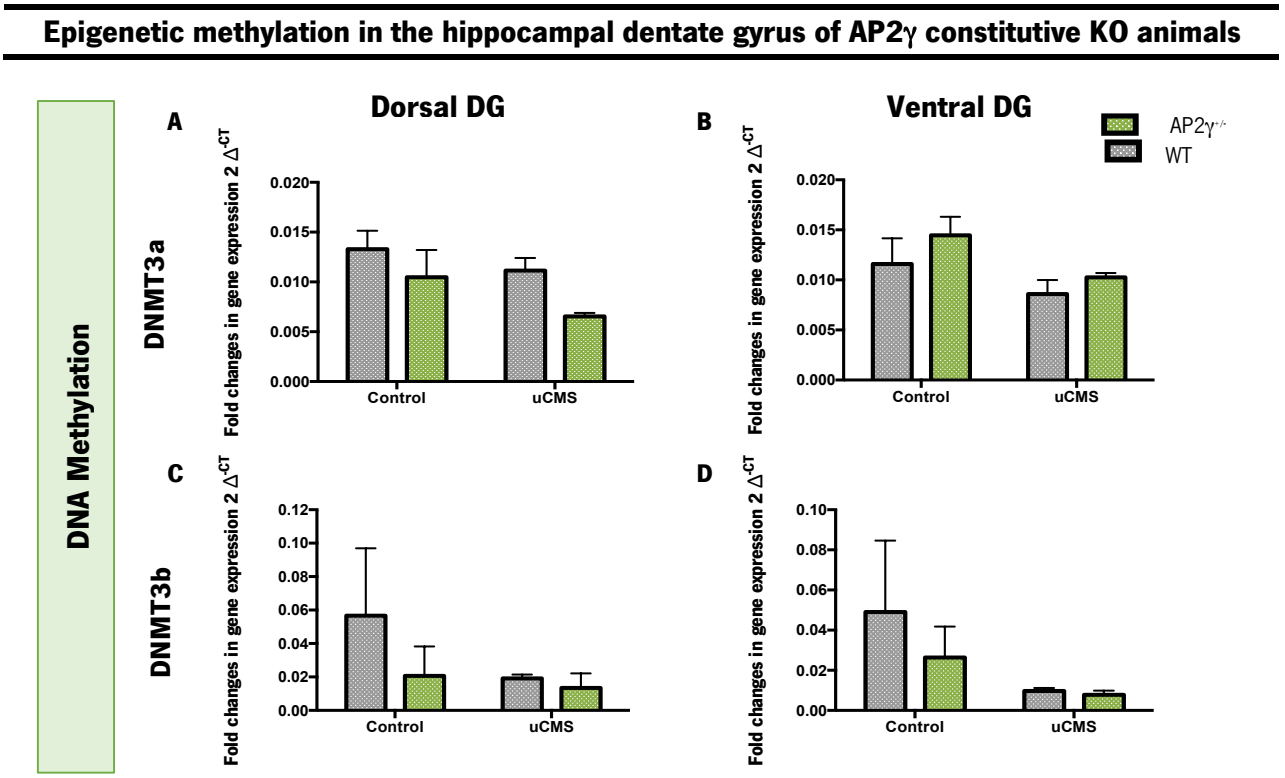



**Figure 21. Epigenetic modulators involved in DNA methylation in the hippocampal DG of AP2γ constitutive KO depressed-like animals.**

DNMT3a (A and B) and DNMT3b (C and D) gene expression was quantified through qRT-PCR. Data is presented with fold change values in the dorsal and ventral DG of AP2γ constitutive KO depressed-like animals. (Control: $n_{WT}$= 3, $n_{AP2\gamma^{+/-}}$= 3; uCMS: $n_{WT}$= 3, $n_{AP2\gamma^{+/-}}$=3; Data presented as mean ± SEM.

**Table 25. Statistical analysis of DNMTs gene expression quantification in the dorsal and ventral DG of the constitutive AP2γ KO animal model.**

Data presented as mean ± SEM.

| | | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|
| | | WT | AP2γ$^{+/-}$ | WT | AP2γ$^{+/-}$ | |
| DNMT3a | Dorsal DG | 0.013 ± 0.003 | 0.11 ± 0.003 | 0.11 ± 0.002 | 0.007 ± 0.0003 | Interaction Genotype vs uCMS: $F_{(1,6)}$= 0.16, p=0.7, $\eta^{2partial}$= 0.03 Interaction Control vs uCMS: $F_{(1,6)}$= 2.90, p=0.14, $\eta^{2partial}$= 0.33 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,6)}$= 1.93, p=0.21, $\eta^{2partial}$= 0.24 |
| | Ventral DG | 0.012 ± 0.003 | 0.14 ± 0.002 | 0.011 ± 0.001 | 0.01 ± 0.0004 | Interaction Genotype vs uCMS: $F_{(1,7)}$= 0.32, p=0.59, $\eta^{2partial}$=0.04 Interaction Control vs uCMS: $F_{(1,7)}$= 1.39, p=0.28, $\eta^{2partial}$=0.17 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,7)}$= 0.52, p=0.06, $\eta^{2partial}$=0.07 |
| DNMT3b | Dorsal DG | 0.057 ± 0.04 | 0.003 ± 0.02 | 0.03 ± 0.006 | 0.014 ± 0.009 | Interaction Genotype vs uCMS: $F_{(1,7)}$= 2.14, p=0.19, $\eta^{2partial}$=0.23 Interaction Control vs uCMS: $F_{(1,7)}$= 0.09, p=0.77, $\eta^{2partial}$=0.01 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,7)}$= 0.54, p=0.49, $\eta^{2partial}$=0.71 |
| | Ventral DG | 0.049 ± 0.04 | 0.026 ± 0.01 | 0.009 ± 0.001 | 0.008 ± 0.002 | Interaction Genotype vs uCMS: $F_{(1,7)}$= 0.22, p=0.65, $\eta^{2partial}$=0.03 Interaction Control vs uCMS: $F_{(1,7)}$= 1.73, p=0.23, $\eta^{2partial}$= 0.19 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,7)}$= 0.31, p=0.59, $\eta^{2partial}$= 0.04 |

**Table 26. Statistical analysis of the TET genes expression quantification in the dorsal and ventral DG of the constitutive AP2γ KO animal model.**

Data presented as mean ± SEM.

| | | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|
| | | WT | AP2γ$^{+/-}$ | WT | AP2γ$^{+/-}$ | |
| TET 1 | Dorsal DG | 0.001 ± 0.0002 | 0.002 ± 0.0001 | 0.001 ± 0.0001 | 0.008 ± 0.0003 | Interaction Genotype vs uCMS: $F_{(1,5)}$= 11.35, p=0.02, $\eta^{2partial}$= 0.70 Interaction Control vs uCMS: $F_{(1,5)}$= 9.86, p=0.02, $\eta^{2partial}$= 0.66 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,5)}$= 0.09, p=0.77, $\eta^{2partial}$= 0.02 |
| | Ventral DG | 0.001 ± 0.0003 | 0.002 ± 0.002 | 0.001 ± 0.0002 | 0.001 ± 0.0001 | Interaction Genotype vs uCMS: $F_{(1,5)}$= 7.83, p=0.04 $\eta^{2partial}$=0.61 Interaction Control vs uCMS: $F_{(1,5)}$= 6.16, p=0.06, $\eta^{2partial}$=0.55 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,5)}$= 1.74, p=0.24, $\eta^{2partial}$=0.26 |
| TET 2 | Dorsal DG | 0.39 ± 1.66 | 0.29 ± 0.01 | 0.55 ± 0.09 | 0.23 ± 0.06 | Interaction Genotype vs uCMS: $F_{(1,4)}$= 6.08, p=0.07, $\eta^{2partial}$=0.60 Interaction Control vs uCMS: $F_{(1,4)}$= 21.52, p=0.009, $\eta^{2partial}$=0.84 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,4)}$= 1.34, p=0.32, $\eta^{2partial}$=0.25 |
| | Ventral DG | 0.37 ± 1.53 | 0.48 ± 0.06 | 0.39 ± 0.07 | 0.45 ± 0.05 | Interaction Genotype vs uCMS: $F_{(1,4)}$= 0.19, p=0.68, $\eta^{2partial}$=0.05 Interaction Control vs uCMS: $F_{(1,4)}$= 0.003, p=0.96, $\eta^{2partial}$= 0.001 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,4)}$= 3.19, p=0.15, $\eta^{2partial}$= 0.44 |

| | | | | | |
|---|---|---|---|---|---|
| TET 3 | Dorsal DG | 0.0004 ± 0.008 | 0.0007 ± 0.0002 | 0.0003± 0.0001 | 0.0003 ± 0.0001 | Interaction Genotype vs uCMS: $F_{(1,4)}$= 6.00, p=0.07, $\eta^{2partial}$=0.60 Interaction Control vs uCMS: $F_{(1,4)}$= 24.0, p=0.008, $\eta^{2partial}$= 0.86 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,4)}$= 6.00, p=0.07, $\eta^{2partial}$= 0.60 |
| | Ventral DG | 0.0004 ± 0.002 | 0.0005 ± 0.0002 | 0.0003 ±0.00001 | 0.0002 ± 0.0001 | Interaction Genotype vs uCMS: $F_{(1,7)}$= 0.98, p=0.36, $\eta^{2partial}$=0.14 Interaction Control vs uCMS: $F_{(1,7)}$= 2.1, p=0.20, $\eta^{2partial}$= 0.26 Interaction WT vs AP2γ$^{+/-}$: $F_{(1,7)}$= 0.15, p=0.72, $\eta^{2partial}$= 0.02 |

## Epigenetic demethylation in the hippocampal dentate gyrus of AP2γ constitutive KO animals

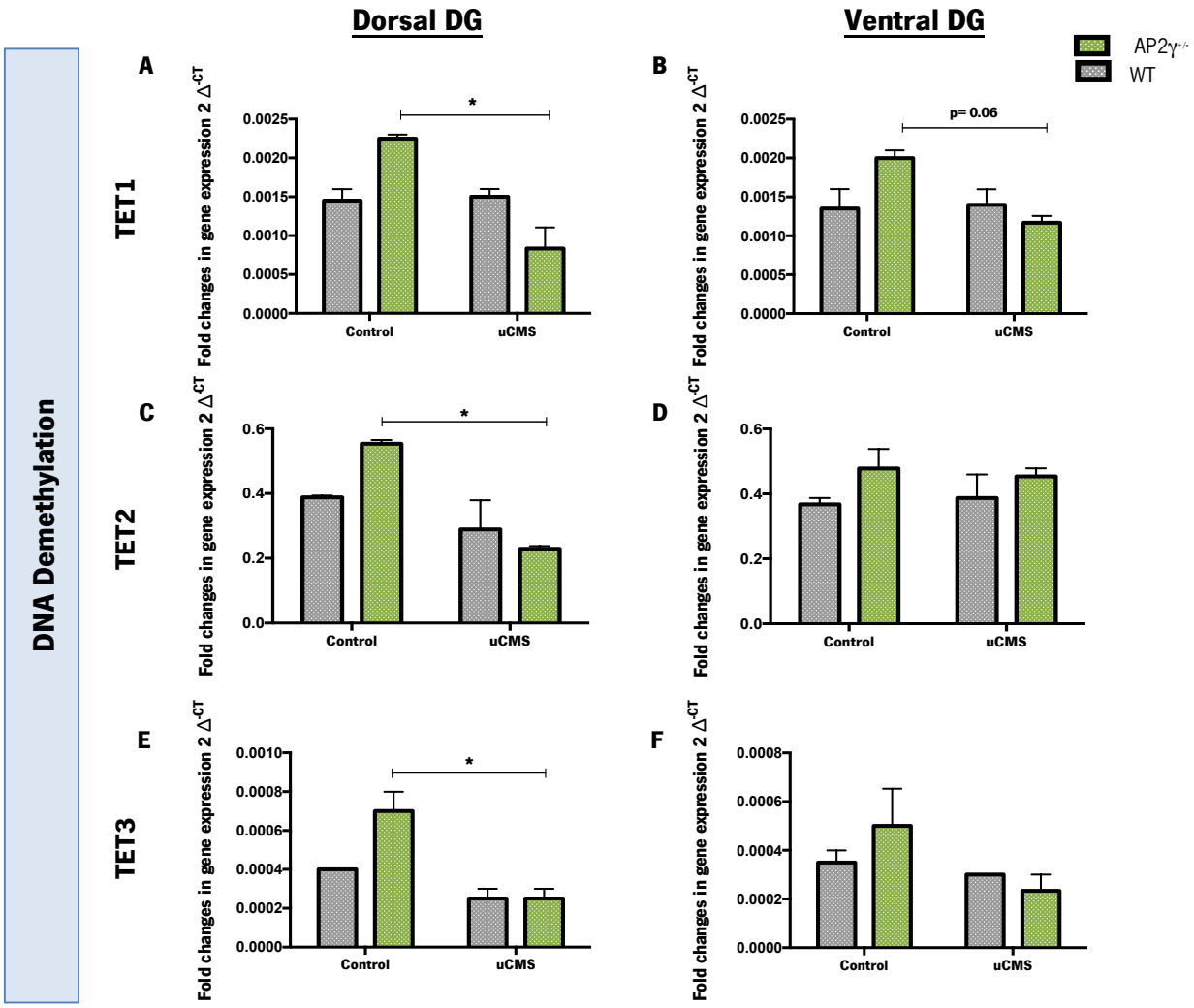



**Figure 22. Epigenetic modulators involved in DNA demethylation in the hippocampal DG of AP2γ constitutive KO depressed-like animals.**

TET1 (A and B), TET2 (C and D) and TET3 (E and F) gene expression was quantified through qRT-PCR. Data is presented with fold change values in the dorsal and ventral DG of AP2γ constitutive KO depressed-like animals. (Control: $n_{WT}$= 2, $n_{AP2\gamma^{+/-}}$= 2; uCMS: $n_{WT}$= 2, $n_{AP2\gamma^{+/-}}$=2 Data presented as mean ± SEM; *p<0.05).

