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Differential Roles of Specific Sub-Regions of the Longitudinal Axis of the Hippocampus in the Behavioural and Neurogenesis Responses to Stress and Antidepressant Drugs

Thesis presented by

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Declaration

All work presented in this PhD thesis is original and my own. The work was carried out under the supervision of Dr. Olivia F. O'Leary and Prof. John F. Cryan between June 2014 and March 2018 in the Department of Anatomy and Neuroscience, University College Cork, Ireland. This thesis has not been submitted in whole or in part for another degree, either at University College Cork or elsewhere.

Author contribution:

The author conducted all the work in the thesis independently with some help as follows:

- Chapter 2: Livia Morais and Clara Seira assisted with the forced swim test.
- Chapter 3: Patrick Fitzgerald, Sofia Cussotto and Dr. Emmanuela Morelli helped with the forced swim test.
- Chapter 4: Lorraine Conroy, Jack O'Leary and Thanida Panyavorakunchai helped imaging the cells and analysing the data.
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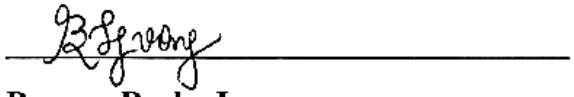

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List of abbreviations

3-Ch - Three chamber test
5-HT - Serotonin
AHN - Adult hippocampal neurogenesis
ANOVA - Analysis of variance
bFGF - Basic fibroblast growth factor
BrdU - Bromodeoxyuridine
CA - Cornu Ammonis
CORT - Corticosterone
DA - Dopamine
DAPI - 4',6-diamino-2-phenylindole
DG - Dentate gyrus
dCA1 - Dorsal Cornu Ammonis 1
DCX - Doublecortin
dDG - Dorsal dentate gyrus
DEX - Dexamethasone
DG - Dentate gyrus
dHi - Dorsal hippocampus
DIV - Days *in vitro*
DS - Donkey serum
EGF - Epidermal growth factor
EPM - Elevated plus maze
FGF - Fibroblast growth factor
FLX - Fluoxetine
FST - Forced swim test
FUST - Female urine sniffing test
GC - granular cells
GCL - granular cells layer
GFAP - Glial fibrillary acidic protein
GR - Glucocorticoid receptor
HBSS - Hank's balanced salt solution
HPA - Hypothalamus-Pituitary-Adrenal
HPRA - Health Products Regulatory Authority
iCA1 - Intermediate Cornu Ammonis 1
i.p. - Intraperitoneally
iHi - Intermediate hippocampus
IPC - Intermediate progenitor cells
LTD - Long-term depression
LTP - Long-term potentiation
ML - Molecular layer
MR - Mineralocorticoid receptor
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA - Noradrenaline
NeuN - Neuronal Nuclei
NIH - Novelty-induced hypophagia test
NPC - Neural progenitor cell
OF - Open field
PBS - Phosphate buffered saline
PBS-T - Phosphate buffered saline with Triton X
PFA - Paraformaldehyde
QNP - Quiescent neural progenitor cells
SP - Saccharin preference test
PTSD - Post-traumatic stress disorder
SGZ - Subgranular zone
SSRI - Selective serotonin reuptake inhibitor
TST - Tail suspension test
vCA1 - Ventral Cornu Ammonis 1
vDG - Ventral dentate gyrus
vHi - Ventral hippocampus
WHO - World Health Organization

Abstract

Accumulating evidence suggests that the hippocampus is functionally segregated along its longitudinal axis into a dorsal (dHi) and a ventral sub-region (vHi). Indeed, recent gene expression studies suggest that the hippocampus has a gradient of gene expression and that the area between the dHi and vHi, the intermediate hippocampus (iHi), may also be functionally independent, but it remains understudied. The hippocampus is also one of few brain regions where neurogenesis, the birth of new neurons, occurs throughout life and this process has been shown to play roles in learning and memory as well as in responses to stress and antidepressants. These diverse roles may be related to the functional segregation of the hippocampus along its longitudinal axis. Indeed, the dorsal hippocampus (dHi) plays a predominant role in spatial learning and memory, while the ventral hippocampus (vHi) is predominantly involved in the regulation of anxiety, a behaviour impacted by stress and chronic treatment with some antidepressants. *In vivo* studies have shown that chronic stress and chronic antidepressant treatment change neurogenesis preferentially in the vHi rather than the dHi. However, whether these findings are due to differential intrinsic sensitivities of neural progenitor cells (NPCs) resident in the dHi, iHi or vHi in response to the stress hormone corticosterone or in response to antidepressants is unknown. Moreover, the roles of the dHi, iHi and vHi in the behavioural responses to chronic stress, a risk factor for depression and anxiety disorders, and in the behavioural responses to acute and chronic antidepressant treatment have not yet been investigated. Thus, the aims of this thesis were to determine whether NPCs isolated from the dHi, iHi and vHi have differential intrinsic sensitivities in response to the stress hormone corticosterone, the glucocorticoid receptor agonist dexamethasone, and the antidepressant, fluoxetine. To this end, we isolated NPCs

from the three hippocampal sub-regions and cultured them for 4 h or 4 days *in vitro* with either corticosterone, fluoxetine, or corticosterone with fluoxetine or for 7 days *in vitro* with either corticosterone or dexamethasone. Cell proliferation, neuronal differentiation and maturation, nuclear GR expression and cell viability were then assessed. Moreover, we also aimed to determine the roles of each hippocampal sub-region on emotional behaviours and neuroendocrine response under baseline conditions, chronic psychosocial stress conditions and under chronic antidepressant treatment conditions. To this end, we performed stereotaxic surgeries in C57BL/6 mice to lesion the dHi, iHi or vHi with ibotenic acid. After recovery, animals were submitted to emotional behaviour and neuroendocrine tests under baseline conditions, or after chronic psychosocial stress, or after acute and chronic fluoxetine treatment. As result, we determined for the first time that NPCs isolated from the iHi and especially vHi are more sensitive to the effects of long-term exposure (7 DIV) to corticosterone and dexamethasone. Long-term (7 DIV) corticosterone and dexamethasone exposure also reduced nuclear GR expression preferentially in cells from the vHi. Fluoxetine alone did not have any effect on cell proliferation or neuronal differentiation or maturation. However, fluoxetine prevented corticosterone-induced reductions in neuronal differentiation after 4 DIV treatment and these effects were observed predominantly in the iHi and vHi cell cultures. *In vivo* experiments showed that vHi lesions reduced anxiety under baseline conditions. Under chronic psychosocial stress conditions, iHi lesions increased stress-induced social avoidance and the lesion of all sub-regions prevented chronic stress-induced anxiety; dHi and vHi lesions prevented stress-induced anhedonia and only vHi lesions caused antidepressant-like behaviour in the forced swim test and promoted active coping behaviour. In the antidepressant experiment, vHi lesions prevented the antidepressant effects of acute fluoxetine treatment while iHi lesions

prevented the antidepressant effects of chronic fluoxetine treatment, and both iHi and vHi lesions prevented the anxiolytic effects of chronic fluoxetine treatment. Taken together, these findings show for the first time that iHi and vHi NPCs have increased intrinsic sensitivity to longer term exposure to the stress hormone corticosterone, and that the vHi seems to be an important sub-region for antidepressant-like effects under chronic stress. Also, both the iHi and vHi but not the dHi seem to modulate the antidepressant and anxiolytic effects of fluoxetine.