CHAPTER 5

# DISCUSSION

# 5. DISCUSSION

## 5.1. Role of the transcription factor AP2γ during postnatal development

The AP2γ gene belongs to the AP2 family of transcription factors, which in mammals are largely associated with embryonic development. These transcription factors play crucial roles in various systems and biological processes, such as cell proliferation, cell adhesion, developmental morphogenesis, tumor progression and cell fate determination, through the regulation of a large number of target genes with different biological functions (Batsche et al. 1998; Ebert et al. 1998; Maconochie et al. 1999; Kuckenberg et al. 2012; Cyr et al. 2015). In mice, AP2γ is expressed during developmental stages, both in the central and peripheral nervous system (Pinto et al. 2009), as well as in the adult mouse forebrain (Pinto et al. 2009) and also in the SGZ of the hippocampal DG and the SEZ of the lateral ventricles (Mateus-Pinheiro et al. 2016). Perceiving the relevancy of this specific transcription factor in the primordial embryonic developmental stages makes it easy to understand why mice with homozygous deletion of AP2γ since embryonic development are not viable.

Therefore, knowing the importance of this transcription factor in embryonic development and in the adult neurogenesis process, combined with previous results from the group suggesting impairments in hippocampal glutamatergic neurogenesis (Mateus-Pinheiro et al. 2016), we wanted to understand if during post-natal neurodevelopment there were already deficits in the heterozygous AP2γ constitutive KO mice. To do so, we used the developmental milestones protocol, a simple procedure that is easy to measure, requires little equipment and includes a vast range of assays as indicators of both neurological, strength and motor development (Hill et al. 2008).

In general, all litters that have been tested for the developmental milestones protocol presented a normal development and no particular traits were noticed in body weight and anogenital distance, which are mainly physical and somatic parameters. Also, any relevant alterations were observed in motor development as assessed by the open field and walking tests, or in the sensorial parameters, as evaluated by the grasping, auditory startle, rooting and ear twitch tests. The only somatic and neurobiological differences found between groups were on the eye opening day, in the negative geotaxis and in the wire suspension tests, as seen by the delay and anticipation in the timing (measured as days) to acquire a mature response by the AP2γ$^{+/-}$ group. These AP2γ$^{+/-}$ animals showed a delay for 1 day in the negative geotaxis test, and 1, 2 days of anticipation for the eye opening day and the wire suspension mature response, respectively. Regarding the somatic anticipation in the AP2γ$^{+/-}$ group we

do not have a clear cause for this occurrence, and we believe that probably there is no biological meaning in this difference between the two groups. One aspect that we have to consider in this protocol, and that might be causing this small difference between the groups, is the different time of birth of the litters. Our strategy to control animal's birth was to check twice a day (in the morning, and at the end of the afternoon) if there were already pups in the animals' cage. These discrepancies in time between litters may lead in some milestones tests to discrete differences, and we do believe that this is the case for the eye opening test. Also corroborating the normality of the eye opening day is the fact that both groups opened both of their eyes within the normal range of maturity in this somatic test, which is between PND7-17 (Heyser 2001).

If we would only take in consideration the two obtained neurobiological differences in the negative geotaxis and wire suspension tests, it would be possible to indicate some sort of deficit associated with strength or muscular rigidity. However, in other developmental milestones protocol parameters, which also evaluated strength domains (like the surface righting, cliff aversion and air righting tests) we did not observe any kind of impairment between groups, indicating no deficits in this evaluated domain. Moreover, both results are within the normal range for rodents to acquire the mature response: defined for the negative geotaxis test to be between PND6-PND15 and for the wire suspension test between PND10-PND16 (Heyser 2001). In the future, it would be interesting to confirm these results through a more sensitive molecular approach. One way to do so could be through high-performance liquid chromatography (HPLC) in which we could quantify different neurotransmitters involved in strength, such as dopamine, which has been associated with muscular rigidity when present in lower percentages (Hemsley and Crocker 2001; Konieczny et al. 2009).

## 5.2. The role of AP2γ in juvenile mice

### 5.2.1. Impact of AP2γ on behavioral performance of juvenile mice

In previous studies from the group, we performed a multidimensional behavioral characterization of the AP2γ$^{+/-}$ adult mice in basal conditions. However, it was not analyzed if the modulatory effects of the transcription factor AP2γ on behavior, are indeed specifically observed at adult stages, or if during the developmental and maturity stages there is already an effect of the constitutive deletion of one allele of the AP2γ gene. Therefore, to start dissecting the precise role of AP2γ throughout postnatal development, in this thesis we used a set of established behavioral tests to

analyze WT and AP2γ$^{+/-}$ animals and study the modulatory effect of AP2γ deletion already in juvenile stages.

There is some information available regarding the proper age to test juvenile mice, but one of the most accepted and published ages is between 3 to 8 weeks. Taking this in consideration, the behavioral profile of the animals was assessed between PND25 to PND31, and as such we had to adapt in some situations the behavioral test to overcome limitations of the young age of the animals. We started the juvenile behavior characterization by evaluating if AP2γ deletion has an impact on the anxious-like behavior of the juvenile mice through the OF test. Although it is not described as a classical test for anxiety, the OF test can also be used as an indicator of anxious-like behavioral states of rodents, by evaluating the ratio of time spent and distanced traveled in the center versus the periphery of the arena (Prut and Belzung 2003). After a first trial with a small group of animals, we noticed that the habitual 5 min per trial in the OF arena was not sufficient to understand any kind of phenotype, as the majority of the animals did not perform the test, spending almost the total time in a resting state. We decided to adapt the time session for 20 min per trial, to allow the young animals to acclimatize to the light stimulus of the arena. Under these conditions we observed that the AP2γ$^{+/-}$ mice traveled lower distances and spent less time in the center of the arena, suggesting higher anxiety levels when compared to the WT group. Interestingly, the same pattern was found in the adult AP2γ$^{+/-}$ group at basal conditions, suggesting that this susceptibility to an anxious-like behavior already occurs during juvenile development.

Then we assessed if in basal conditions there are any signs of susceptibility to behavioral despair, through the TST. We did not find any difference between AP2γ$^{+/-}$ and WT juvenile mice in the percentage of time that the mice stay immobile during the test, suggesting the absence of depressive-like symptoms in AP2γ$^{+/-}$ juvenile mice. Also in the splash test we did not find differences between groups in the time that the animals spent grooming after applying a sucrose solution to their coat, further indicating that AP2γ$^{+/-}$ juvenile mice do not present core alterations in motivational self-care. Together, both results indicate that AP2γ deletion does not cause any predisposition towards a depressive-like behavior in juvenile ages. The same pattern was found during adulthood in the FST, thus corroborating this hypothesis.

One of our main goals with the juvenile behavior assessment was also to understand if AP2γ could have a modulatory role in the cognitive performance of juvenile mice, since in previous studies we obtained results suggesting its importance in modulating cognition of adult mice. Although Carr and colleagues already assessed cognition in juvenile mice (from PDN16 to PND22) (Carr et al. 2016), we

tried to assess it in our juvenile animals through the same novel object recognition (NOR) protocol but the animals did not perform this behavioral test. We further tried to explore this dimension through the social interaction and Y-maze tests, and once again animals did not perform these behavioral paradigms. Thus, until this moment we were not able to evaluate how AP2γ transcription factor is impacting on the cognitive performance of juvenile mice.

### 5.2.2. AP2γ modulation of hippocampal proliferation and neurogenesis in juvenile mice

In previous studies from the group hippocampal dentate gyrus (DG) cell proliferation and glutamatergic neurogenesis in the brain of AP2γ$^{+/-}$ adult mice was already characterized. However, it was not analyzed if these effects in the adult mice promoted by deletion of AP2γ are indeed specifically observed at adult stages, or if during the developmental and maturity stages there is already an effect of the constitutive deletion of one allele of AP2γ in hippocampal neurogenesis. To comprehend if the reduced expression of AP2γ could have already an impact on hippocampal glutamatergic neurogenesis during development and maturity stages, we injected juvenile animals with BrdU and sacrificed them 24h later (at PND31), allowing us, through immunostaining, to assess the proliferation of progenitor cells and newborn neurons (neuroblasts expressing doublecortin – DCX). Analysis of total cell proliferation in the DG of WT and AP2γ$^{+/-}$ animals revealed no significant differences between groups, although the proliferation values of AP2γ$^{+/-}$ animals were slightly reduced. In addition, we analyzed the number of newborn neuroblasts with double staining for BrdU and DCX, revealing a significant difference between WT and AP2γ$^{+/-}$ animals. In AP2γ$^{+/-}$ mice, a decreased number of neuroblasts was observed as shown by the reduced number of double-positive BrdU/DCX cells, when compared to the WT control group.

These deficits in newborn neurons, observed in juvenile animals with constitutive deletion of one allele of AP2γ, confirm the modulatory involvement of AP2γ in the modulation of post-natal glutamatergic neurogenesis in the healthy brain, already during development and maturity of these animals. Impairments in the generation of new hippocampal neurons have been largely associated with a more anxious phenotype (Revest et al. 2009; Hill et al. 2015). In our juvenile behavioral results we could observe through the OF test a higher anxiety level in the AP2γ$^{+/-}$ animals, since this KO mice traveled lower distances and spent less time in the center of the arena. Through these results we can suggest that the modulation exerted by the reduce expression of AP2γ transcription factor in the constitutive KO mice, is impairing the glutamatergic neurogenic process and causing a striking increase

in anxiety-related behavior. In the future, it would be interesting to quantify the expression of different AP2γ targets, to understand if at this point of development, it is already noticeable an altered presence of different transcripts involved in the post-natal glutamatergic process, such as Sox2, Pax6 and Tbr2. Also associated with impaired generation of new glutamatergic neurons in the hippocampus are the cognitive deficits (Shen et al. 2006; Deng et al. 2009; Jessberger et al. 2009). Thus, it becomes relevant to find suitable behavioral cognitive tests to assess if indeed this decreased neuronal generation leads to impaired cognitive performance of juvenile mice, as also seen by the deficits in the contextual-hippocampal associated memory and behavioral flexibility during adulthood (Mateus-Pinheiro 2016). Although in the duration of this thesis we were not able to successfully assess the cognitive performance of juvenile mice, in later results we adapted the NOR protocol to the night time period, since it is the active phase of the animals. In a preliminary trial with WT animals we observed that animals are indeed more actively performing the test when compared to the diurnal results. As such, in the future we plan to assess the cognitive behavior of both WT and AP2γ$^{+/-}$ juvenile mice in the nocturnal period.

The anxious and cognitive domains are largely associated with the post-natal glutamatergic neurogenic process, being the impairments in the emotional dimension more associated to deficits in the neurogenic process of the ventral DG, and the cognitive deficiencies more related to deficits in the glutamatergic neurogenesis occurring in dorsal DG (Kheirbek and Hen 2011). As such, in the future it would be interesting to discriminate the neurogenic alterations in these two areas of the DG, in order to better understand the behavioral phenotype, as previously performed in the adult mice (Mateus-Pinheiro et al. 2016).

## 5.3. Functional impact of the transcription factor AP2γ in depression

### 5.3.1. The uCMS protocol as a model of depression

To validate the uCMS protocol implemented in this master thesis study two major measures were used to control its efficacy: the body weight gained throughout the six weeks of uCMS protocol and the levels of corticosterone in blood at the end of the chronic stress exposure. Through the body weight gain, we already were able to observe imbalances between control and animals exposed to chronic stress in both models. During uCMS protocol, the control animals were gaining and maintaining the gained weight whereas the mice exposed to stress from both genotypes and models were first loosing and maintaining the weight lost throughout the six weeks of the chronic stress protocol. These findings

alone already are good validators of the efficacy of the uCMS protocol as a model to induce depressive-like features (Retana-Marquez et al. 2003; Bhatnagar et al. 2006). However, we wanted to perceive if these differences in body weight were really an indicator of a depressive-like state, or if it was a consequence of the food deprivation during some mild stressors. For that, we proceeded to a molecular analysis quantifying the levels of corticosterone in blood of the animals at the final week of the uCMS protocol.

As previously described, the circadian distribution of corticosterone in control rodents is characterized by a basal quantity at day time (nadir) and a night time peak (zenith) (D'Agostino et al. 1982). This circadian regulation of corticosterone secretion was found to be altered in animals exposed to stressful events, suggesting a disruption of the HPA axis (Ottenweller et al. 1994). In healthy conditions, the HPA axis is the major neuroendocrine system that controls organisms' reactions. Upon acute stress stimulation, the axis releases corticotrophin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, which in turn acts on the pituitary gland inducing adrenocorticotropic (ACTH) release from the pituitary into the circulatory system. In the periphery, corticosteroids are then secreted from the adrenal gland. A negative feedback mediated by corticosteroids on the hypothalamus, pituitary and higher brain centers ensures the equilibrium of the system (Bhatnagar et al. 2006; Femenia et al. 2012).

It is known that chronic stress exposure is accompanied by HPA axis hyperactivity, resulting in deregulated glucocorticoid secretion-pattern into the blood (Schoenfeld and Gould 2012). In our results, we observed that both constitutive and conditional KO control groups maintained a normal circadian rhythm of corticosterone secretion, since there is a significantly peak of corticosterone at the zenith time-point. The uCMS exposed animals showed a disruption of the HPA axis by presenting a significantly higher nadir levels of corticosterone than the control groups, and no circadian regulation, as there are no differences between nadir and zenith in both constitutive and conditional KO uCMS groups.

Together, the imbalances in the weight gain and the disruption of the corticosterone circadian rhythm, validate the efficacy of the uCMS protocol in both animal models, a shoring in this way, that the animals presented core symptoms of depressive-like disorder.