Keywords: ventral hippocampus, intermediate hippocampus, dorsal hippocampus, corticosterone, stress, neurogenesis, fluoxetine, antidepressant, anxiety, depression.

CHAPTER 1

General Introduction

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1.1. The Hippocampus: General Structure and Functions

The hippocampus is a large structure of the human and other vertebrates brain. It is a bilateral limbic structure located in the medial temporal lobe of the brain. This brain region has a long-established role in certain forms of learning and memory (Scoville and Milner 1957, Milner and Klein 2016), playing a special role in declarative memory; and as the memories consolidate, they become hippocampus-independent. Indeed, the hippocampus plays a role in the consolidation of information from short-term to long-term memory. In addition to that, the hippocampus has also been shown to play a role in spatial memory. Many previous and recent studies have reported that the hippocampus is actually heterogeneous in function, also playing central roles in emotional behaviour, response to stress, and the regulation of the release of stress hormone.

1.1.1. Neuroanatomical structure of the hippocampus

The hippocampal formation is a large curved shaped structure along its longitudinal axis, which comprises different areas, including the dentate gyrus (DG), Cornu Ammonis (CA1, CA2, CA3 and CA4) and the subiculum (see Fig1.1). These areas have mostly excitatory pathways that repeat themselves along the longitudinal axis of the hippocampus (Andersen *et al.* 1969). The hippocampus receives major inputs from the entorhinal cortex (medial and lateral), perirhinal cortex, postrhinal cortex, medial septum, locus coeruleus, raphe nucleus, nucleus reuniens and amygdala while its main outputs are to the lateral septum, nucleus accumbens, amygdala and prefrontal cortex.

The entorhinal cortex provides the major cortical inputs to the hippocampus, passing though the perforant path on its way to the DG. Via the mossy fibre

pathway, the granule cells of the dentate gyrus projects to the CA3 area of the hippocampus, which in turn projects to the CA1 area through the Schaffer collateral pathway. Finally, the CA3 projects to the entorhinal cortex, closing the circuit called the trisynaptic loop of the hippocampus (Knierim 2015). Granule neurons compose the DG of the hippocampus, and they are organised into three main layers: the molecular layer (ML), which is occupied by the dendrites of the dentate gyrus granule cells, the fibres of the perforant pathway and some interneurons; the granular cell layer, which contains the cell bodies of the granule cells (Amaral *et al*. 2007) and comprises also the subgranular zone (SGZ). The SGZ is also the home for neural progenitor cells, as discussed later in this introduction. The anatomy of the hippocampus is illustrated in the Fig 1.1.

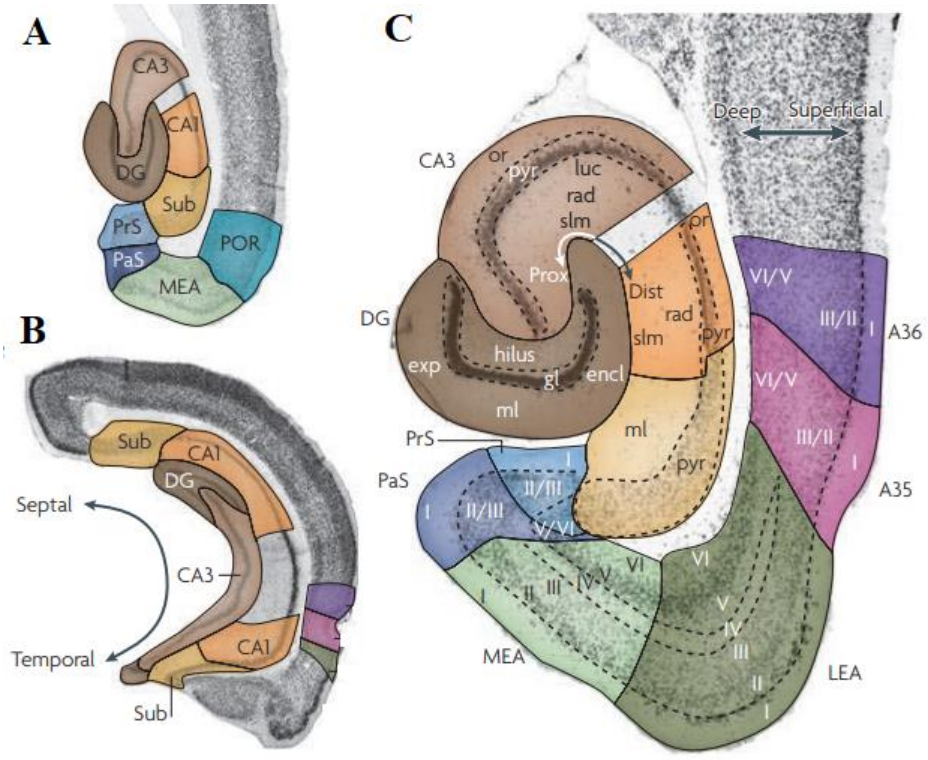

Figure 1.1*: Anatomy of the hippocampus. The hippocampal formation is composed of dentate gyrus (DG) and the hippocampus proper, which include the Cornu Ammonis (CA1, CA2, CA3 and CA4). In A and C, a transverse section and in B, a coronal section of the rat hippocampus. Figure adapted from: (van Strien et al. 2009).*

1.1.2. Functions of the hippocampus

Since the mid-20th century, when the famous case of the patient H.M. was studied, it became well established that the hippocampus plays a role in the consolidation of memory. There are two main types of memory: declarative (explicit) and procedural (implicit). While procedural memory refers to the unconscious memory of skills which normally involve automatic sensorimotor behaviour, declarative memory is the memory of facts and events which are stored and then retrieved. Declarative memory can be subdivided into episodic and semantic memories. Episodic memory represents the memory of experiences, and semantic memory is a structured record of facts and accumulated knowledge. While the hippocampus has been shown not to be involved in procedural memory, it is known to play a key role in declarative memory (Eichenbaum 2001, Corkin 2002, Eichenbaum 2004, Wixted *et al.* 2018).