### 5.3.2. Role of AP2γ in the modulation of behavioral domains affected by depression

The confirmation of the modulatory role of AP2γ on the adult hippocampal neurogenic process opens exciting perspectives regarding the importance of these gene in neuropathological contexts in which neurogenesis is impaired, such as depression. Having this in mind, we decided to study if AP2γ deletion also modulates adult hippocampal neurogenesis and behavior in depressive-like animals. For this, we exposed both AP2γ KO constitutive and conditional animal models to an uCMS protocol.

The behavioral profile of the animals was assessed immediately after the six weeks of chronic stress exposure. The uCMS protocol successfully induced depressive-like behavior in the chronic stressed WT (WT and cAP2γ$^{+/+}$) animals from both models, reflected in a substantial increase of immobility in the FST (for both models) and in the TST (for the conditional model). However, no differences were found between the KO (AP2γ$^{+/-}$ and cAP2γ$^{-/-}$) and the WT (WT and cAP2γ$^{+/+}$) groups, suggesting that the reduced expression of AP2γ did not affect the mood of mice. Interestingly, and considering that the hippocampal neurogenic process of these two different groups of AP2γ KO mice is partially compromised (Mateus-Pinheiro et al. 2016), this finding matches the results reported by Bessa et al (Bessa et al. 2009a), in which it was shown that stressed animals with and without suppressed neurogenesis spend higher periods of time immobile in the FST comparing to control animals, but that these two groups did not differ between them. Furthermore, anxiety-related behavior assessed by the OF and the EPM tests, was found to be affected by the uCMS protocol in both mice models analyzed, reflected in a substantial decreased distance traveled and time spent in the center of the arena in the OF test by the WT uCMS groups, and the increased time in enclosed arms of the EPM apparatus by the same referred animals. Notably, in these two behavioral tests, different trends regarding the control situations were observed, since in the OF test we could observe an anxious-like behavior in the AP2γ KO groups, which was not the case in the EPM test. However, we have to take in consideration that the different behavioral tests employed to assess the anxiety-related behavior have advantages and limitations such as all the paradigms used to assess animal's behavior. Moreover, these different behavioral tests measure different fractions of an animal's emotional profile, making it possible to obtain distinct phenotypes from both tests. To further dissect the role of AP2γ in anxiety-like behavior we may in the future perform additional behavioral tests, like the light-dark box test, which can also provide valuable insights regarding this behavioral dimension (Bourin and Hascoet 2003).

The most interesting findings in the characterization of the behavioral profile of AP2γ KO adult mice are related to cognition. Indeed, the constitutive deletion of AP2γ exerted significant impacts on

mice cognitive performance in the working and spatial reference memory, and also in the reversal learning task. Exposure to uCMS induced highly significant deficits on working memory and reference memory of the constitutive WT mice, an effect that was completely absent in the AP2γ$^{+/-}$ stressed mice. This is an interesting observation that seemed to be associated to some compensatory mechanism in AP2γ$^{+/-}$ animals exposed to stress, that lead to the reinforcement of the hippocampal neuronal population, thus counteracting the deleterious effects of stress in this cognitive dimension. Interestingly, this proved to be case, as data from the protein levels of different transcripts involved in adult glutamatergic neurogenesis, namely Sox2, Pax6 and Tbr2 seem to be highly expressed in uCMS AP2γ$^{+/-}$ animals when compared to the WT stressed mice.

Moreover, in the reversal learning task in the water maze test and in the context probe from the CFC test, AP2γ$^{+/-}$ mice already showed significant deficits in contextual hippocampal-associated memory and impaired hippocampal-to-PFC network in basal conditions, independently of stress exposure. Likewise, in the conditional AP2γ KO model a tendency was found in the reversal learning task in which cAP2γ$^{-/-}$ presented lower percentage of time in the new quadrant when compared to the cAP2γ$^{+/+}$ control group. Regarding the deleterious effect of stress in the reversal learning task, in the conditional model, chronic stress induced detrimental effects on the cAP2γ$^{+/+}$ uCMS animals, and interestingly this result was not observed between the cAP2γ$^{-/-}$ groups. However, no differences were found between control and uCMS groups in the constitutive model.

Taking into consideration that this deficiency in AP2γ in the constitutive model is present since embryonic development, most likely it has affected cortical basal progenitors specification, accounting for deficits in neocortical development, as previously shown (Pinto et al. 2009). As the reversal learning task is closely related to PFC functions, deficits observed in the control AP2γ$^{+/-}$ are probably due to misspecification of cortical layers in this cortical region, that ultimately leads to defects on cognitive activities in which PFC is actively involved. Interestingly, in previous electrophysiological studies from the group, we showed that in basal conditions, the AP2γ deficiency in the adult brain of the conditional cAP2γ$^{-/-}$ mice led to a significant decrease of coherence between the ventral hippocampus and the PFC (Mateus-Pinheiro et al. 2016), indicating a decrease in the ability of these two region to functionally interact, previously shown to be critically related with behavior outputs dependent on cortico-limbic networks (Fell and Axmacher 2011; Gordon 2011). Moreover, the integrity of the ventral hippocampus-to-PFC link has been recently described to be important to the antidepressant action of drugs, such as ketamine (Carreno et al. 2016), raising the possibility that AP2γ may play an important role in the preservation of this neuronal circuit (Mateus-Pinheiro et al. 2016). In the future, it would be interesting

to explore this electrophysiological measurements in the context of depression, in order to better perceive how AP2γ influences interregional communication in the pathogenesis of psychiatric disorders.

### 5.3.3. The modulatory action of AP2γ in the hippocampal neurogenic niche

The behavioral profile of the constitutive and conditional AP2γ KO groups was altered in the assessed cognitive domains. As previously discussed, the evident decline in the performance of AP2γ KO animals in the reversal learning tasks is likely a consequence of defects on cortical development, and also impairments in neuronal circuits that create deficits in interregional communication. At this point, further studies are needed to fully assess if this is indeed the cause underlying the observed behavioral phenotypes. However, driven by the observed resilience to the deleterious effects of uCMS exposure on the reference memory – an hippocampal-dependent function – we hypothesized whether such phenotype could be also related to alterations in the hippocampal neurogenic niche. To prove so, we started to quantify the impact of AP2γ on the protein levels of different transcripts involved in the adult glutamatergic neurogenic process in both depressed constitutive and conditional animal models.

Although we are aware, that further studies are needed to fully comprehend the effect of AP2γ in the hippocampal dentate gyrus and its repercussion on the altered cognitive function, we started this hippocampal neurogenesis characterization through western blots quantification. As it was possible to observe, both models showed a significant reduction on AP2γ protein levels in the hippocampal DG, wherein the conditional model was highly impacted by the tamoxifen-inducible deletion of AP2γ. Moreover, AP2γ reduction in the constitutive animal model triggered a reduction of Tbr2 protein levels, but had no impact on its upstream regulators such as Sox2 and Pax6. However, in the conditional AP2γ model, all the transcripts measured seem to be highly impacted by the deficits in AP2γ since cAP2γ$^{-/-}$ control mice have reduced expression of Pax6, DCX and also Tbr2.

Regarding the uCMS conditions we had an interesting finding in the AP2γ constitutive KO model that lead us to hypothesize that compensatory mechanisms during developmental stages may happen. After uCMS exposure the AP2γ$^{+/-}$ animals showed a high bolstering of Sox2, Pax6 and Tbr2, which, like previously reported, are highly involved in the modulation of adult hippocampal glutamatergic neurogenesis. We may hypothesize that by having a developmental defect in neuronal progenitor cells since embryonic development, this neuropathological target is already affected, thereby contributing to a minor extent to the pathological repercussions of chronic stress exposure. This phenomenon, can be attributable to compensatory mechanisms that emerged by the constitutive deletion of one allele of the AP2γ gene since embryonic development, similarly to what happens with several cell proliferation

regulatory molecules and their binding partners cyclins (Satyanarayana and Kaldis 2009). Another possibility is that functionally redundant members of the AP2 transcription factors family may also interplay the lack of AP2γ, reinforcing hippocampal cell proliferation both in basal and in neuropathological contexts. Interestingly, and although in the conditional AP2γ model the WB quantifications are not yet fully concluded in uCMS conditions, the same pattern was not observed, further supporting the contribution of putative compensatory mechanism that emerged since early development in the constitutive KO model.

To better understand the modulatory impact of AP2γ in hippocampal neurogenesis and its repercussions on the cognitive dimension, further complementary studies are required in both KO models, such as DG immunostainings for proliferative markers, analysis of the dendritic morphology of DG granular neurons, spine densities and morphology. Moreover, complementing these results with electrophysiological characterization of the hippocampus-to-PFC network in uCMS conditions would bring some insights if the interregional communication dysfunction emerges as a consequence of glutamatergic network malfunction triggered by the lack of AP2γ regulation in the adult neurogenic niche.

## 5.4. AP2γ impacts on epigenetic regulators of adult hippocampal glutamatergic neurogenesis – A preliminary perspective

Epigenetic mechanisms have been proposed to play pivotal roles in different stages of both embryonic and adult neurogenesis, being involved in the regulation of NSCs proliferation, fate specification and differentiation. These mechanisms are now becoming to be recognized as fundamental for the balanced production of new neuronal and glial cells needed for the homeostatic brain function (Mateus-Pinheiro et al. 2011). Cumulative evidence now suggests that epigenetic dysregulation also plays an important part in neurodegenerative disorders, and more interestingly, in psychiatric disorders (Yao et al. 2016). It is possible to say that epigenetic mechanisms are becoming gradually accepted to play dynamic roles in adult neurogenesis, and actually in a recent study from Amador-Arjona and colleagues (Amador-Arjona et al. 2015) Sox2 transcription factor was proposed to set a permissive epigenetic state in NSCs, enabling in this way the neuronal differentiation under neurogenic cue.

Having in mind the cumulative evidences of epigenetic modifications in the transcriptional sequence underlying adult glutamatergic neurogenesis, and the crucial role of AP2γ in this process we

decided to perform a broad analysis of DNA methylation and demethylation – two well-known epigenetic mechanisms that play important roles in the adult glutamatergic neurogenic process. Although we still did not obtain a clear interpretation of how deficits in AP2γ impact on the epigenetic regulation of adult glutamatergic neurogenesis in basal conditions, it was possible to observe alterations in the uCMS AP2γ$^{+/-}$ mice.

In this thesis, we decided to measure the expression levels of different enzymes involved in the methylation and demethylation of DNA. No differences were observed in the methylation enzymes in WT and AP2γ$^{+/-}$ animals both in basal and uCMS conditions. We measured the activity of DNMT3a and DNMT3b which are largely responsible for the *de novo* methylation, a process highly important for embryogenesis, neural development and the establishment of methylation patterns (Montalban-Loro et al. 2015). However, there is another type of methylation reaction, responsible to copy the existing methylation patterns, promoted by DNMT1, which we still did not assess. This enzyme is abundantly expressed in the embryonic, perinatal and adult CNS in both dividing NSCs and mature neurons, thus it is highly relevant to assess in the near future if DNMT1 is affected by the reduced expression of AP2γ. Unlike DNA methylation, when we assessed the epigenetic demethylation process we found differences between AP2γ$^{+/-}$ control and uCMS groups in the dorsal DG. We measured the activity of the TET family of enzymes which are largely responsible for the demethylation process promoting the removal of the methyl mark and leaving in this way the gene in a more activated state (Maeder et al. 2013). We did obtain a similar pattern among all three TET enzymes (TET1, TET2, TET3) in the dorsal DG, presenting a reduced expression after uCMS exposure. Furthermore, in the ventral DG we only found the same pattern of expression for the TET1 enzyme.

These results suggest a reduction in the demethylation process in the AP2γ$^{+/-}$ chronic stressed animals, which can be related with the cognitive alterations that we did observe in the constitutive AP2γ KO animal model. However, further studies are required to understand which genes have an altered demethylated process, perceive if its function are related to neuroplasticity, and if indeed an altered epigenetic mechanism, induced by chronic stress exposure, in adult hippocampal neurogenesis can lead to significant behavioral adaptations. Moreover, we need to understand how is AP2γ being modulated by the demethylation process, if its expression is increasing or decreasing by this modulatory action, and how it rebounds in other transcription factors involved in the glutamatergic neurogenic process, such as Tbr2, which is highly influenced by the AP2γ (Mateus-Pinheiro et al. 2016).

The techniques used in this thesis to analyze these specific epigenetic mechanisms gave only a broad analysis, allowing to explore if indeed the reduced expression of AP2γ could cause epigenetic alterations in the hippocampal neurogenic niche. In the future, and to perform a more specific analysis it would be interesting to use different techniques such as DNA bisulfite conversion which allows the evaluation of the methylation and demethylation status of individual CpG dinucleotides in the genomic DNA, understanding at the same time which genes are being methylated or demethylated in depression (Booth et al. 2013).

## 5.5. AP2γ transcription factor – An integrated perspective

The work developed and presented here was designed to further explore the impact of the transcription factor AP2γ in brain neurophysiology and behavior during development and at adult stages both in basal and depressive-like conditions. Using two animal models with different mechanical targeted AP2γ deletion, we were able to better understand some of the contributions of this transcription factor in varied brain processes, from the developmental period until adulthood. One of the best characterized function of the AP2γ transcription factor, shown by previous studies of the group, is its involvement in the transcriptional network that underlies glutamatergic neurogenesis during embryonic development, which integrates several transcription factors sequentially expressed (Sox2→Pax6→Ngn2→ Tbr2→NeuroD→Tbr1). AP2γ participates in this transcriptional network that hallmarks embryonic glutamatergic neurogenesis acting as a downstream target of Pax6, with modulatory effects upon basal progenitors such as Tbr2 (Pinto et al. 2009). Interestingly, it was found that during adult glutamatergic neurogenesis, neurons recapitulate this transcriptional sequence that hallmarks the embryonic glutamatergic neurogenesis in the developing cerebral cortex (Brill et al. 2009). As such, this transcriptional sequence is maintained postnatally, and actually, more recently we were able to identify the presence of AP2γ in the adult hippocampal DG, both in Tbr2-positive glutamatergic progenitor cells and neuroblasts. We further revealed that AP2γ is also a positive regulator of adult glutamatergic neurogenesis in the adult hippocampal DG, as its overexpression increments the generation of new neurons in this region and its deletion, both *in vivo* and *in vitro*, results in marked reduction of the neuroblasts population (Mateus-Pinheiro et al. 2016).