Many studies have also shown that the hippocampus plays a key role in spatial learning and place discrimination. The Nobel Laureate in Physiology or Medicine, John O'Keefe, was the first to determine a relationship between the hippocampus and space learning, by showing that pyramidal cells of the hippocampus fire selectively in specific locations, and these were denominated place fields (O'Keefe and Dostrovsky 1971, O'Keefe 1976, Morris *et al.* 1982, O'Keefe and Speakman 1987, Moser *et al.* 1995, O'Keefe *et al.* 1998, Moser *et al.* 2008). Further studies on place fields, head direction cells and grid cells have emphasised a role for hippocampus as cognitive map (Moser *et al.* 1995, Moser and Moser 1998, Moser *et al.* 2008).

In addition to playing a role in spatial learning and memory, the hippocampus also modulates anxiety and the response to stress via control of the release of the stress

hormone, corticosterone (Bannerman *et al.* 2004, Fanselow and Dong 2010, O'Leary and Cryan 2014). Indeed, the hippocampus contains a high concentration of both glucocorticoid and mineralocorticoid receptors (Jacobson and Sapolsky 1991), thus rapidly responding to the effects of cortisol in humans and corticosterone in rodents, as discussed further in this introduction. In agreement, bilateral lesions of the rat hippocampus caused a hypersecretion of glucocorticoids (Sapolsky *et al.* 1984). In parallel, a reduction in the volume of the hippocampus in chronically stressed rats has been reported (Lee *et al.* 2009). Moreover, lesion studies have shown that hippocampal lesions cause changes in emotional behaviours, particularly in anxiety (Bannerman *et al.* 2002, Kjelstrup *et al.* 2002, Bannerman *et al.* 2003, Bannerman *et al.* 2004, Pentkowski *et al.* 2006). Finally, it is well established that the hippocampus plays an important role in the regulation of the response to stress, a risk factor for some psychiatric disorders, including depression.

1.2. Stress

Stress is an event that threatens the homeostasis of the organism and, as a result, causes physiological and behavioural responses that attempt to reinstate equilibrium (McEwen and Wingfield 2003, Day 2005, de Kloet *et al.* 2005). Allostasis can be defined as the collection of processes that are required to achieve internal and external stability in the face of a changing environment thus maintaining homeostasis (McEwen and Wingfield 2003, Day 2005, de Kloet *et al.* 2005). Allostatic load results from excessive stress or a failure to achieve homeostasis and may occur as a result of repeated stress from multiple stressors, poor adaptation and prolonged or inadequate response to stress (McEwen and Stellar 1993, McEwen 2007). While the acute stress response is an important and necessary mechanism to

adapt to environmental changes that occur throughout life thus promoting effective coping, severe or chronic stress can result in allostatic load and it is also a contributing risk factor for the development of several psychiatric disorders such as depression and post-traumatic stress disorder (PTSD) (McEwen and Wingfield 2003, McEwen 2007). However, it is also important to note that many stress-exposed individuals do not develop stress-related psychiatric disorders (Caspi *et al.* 2003, Charney and Manji 2004, Yehuda and LeDoux 2007) and are thus more resilient to the negative consequences of stress than others.

Resilience to stress is the ability to cope with environmental challenges, ensuring survival, while susceptibility to the negative consequences of stress seems to result from an improper functioning of the systems of resilience or an amplification of the stress experience (Karatsoreos and McEwen 2013), which in turn can result in maladaptive physiological and behavioural responses. Such maladaptive responses to stress may increase the risk for the development of stress-related psychiatric disorders, and as such great effort is being made to elucidate the neural processes that underlie stress-resilience in the hope that these might be then exploited for drug development (Franklin *et al.* 2012, Hughes 2012, Russo *et al.* 2012, Wu *et al.* 2013).

The response to stressors involves the activation of the hypothalamus-pituitary-adrenal axis (HPA), the system within the body responsible for the release of glucocorticoid stress hormones. The activation of the HPA axis rapidly stimulates the secretion of corticotropin-releasing factor and vasopressin from parvocellular neurons of the paraventricular nucleus of the hypothalamus and this stimulates the release of adrenocorticotropic hormone from the anterior pituitary, which in turn stimulates the release of glucocorticoids from the adrenal cortex into the circulation

(Cullinan *et al.* 1995). These glucocorticoids, cortisol in humans and corticosterone in rodents (Herman and Cullinan 1997), feedback onto two types of receptors in the brain: the mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), which are highly expressed in limbic structures of the brain, including the hippocampus (Morimoto *et al.* 1996). Following stress termination, glucocorticoid concentrations slowly decrease to pre-stress levels and this recovery is primarily controlled by negative feedback of glucocorticoids onto their receptors in the anterior pituitary and the paraventricular nucleus of the hypothalamus as well as the hippocampus (Herman and Cullinan 1997, De Kloet *et al.* 1998). The role of the hippocampus in the negative feedback of the HPA axis is shown in Fig 1.2.

1.2.1. Stress & the hippocampus

The hippocampus is a key brain area involved in the regulation of the stress response, exerting negative feedback on the hypothalamic-pituitary-adrenal (HPA) axis (Fig 1.2) (Jacobson and Sapolsky 1991). While hippocampal MR mediates the effects of glucocorticoids on assessment of the stressor and initiation of the stress response, GR acts in the consolidation of acquired information (Morimoto *et al.* 1996, De Kloet *et al.* 1998, de Kloet *et al.* 2005). Activation of these receptors in the hippocampus exerts negative feedback on the HPA axis, suppressing further release of glucocorticoids following stress termination. Thus, inappropriate functioning of the hippocampus could disrupt proper functioning of the HPA axis (De Kloet *et al.* 1998).

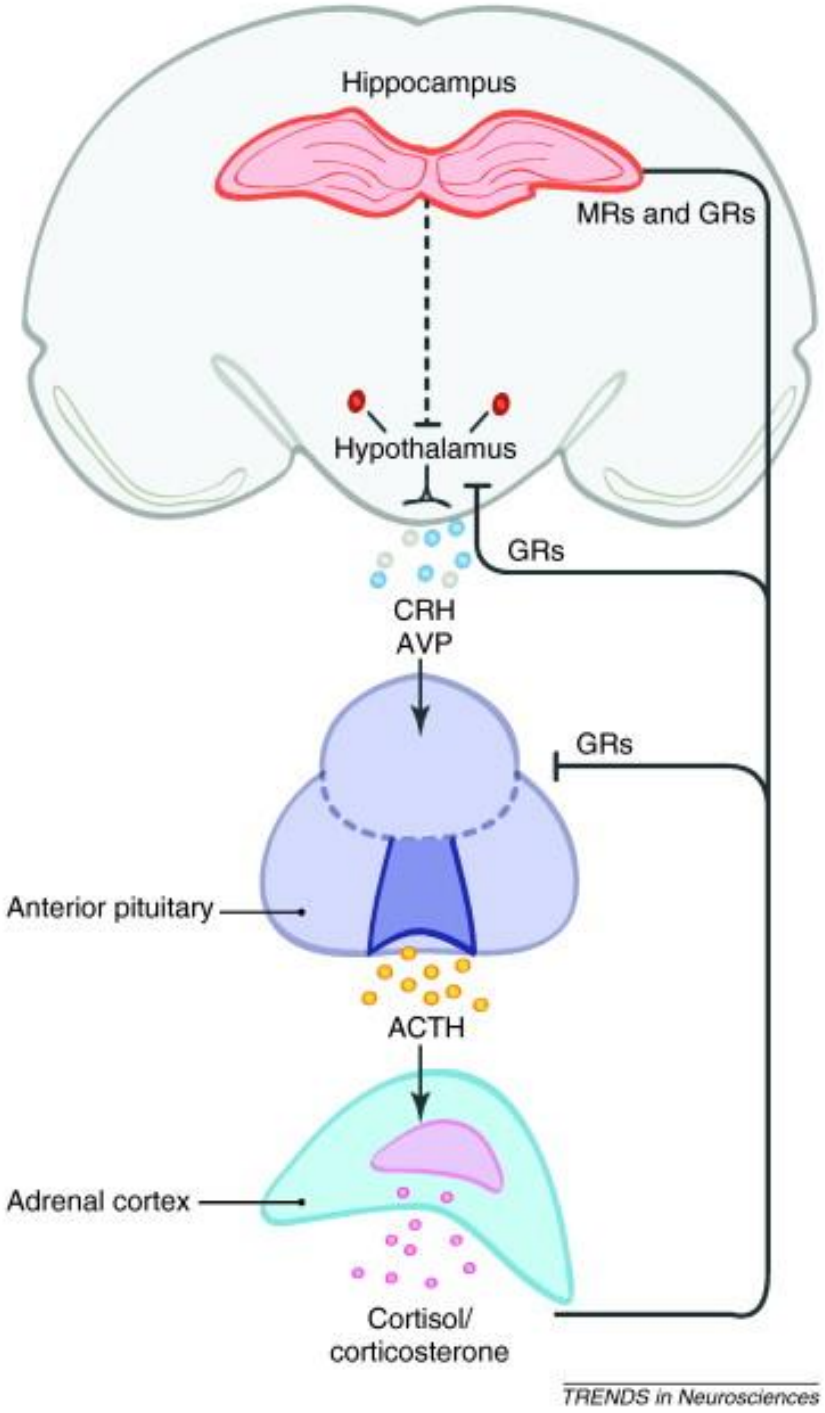

Figure 1.2*: Stress and the activation of the hypothalamic–pituitary–adrenal (HPA) axis. Image from: (Schloesser et al. 2012). Abbreviations: ACTH: adrenocorticotropic hormone; AVP: arginine vasopressin; CRH: corticotropin-releasing hormone; GRs: glucocorticoid receptors; MR: mineralocorticoid receptors.*

In addition to playing a key role in the regulation of stress response, the hippocampus is also particularly vulnerable to the effects of stress (Sapolsky 1986, McEwen *et al.* 1992, McEwen and Sapolsky 1995). Accordingly, volumetric

reductions in the hippocampus have been reported in PTSD patients (Bremner *et al.* 2003, Smith 2005, Felmingham *et al.* 2009) and PTSD patients exhibit dysfunction of the HPA-axis with high levels of corticotropin-releasing hormone in the cerebrospinal fluid (Bremner *et al.* 1997) and low levels of cortisol in urine (Yehuda *et al.* 1995), indicating an enhanced HPA-axis feedback regulation (de Kloet *et al.* 2006). On the other hand, plasma concentrations of cortisol are often increased in depressed adults (Westrin *et al.* 1999) and it has been suggested that elevated glucocorticoid concentrations contribute to stress-induced atrophy of the hippocampus (McEwen and Sapolsky 1995) and its correlation with cognitive dysfunction (Lupien *et al.* 1998). Similarly, neuroimaging studies also report volumetric reductions in the hippocampus in depression (Sheline *et al.* 1996, Bremner *et al.* 2000, Frodl *et al.* 2002, Videbech and Ravnkilde 2004). In parallel, studies in animals report that chronic stress, which is a risk factor for depression, reduces hippocampal volume (Lee *et al.* 2009, Tse *et al.* 2014), causing dendritic atrophy (Lee *et al.* 2009, McEwen 2016) and reducing adult hippocampal neurogenesis (Gould *et al.* 1997, Simon *et al.* 2005, Dranovsky and Hen 2006, Schoenfeld and Gould 2012, Levone *et al.* 2015). Taken together, it is clear that there is a reciprocal relationship between the hippocampus and glucocorticoids and that disrupted HPA-axis activity might impact hippocampal structure and function which in turn might further impact hippocampal regulation of glucocorticoid concentrations. Also, stress-induced changes in the hippocampus seem to mirror the hippocampal changes that have been reported in depression. Indeed, stress is a risk factor for depression.

1.3. The Role of the Hippocampus in Depression and Antidepressant Treatment

According to the World Health Organisation (WHO), depression is a common mental disorder, currently being the leading cause of disability worldwide, with more than 300 million people of all ages affected (WHO 2017). Depression is characterized by a persistent sadness, a loss of interest in pleasant activities, loss of energy, a change in appetite; sleeping problems, anxiety, reduced concentration, indecisiveness, guilt, and thoughts of self-harm or suicide may also occur (WHO 2017). Despite the continuous research that is done in an effort to try to understand the pathophysiology of depression, the precise mechanisms through which depression develops is elusive, probably because depression is a heterogeneous disorder. Many studies report that antidepressants are significantly better than placebo on the reversal of the general depressive state (Thase and Denko 2008). However, half of the depressed patients treated with first-line antidepressant treatment fail to achieve remission and a third of these patients remain treatment-resistant (Fava 2003, Trivedi *et al.* 2006).