Taking in consideration these previous findings of the group regarding the AP2γ modulatory effect of embryonic and adult glutamatergic neurogenesis, we wanted to discriminate if the molecular and behavioral deficits seen in the adult constitutive AP2γ KO mice were indeed an adult response or if

there is already an impact of AP2γ reduction on post-natal development and on juvenile stages. We also wanted to investigate the potential role of AP2γ as a key neurogenesis modulator in the neuropathological context of depression, since in this disorder, hippocampal glutamatergic neurogenesis is highly affected. To do so, we used two animals models for AP2γ deletion to understand the impact of AP2γ in depression in two different perspectives. By using the constitutive AP2γ KO model, we intended to understand if the deletion of one allele of AP2γ since embryonic development could lead to a predisposition to deficits in the three behavioral dimensions affected by depression, since hippocampal glutamatergic neurogenesis is impaired in the AP2γ$^{+/-}$ animals. Through the conditional AP2γ KO mice, in which the homozygous deletion of the gene is only induced at adult stages prior to the uCMS protocol, we were able to better elucidate the role of this transcription factor specifically in adult hippocampal neurogenesis both in basal and depressive-like conditions, without the contribution of putative compensatory mechanisms that had eventually emerged since early development.

We observed in the basal conditions results presented in this thesis, molecular deficits in the constitutive AP2γ$^{+/-}$ animals since juvenile development until adulthood, which were largely associated with the altered behavioral phenotypes, namely anxiety and cognitive impairments, obtained both at juvenile and adult stages. However, if we had only used this constitutive KO animal model, it would not have been possible to completely understand if the results observed at the moment of testing were indeed an alteration of the reduced expression of AP2γ, or if there were putative compensatory mechanisms intervening in the analysis. Combining the evidences from both constitutive and conditional AP2γ animals models we were able to better perceive how is this transcription factor modulating post-natal hippocampal neurogenesis, and its repercussions in both basal and uCMS conditions. Between the constitutive and conditional KO animals two major differences were observed: in behavioral tests associated to cognition, and in the expression of the different transcription factors involved in the glutamatergic neurons formation in the adult DG. When analyzing the cognitive dimension of the constitutive animal, and we compare it with the expression profiles obtained by the western blot, it is possible to perceive that there is some sort of compensatory mechanism in the AP2γ$^{+/-}$ mice, bolstering the expression of Sox2, Pax6 and Tbr2, ameliorating in this way the deleterious effects induced by chronic stress. But when we analyzed the same cognitive dimensions in the chronically stressed conditional cAP2γ$^{-/-}$ animals, we did not obtain the same cognitive phenotype, nor the bolstering of transcription factors involved in the glutamatergic neurogenic process. Thus, the

amelioration of cognitive domains observed in the constitutive uCMS AP2γ$^{+/-}$ animals, seem to be determined during the development period.

Regarding the anxiety phenotype, we did obtain similar patterns in both constitutive and conditional animal models, showing both AP2γ KO (AP2γ$^{+/-}$ and cAP2γ$^{-/-}$) control groups a higher anxiety rate in the OF test, but no differences after uCMS exposure. We also assessed the anxious behavior at juvenile ages, showing once again the constitutive AP2γ$^{+/-}$ animals a more anxious-like behavior when compared to the WT control group, in the OF test. Thus, the compensatory mechanisms observed in the neurogenic transcripts in the constitutive model are not influencing the anxiety dimension, since there is no amelioration of the anxious-phenotype in the AP2γ$^{+/-}$ uCMS group. Moreover, when we analyzed epigenetic mechanisms in the constitutive model, the most altered area was the dorsal DG which is largely associated with the cognitive dimension (Fanselow and Dong 2010), being the ventral area (more related to the emotional dimension) less affected by these alterations. Although it is quite early to speculate how AP2γ modulates the epigenetic regulators of the neurogenic process, it is already noticeable that demethylation of some genes is occurring after chronic stress exposure. However, further studies are required to perceive if these alterations in the constitutive model lead to some functional impact.

CHAPTER 6

# CONCLUDING REMARKS

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In the past decades, a large effort has been made to unravel the complex pathological basis that underlies several neuropsychiatric disorders in which adult hippocampal glutamatergic neurogenesis is impaired, such as major depression. The view of adult hippocampal neurogenesis as a causative neurobiological mechanism of depression has been supported by several reports, showing the high comorbidity between the precipitation of depressive symptomatology and the reduction of hippocampal proliferation. Finding a molecule with an important modulatory action in the adult hippocampal neurogenic process is, in the present context, of the outmost importance in order to revert the pathological effects upon adult neurogenesis, observed in depression. It can be stated that the work performed and included in this thesis provided important insights on the potential of AP2γ as a promising candidate to mediate such action. In fact, the transcription factor AP2γ was proved to be actively involved in the promotion of post-natal neurogenesis in the hippocampal DG neurogenic niche. Furthermore, in this thesis, we observed that reduced levels of AP2γ since embryonic development already impacted juvenile behavior and impaired neuronal proliferation at this stage of development and maturity, specifically causing a more anxious-like phenotype and decreased proliferation of immature neurons. Moreover, we submitted both constitutive and conditional animals to an unpredictable chronic mild stress protocol, and the behavioral profile revealed that the reduced expression of AP2γ produced significant deficits in cortical-dependent cognitive tasks, which is likely a consequence of cortical developmental deficits. However, this deletion since embryonic development proved to be beneficial for hippocampal dependent cognitive functions, namely working and spatial memory, possibly due to a compensatory increase of different transcripts important for adult glutamatergic neurogenesis. This compensatory mechanism is further supported by the fact that these transcripts were normally expressed in the DG of conditional KO mice after chronic stress exposure.

Together, these results further support the involvement of AP2γ in the adult glutamatergic neurogenic process, and that it may be a strong candidate to be used as a therapeutical tool in future therapies directed to the modulation of the neurogenic niche, mostly in patients with cognitive deficits. However, further analyses are needed to elucidate what is the functional role of this transcription factor in the context of depressive, and to fully understand the different mechanisms by which AP2γ is exerting its modulatory actions.