Antidepressant drug development has been hampered by the fact that we do not yet have a complete understanding of the neurobiology underlying the pathophysiology of depression and its successful treatment by current antidepressants. However, one of the main theories is the monoamine hypothesis of depression. In this hypothesis, depression is suggested to be a result of a dysregulation in monoamine neurotransmitter systems including those of serotonin, noradrenaline, and dopamine, and most currently available antidepressants have been designed to increase extracellular concentrations of these neurotransmitters particularly serotonin and/or noradrenaline (Manji and Duman 2001, O'Leary *et al.* 2015). The

most commonly used class of antidepressants are the selective serotonin reuptake inhibitors (SSRI), which selectively inhibit the serotonin transporter, thus increasing the availability of serotonin in the synaptic cleft. The main SSRI drugs include fluoxetine, paroxetine, citalopram, escitalopram, sertraline and fluvoxamine. Tricyclics antidepressants (TCA) were one of the first types of antidepressants to be developed and these block noradrenaline and/or serotonin transporters thus inhibiting serotonin/noradrenaline reuptake and increasing their availability in the synaptic cleft. Some TCA drugs include imipramine, desipramine and amitriptyline. Monoamine oxidase inhibitors (MAOI) prevent the degradation of serotonin, noradrenaline and dopamine, by acting on the enzyme that degrades them. Examples include phenelzine, tranylcypromine and moclobemide. Many other drugs are specific for blocking the reuptake of noradrenaline (NRIs) like reboxetine, or for blocking both noradrenaline and serotonin reuptake (SNRI), like venlafaxine. Other antidepressants include multi-target antidepressants which target both monoaminergic transporters and receptors, e.g. vortioxetine

Although the pathophysiology of depression is still under debate, clinical neuroimaging studies report volumetric reductions in the hippocampus in depression (Sheline *et al.* 1996, Bremner *et al.* 2000, Frodl *et al.* 2002, Videbech and Ravnkilde 2004, Koolschijn *et al.* 2009). Interestingly, when exposed to chronic stress, animals can vary in their response to stress, and thus can be separated into two groups: stress-resilient or stress-susceptible. This variation in the stress response has been linked to hippocampal volumes whereby resilient animals exhibit increased hippocampal volume (by 4%), even after stress, while susceptible animals exhibit decreases in volume (by 1%) (Tse *et al.* 2014), findings which parallel the volumetric losses in the hippocampus of individuals with depression or PTSD

(Sheline *et al.* 1996, Felmingham *et al.* 2009), both of which are stress-related disorders. Interestingly, chronic treatment with different classes of antidepressant treatment has been shown to reverse stress-induced reductions in adult hippocampal neurogenesis (Malberg *et al.* 2000, Malberg and Duman 2003, Rainer *et al.* 2012). Correspondingly, human neuroimaging studies report that hippocampal volumetric reductions are more evident in unmedicated depressed individuals (Sheline *et al.* 2003) and in poor responders to antidepressant treatments (Frodl *et al.* 2008). Taken together, it is clear that the hippocampus is recruited in antidepressant action.

1.4. Adult Hippocampal Neurogenesis

An intriguing feature of the hippocampus is that it is just one of a few areas of the adult brain where new neurons are born throughout adult life, through a process called adult hippocampal neurogenesis (AHN) (Kempermann *et al.* 2004, Ming and Song 2011, Kempermann *et al.* 2015). It was not until recently that this was accepted and recognised by the scientific community (Gage 2002). In fact, it is suggested that hundreds of new neurons are born in the human dentate gyrus of the hippocampus everyday (Spalding *et al.* 2013). However, recently, an important study did not find any immature neurons in the adult human brain, suggesting the absence of adult neurogenesis (Sorrells *et al.* 2018). However, another study reported that the quiescent progenitors pool does remain active throughout aging (Boldrini *et al.* 2018), thus leaving room for much further debate (Kempermann *et al.* 2018). Such studies also highlight the difficulties researchers face when trying to measure AHN in the human brain. Indeed, we do not yet have neuroimaging tools to measure the dynamic process of AHN in humans. Nevertheless, we have learned a lot about the process of AHN from animal studies.

1.4.1. Adult hippocampal neurogenesis: Historical perspective

Until the middle of the 20th century, neuroanatomists such as Santiago Ramón y Cajal, believed that the nervous system was something fixed, immutable and incapable of regeneration. In 1962, however, Joseph Altman found the first evidence of adult neurogenesis in the cerebral cortex (Altman 1962) and then, in 1963, in the dentate gyrus of the hippocampus (Altman 1963). It was not until the 1980s, when adult neurogenesis was shown to exist in rats (Bayer *et al.* 1982), and in birds (Goldman and Nottebohm 1983) that the interest in AHN started growing in the scientific community. In the 1990s, neural progenitor cells were isolated in the adult mammalian brain (Reynolds and Weiss 1992, Gage *et al.* 1995) and adult neurogenesis was first shown in non-human primates (Gould *et al.* 1999) and humans (Eriksson *et al.* 1998). Human neural progenitor cells were also successfully isolated and cultured in an *in vitro* neurogenesis assay (Roy *et al.* 2000). Nowadays the presence and importance of AHN is well recognised by the scientific community (Boldrini *et al.* 2018), although there are still some controversies surrounding its presence and role in the human brain (Sorrells *et al.* 2018).

1.4.2. Adult hippocampal neurogenesis: A series of sequential developmental events

AHN is a complex process; neural progenitor cells (NPCs) go through diverse developmental stages (Fig 1.3), including cell proliferation, neuronal differentiation, maturation and integration onto the neural circuitry of the hippocampus (Christie and Cameron 2006). Multipotent neural stem cells (NSCs) can give rise to NPCs, although there is no clear definition of the differences between NPCs and NSCs, NPCs are thought to be an intermediary between NSCs

and differentiated cells, having a more limited capacity for self-renewal, when compared to NSCs. The NPCs are located in a neurogenic niche in the SGZ of the DG of the hippocampus. In the DG, these cells are quiescent radial glia-like neural progenitor cells (QNP), also known as type 1 NPC or early progenitor cells, and they express glia fibrillary acidic protein (GFAP), Nestin and Ki67. These QNP divide very slowly, but upon specific stimuli, QNP enter the cell cycle, self-renewing, dividing and producing new radial glia-like neural progenitor cells. These radial glia-like neural progenitors, in turn divide to generate the proliferating intermediate progenitor cells (IPCs), also known as transiently amplifying progenitors, type 2a which expresses only nestin, and type 2b which expresses nestin and doublecortin - DCX). Depending upon a stimulus these IPCs eventually turn into neuroblasts (type 3), which expresses doublecortin (DCX). These neuroblasts in turn differentiate into immature neurons, which express DCX and Prospero Homebox 1 (Prox1). These immature neurons, once mature, express neuron-specific nuclear protein (NeuN) and Calbindin (Ming and Song 2011). These stages of the neurogenesis are shown in Fig 1.3.

It takes about 1-3 days for the IPCs to differentiate into neuroblasts, which further develop into new immature neurons. These newly generated immature neurons migrate short distances into the granular cell layer (GCL) of the DG and mature into dentate granule cells (GCs) within approximately 1.5 to 2 weeks. Newly generated GCs project their dendrites to the molecular layer (ML) of the DG, and their axons to the CA3 of the hippocampus through the hilus. Fully integrated adult-born granule cells can be found within 7 weeks. Newly-born neurons are silent until 3 days from differentiation, but slow GABAergic neurotransmission is formed by day 7; then fast glutamatergic transmission is developed and finally fast

GABAergic neurotransmission (Esposito *et al.* 2005, Ge *et al.* 2006). In this first month from differentiation, newly-born neurons display enhanced LTP, and increases in this LTP promotes further proliferation of NPCs (Bruel-Jungerman *et al.* 2006). In turn, depolarizing GABAergic effect promotes synaptic integration of the newly-born neurons (Ge *et al.* 2006).

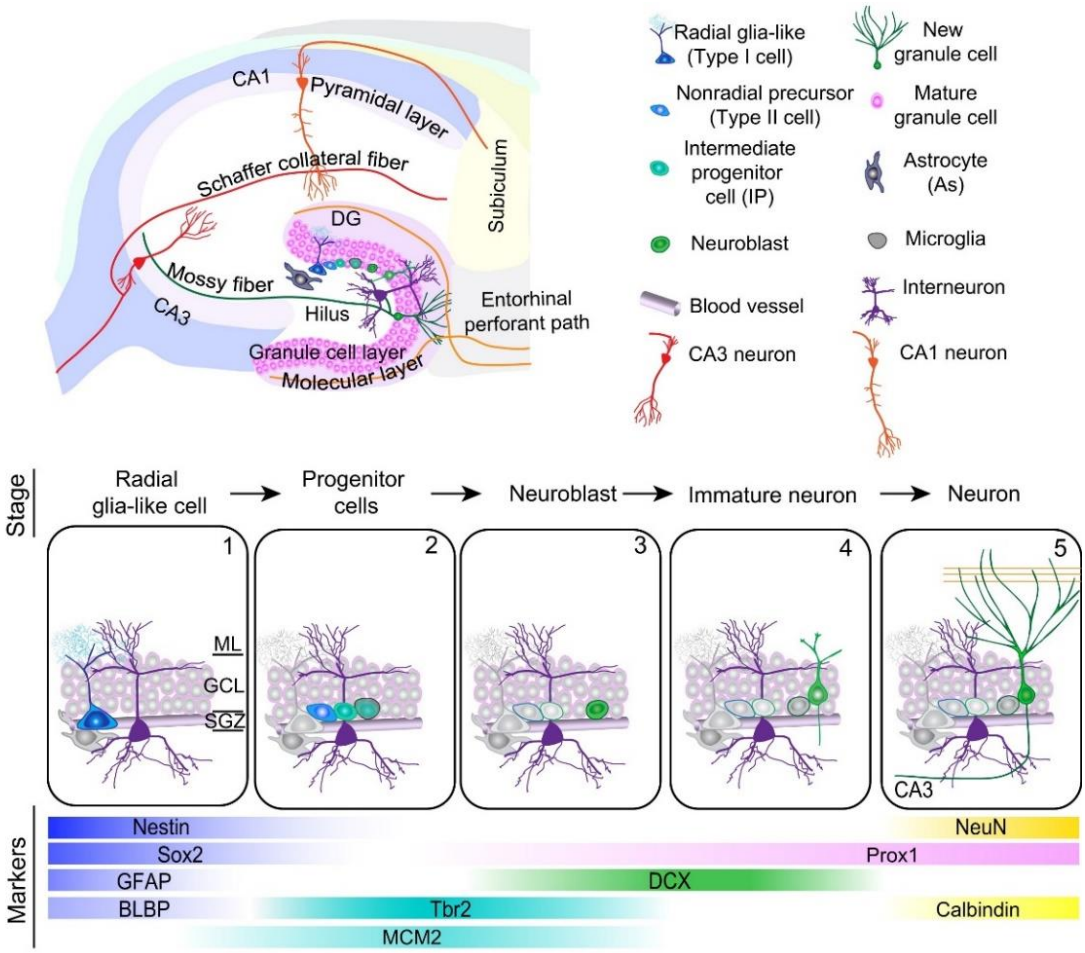

Figure 1.3: *Hippocampal trisynaptic circuit and stages of adult hippocampal neurogenesis. Image from: (Ming and Song 2011). Abbreviations: BLBP: brain lipid-binding protein; CA1,3: Cornu Ammonis 1,3; DCX: doublecortin; DG: dentate gyrus; GFAP: glial fibrillary acidic protein; MCM2: minichromosome maintenance complex component 2; Prox1: prospero homebox 1; Sox2: SRY-box2; Tbr2: T-box brain protein 2.*

Many of the newly-born neurons do not survive to become mature GCs. Indeed, the proliferation and survival of neural progenitor cells are regulated by many factors including endogenous intrinsic factors and external influences including stress,

antidepressant treatment, environmental experience, physical activity and learning and memory (Kempermann *et al.* 1997, Gould *et al.* 1999, Malberg *et al.* 2000, Dobrossy *et al.* 2003, Simon *et al.* 2005, Fabel and Kempermann 2008, Schloesser *et al.* 2010, Surget *et al.* 2011, Tanti *et al.* 2012, Hueston *et al.* 2017).

1.4.3. Current methodologies for interrogating adult hippocampal neurogenesis

1.4.3.1. Endogenous markers for immunohistochemical analysis of different stages of neurogenesis

The cells involved in the different stages of AHN express different markers and these endogenous markers can be used to identify the presence and stage of neurogenesis. Neural progenitor cells are defined by their ability to proliferate and self-renew. These proliferative cells can typically be identified by the following markers: glia fibrillary acidic protein (GFAP), Nestin, Ki67, Aldoc, Apoe, ID4, Hopx, Sox2, Slc1a3, Pcna and Mcm2. Once the cells obtain a neuronal progenitor fate, they can be identified using markers for immature neurons, such as Neurod1, DCX, Tbr1, Tbr2, Neurog2, Ccnd2 and Sox11, while mature neurons can be identified using the markers NeuN, MAP2, Synaptophysin, PSD95, Prox1 and calbidin. Oligodendrocyte progenitors express Olig1, Olig2, Sox10, Pdgfra and Cspg4. To identify astrocytes, S100b and Fzd2 markers are used.