CHAPTER 7

# REFERENCES

# 7. REFERENCES


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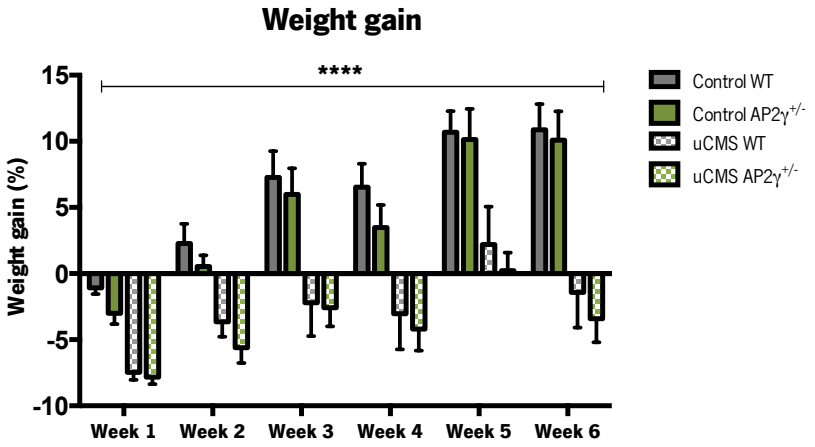
CHAPTER 8

# ANNEXES

## 8.1. Supplementary figures

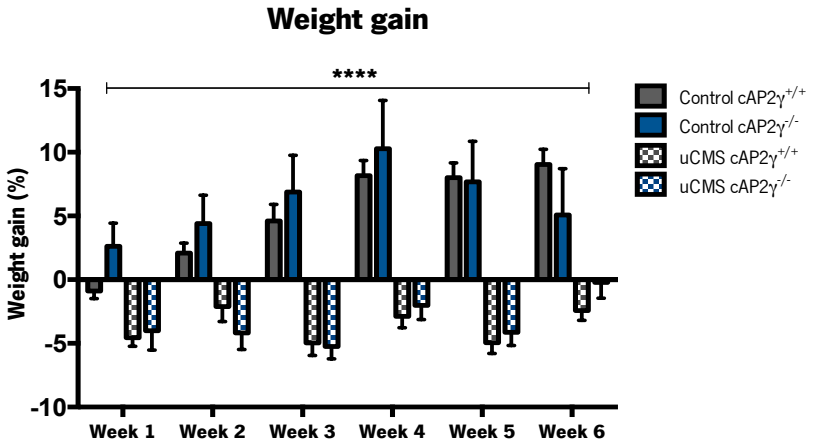



**Supplementary figure 1: Percentage of weight gain in the AP2γ constitutive KO animal model throughout the 6 weeks of uCMS protocol, divided by individual groups.**

During the uCMS protocol, we controlled the body weight in order to understand if the chronic stress exposure was inducing alterations in the animals exposed to it. It was observable that the uCMS was indeed inducing highly different directions regarding the percentage weight gain/loss, since the the control groups were gaining and maintaining the weight gain, and the uCMS exposed groups lost and maintained the weight loss. However, no differences were found between WT and AP2γ$^{+/-}$ groups, proving that there are no differences between the genotypes concerning to eating patterns. (Controls: $n_{WT}$= 8, $n_{AP2\gamma^{+/-}}$= 7 ; uCMS: $n_{WT}$= 8, $n_{AP2\gamma^{+/-}}$=7; Data presented as mean ± SEM; ****p<0.0001).

**Supplementary table 1: Percentage of weight gain in the AP2γ constitutive KO animal model throughout the 6 weeks of uCMS protocol, divided by individual groups.** Data presented as mean ± SEM. In grey shading are the tests were it was observable differences between the variables.

| Test | | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|
| | | WT | AP2γ$^{+/-}$ | WT | AP2γ$^{+/-}$ | |
| Weight | Week 1 | -1.06 ± 0.44 | -3.02 ± 0.76 | -7.48 ± 0.52 | -7.83 ± 0.49 | $F_{(1,24)}$=1.75, p< 0.0001, $\eta^2_{partial}$= 0.55 |
| | Week 2 | 2.28 ± 1.39 | 0.53 ± 0.79 | -3.63 ± 1.05 | -5.60 ±1.08 | |
| | Week 3 | 7.28 ± 1.86 | 5.99 ± 1.83 | -2.22 ± 2.36 | -2.59 ± 1.31 | |
| | Week 4 | 6.54 ± 1.65 | 3.48 ± 1.57 | -3.05 ± 2.52 | -4.19 ± 1.51 | |
| | Week 5 | 10.70 ± 1.49 | 10.13 ± 2.15 | 2.20 ± 2.70 | 0.22 ± 1.26 | |
| | Week 6 | 10.88 ± 1.82 | 10.09 ± 2.02 | -1.42 ± 2.49 | -3.41 ± 1.6 | |




**Supplementary figure 2: Percentage of weight gain/loss in the AP2γ conditional KO animal model throughout the 6 weeks of uCMS protocol, divided by individual groups.** During the uCMS protocol, we controlled the body weight in order to understand if the chronic stress exposure was inducing alterations in the animals exposed to it. It was observable that the uCMS was indeed inducing highly different directions regarding the percentage weight gain/loss, since the the control groups were gaining and maintaining the weight gain, and the uCMS exposed groups lost and maintained the weight loss. However, no differences were found between cAP2γ$^{+/+}$ and cAP2γ$^{-/-}$ groups, proving that there are no differences between the genotypes concerning to eating patterns. (Controls: $n_{cAP2\gamma^{+/+}}$= 12, $n_{cAP2\gamma^{-/-}}$=5; uCMS: $n_{cAP2\gamma^{+/+}}$=12, $n_{AP2\gamma^{-/-}}$=8; Data presented as mean ± SEM; ****p<0.0001].

**Supplementary table 2: Percentage of weight gain in the AP2γ conditional KO animal model throughout the 6 weeks of uCMS protocol, divided by individual groups.** Data presented as mean ± SEM. In grey shading are the tests were it was observable differences between the variables.

| Test | | Control | | uCMS | | Statistical test, significance, effect size |
|---|---|---|---|---|---|---|
| | | cAP2γ$^{+/+}$ | cAP2γ$^{-/-}$ | cAP2γ$^{+/+}$ | cAP2γ$^{-/-}$ | |
| Weight | Week 1 | -0.62 ± 0.62 | 2.63 ± 1.82 | -4.22 ± 0.60 | -4.00 ± 1.53 | $F_{(1,36)}$=3.16, p< 0.0001, $\eta^2_{partial}$= 0.64 |
| | Week 2 | 2.41 ± 0.81 | 4.40 ± 2.23 | -1.62 ± 0.60 | -4.18 ± 1.31 | |
| | Week 3 | 3.01 ± 2.00 | 6.88 ± 2.87 | -3.63 ± 1.31 | -5.24 ± 0.97 | |
| | Week 4 | 8.23 ± 1.13 | 10.27 ± 3.81 | -1.54 ± 1.28 | -2.02 ± 1.12 | |
| | Week 5 | 8.14 ± 1.10 | 7.70 ± 3.17 | -3.38 ± 1.35 | -4.13 ± 1.04 | |
| | Week 6 | 8.89 ± 1.13 | 5.07 ± 3.64 | -0.71 ± 1.31 | -0.21 ± 1.25 | |