1.4.3.2. In vitro approaches: The isolation and culture of neural progenitor cells

The neurosphere and monolayer culture of neural progenitor cells are the gold standard for the study of neurogenesis *in vitro*. Neural progenitor cells (NPCs) can be obtained from the brain of the rodent embryo or from the dentate gyrus or subventricular zone in postnatal animals. In embryonic hippocampal NPC cultures,

the protocol for the culture of NPCs does not involve the isolation of these cells, because most of the cells are undifferentiated; however, in postnatal cultures, a protocol for the isolation of the NPCs brain tissue must be used. Isolating and culturing NPCs is a tool to study intrinsic characteristics of these cells when removed from their surrounding brain environment and can be a useful tool to study their intrinsic sensitivities and intracellular signalling pathways in response to stimuli that have direct effects on NPCs. To avoid NPCs differentiating in culture, these cells should be supplemented with epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2), and a retinoic-acid (vitamin A) free medium is also recommended, because vitamin A is known to induce NPC differentiation (Janesick *et al.* 2015). NPCs have the ability to self-replicate and to differentiate into other neural cells, including neurons, astrocytes and oligodendrocytes. To promote differentiation of NPCs *in vitro*, changing the medium to a modified medium in the absence of growth factors and in the presence of vitamin A is sufficient. To selectively reduce the number of astrocytes and increase the number of neurons in culture, a serum-free medium is recommended, given that the serum is a potent astrocytic differentiation inducer (Chiang *et al.* 1996, Brunet *et al.* 2004).

Although very useful, there are many limitations for the use of cultured NPCs, particularly if they are allowed to grow as neurospheres in cell suspension. It is difficult monitor the properties of the individual cells during this culture period, and, besides that, it has been reported that as the neurospheres grow, the cells in its centre become deprived of nutrients and die; the neurogenic capacity of the NPCs has also been shown to decline after a number of passages (Fricker *et al.* 1999). Moreover, neurospheres are not homogeneous cultures as many would expect; in fact, not a large percentage of the cells have neurosphere-forming capacity

(Reynolds and Weiss 1996), and even fewer can be considered neural stem cells (Reynolds and Rietze 2005). Monolayer cultures of NPCs, as opposed to neurospheres, are adhesive cultures, grown on coated substrates and overcome many of the problems and difficulties found in the neurospheres cultures. While monolayer NPCs are more isolated and more homogeneous, they are not completely homogeneous however as asymmetric divisions and some differentiation may also occur.

1.4.3.3. In vivo approaches

In the mid-20th century, the use of tritiated ([3H]) thymidine revolutionised the study of the nervous system (Sidman *et al.* 1959, Altman 1962, Altman 1963). Some years after its discovery, the use of [3H]-thymidine autoradiographic assays lead to the discovery of AHN in the subventricular zone and in the dentate gyrus of the rat hippocampus (Altman and Das 1965, Altman 1969). More recently, bromodeoxyuridine (BrdU), a thymidine analogue, has become the most widely used technique for labelling recently generated neurons and thus studying AHN. BrdU is an exogenous marker that should be injected into the animal prior its detection. Specifically, BrdU is not a marker of cell proliferation, but rather a marker of DNA synthesis; it becomes incorporated into the DNA during cell division. The use of BrdU staining technique allowed us to find that, in an adult rodent, approximately 9000 new neurons are generated per day in the DG (Kempermann *et al.* 1997, Cameron and McKay 2001), while 30000 are generated per day in the SVZ (Alvarez-Buylla *et al.* 2001). It is also important to note that BrdU is a toxic substance and high concentrations may lead to death or may lead to some heath complications and thus currently cannot be used in humans. For the study of adult neurogenesis in rodents however, most studies use a dose of 50-150

mg/kg (intraperitoneally). The use of 50 mg/kg per day for 12 days, and the use of higher doses as 300 mg/kg have shown no apparent complications or toxicity (Cameron and McKay 2001). Depending upon the time interval between BrdU injection, the experimental intervention and time of death, BrdU labelling can be used to measure either the proliferation or survival of newly born neurons. To measure proliferation, an animal is injected with 50 mg/kg 1-3 times with an interval of 2 hours, and the animal is perfused 2 hours after the last injection; the number of BrdU-positive cells is then used as a measure of cell proliferation. On the other hand, to study the impact of experimental interventions on the maturation and survival of newly born neurons, 50 mg/kg is normally injected 1 to 3 times with 2 hours interval and the animal is perfused at least 3-4 weeks later. When coupled with neuron-specific markers, BrdU labelling permits the survival of newly born neurons to be measured. Many studies have used this technique to show that hippocampal neural progenitor cells proliferate throughout adult life in many mammalian species (Gould *et al.* 1997, Eriksson *et al.* 1998, Gould *et al.* 1999, Ming and Song 2011). Other thymidine analogues including chlorodeoxyuridine and iododeoxyuridine have also been used for to study adult neurogenesis, but BrdU is still the most commonly used one. Nevertheless, these other markers are useful when studying newly generated neurons born at different timepoints within the same animal.

Besides the use of markers to study AHN, the use of viral vectors, injected by stereotaxic surgery directly into the hippocampus has also revolutionised the study of AHN. While the adenoviruses infect the cells of interest without integrating to their genome, lentiviruses are transcribed into the genome, being permanently inserted into the host DNA (Thomas *et al.* 2003). Cells infected by these viruses

can be labelled using a reporter gene, such as EGFP; moreover, a siRNA can be used to knockdown a particular protein of interest. Using this single-cell gene knockout approach, it has been shown that the survival of new neurons is regulated by their own NMDA-type glutamate receptor during a critical period after neuronal birth. (Tashiro *et al.* 2006). Moreover, the use of promoters for genetically-engineered animals (e.g. nestin-GFP expressing) are also useful for gain- or loss-of-function.

1.4.3.4. Methods for experimental manipulation of adult hippocampal neurogenesis

One of the methods used to study AHN *in vivo* is to inhibit or ablate adult neurogenesis and determine the impact of this on brain and behaviour. Inhibition of AHN can be achieved by chemical (i.e. methylazoxymethanol - MAM) (Jayatissa *et al.* 2009, Mateus-Pinheiro *et al.* 2013), genetic (Yu *et al.* 2008, Schloesser *et al.* 2010, Snyder *et al.* 2011) and irradiation-based methods (Santarelli *et al.* 2003, Wu and Hen 2014). Nestin-TK mice are also useful in the study of the AHN (Snyder *et al.* 2016). These mice express the herpes simplex virus thymidine kinase (HSV-tk) under the control of the nestin and thus expressing HSV-tk in NPCs. The administration of ganciclovir (GCV) kills actively HSV-tk expressing cells (dividing cells expressing this transgene). This method can be used to impair neurogenesis *in vivo* at a given time (time of the injection of GCV) and then the effects of the reduction of neurogenesis on a variety of behavioural tests. Recent studies have shown that the use of this model of mice to ablate neurogenesis did not alter anxiety or depressive-like behaviour (Santarelli *et al.* 2003, Lagace *et al.* 2010, Deng and Gage 2015). Interestingly, using a method very similar to this, a Nestin-CreER^{T2} mice, in which the injection of tamoxifen reduces neurogenesis, a study recently showed that reducing neurogenesis increased anxiety in the novelty-

induced hypophagia and depressive-like behaviour in the tail suspension test in mice (Yun *et al.* 2016).

Another useful tool is the use of the transgenic mouse line iBax, in which the pro-apoptotic gene Bax can be selectively ablated in neural progenitor cells, following tamoxifen injection. Thus, this enhances the survival and the functional integration of newly born neurons at any specific timepoint (Sahay *et al.* 2011, Hill *et al.* 2015, Culig *et al.* 2017). The use of these mice showed that, increasing neurogenesis counteracts some effects of stress (Culig *et al.* 2017), reducing anxiety and depressive-like behaviour (Hill *et al.* 2015) and improving pattern separation (Sahay *et al.* 2011). Taken together, this suggests that the transgenic studies could be very useful to study the roles of AHN, however, special attention should be given to the model of animal used and timing since the inhibition of neurogenesis and the initiation of the behavioural testing.

1.4.4. Functional roles of adult hippocampal neurogenesis

1.4.4.1. Properties that distinguish immature from mature granule cells

In their first month after neuronal differentiation, immature newly-born neurons display diverse properties from those exhibited by mature, integrated granule cells (GC) (Lopez-Rojas and Kreutz 2016). Specifically, immature GC show enhanced long-term potentiation (LTP) and reduced threshold for LTP induction (Wang *et al.* 2000, Schmidt-Hieber *et al.* 2004, Ge *et al.* 2007), lower action potential amplitude (Liu *et al.* 1996, Mongiat *et al.* 2009), lower GABAergic inhibition (Esposito *et al.* 2005) and lower dendritic complexity (Schmidt-Hieber *et al.* 2004, Esposito *et al.* 2005). These distinct characteristics can be used to distinguish an immature from a

mature GC, and to assess AHN *in vivo* and suggest that immature neurons may play distinct functional roles compared to fully integrated mature GCs.

1.4.4.2. Role of adult hippocampal neurogenesis in learning and memory

As discussed before, the hippocampus plays important roles in learning and memory. However, the roles of AHN in spatial learning and memory are not yet fully understood. Interestingly, learning and memory have been shown to increase AHN in rats (Gould *et al.* 1999, Dobrossy *et al.* 2003) and in turn, AHN is required for hippocampal-dependent learning (Marin-Burgin and Schinder 2012). Indeed, spatial learning has been shown to cause LTP in rats (Whitlock *et al.* 2006), while LTP facilitates cell proliferation and survival in the hippocampus (Bruel-Jungerman *et al.* 2006). Moreover, using transgenic mice, the selective ablation of the pro-apoptotic gene Bax in NPCs increased NPC survival and also promoted an improvement in pattern separation (Sahay *et al.* 2011).

Due to their high excitability and increased plasticity, immature newly-born GC are suggested to have key roles in learning and memory (Cameron and McKay 2001, Ninkovic *et al.* 2007), while mature GC are less excitable and not as plastic. Indeed, AHN has been linked to formation of contextual fear memory (Saxe *et al.* 2006, Ko *et al.* 2009, Gao *et al.* 2018). AHN has also been shown to regulate forgetting (Akers *et al.* 2014). Interestingly, increasing AHN immediately after the training of the fear conditioning reduced conditioned freezing, indicating memory erasure (Gao *et al.* 2018). However, the role of AHN in memory erasure seems to be timing dependent, as the modulation of AHN at a later timepoint after learning (after consolidation) did not change the fear conditioning response (Gao *et al.* 2018). Taken together, the AHN seems to have an important role in cognition, from spatial

learning and memory to forgetting. In addition to playing these roles, AHN is also known to play a role in stress-related disorders.

1.4.4.3. The role of adult hippocampal neurogenesis in stress and stress-related psychiatric disorders

Stress and high levels of glucocorticoids in adulthood impact not only the hypothalamus-pituitary-adrenal (HPA) axis, but they also have been shown to decrease all stages of AHN (Simon *et al.* 2005, Dranovsky and Hen 2006, Jayatissa *et al.* 2006, Mitra *et al.* 2006, Jayatissa *et al.* 2009, Surget *et al.* 2011, Schoenfeld and Gould 2012, Lehmann *et al.* 2013). Specifically, different forms of stress reduce AHN, including predator odour (Galea *et al.* 2001), social stress (Gould *et al.* 1997, Czeh *et al.* 2001), and chronic mild stress (Alonso *et al.* 2004). Moreover, the neurogenic niche in which NPCs reside is located near blood vessels (Wurmser *et al.* 2004), which makes these cells vulnerable to blood-transported molecules and hormones, such as cytokines (Bauer 2009), growth factors (Oliveira *et al.* 2013) and the stress hormone, corticosterone. Indeed, it has been shown that chronic treatment with corticosterone reduces AHN in mice (David *et al.* 2009) and rats (Ekstrand *et al.* 2008, Brummelte and Galea 2010). Adrenalectomy, and the consequent removal of circulating plasma glucocorticoids, is also known to increase hippocampal cell proliferation (Krugers *et al.* 2007). In turn, AHN facilitates normalization of glucocorticoid levels after stress in mice (Snyder *et al.* 2011, Surget *et al.* 2011, Culig *et al.* 2017). Interestingly, it has been reported that a transgenic mouse model of neurogenesis inhibition exhibits a transient increase in the corticosterone response to stress as well as an attenuated dexamethasone-induced suppression of corticosterone release (Snyder *et al.* 2011), thus suggesting that these mice exhibit a modest overactivation of the HPA axis. On the other hand,

it has been reported that ablation of AHN by X-ray irradiation does not impair basal HPA axis activity (Surget *et al.* 2011).

Similarly, prenatal stress may influence adult phenotypes and early-life stress has been implicated in susceptibility to depression and anxiety in later life (Seckl and Holmes 2007). Accordingly, the exposure of pregnant animals to stress or glucocorticoids may affect foetal brain development of the offspring (Brummelte *et al.* 2006, Lucassen *et al.* 2009) and it may also lead to anxiety and depressive behaviour, increased HPA axis activity, memory impairment (Henry *et al.* 1994, Vallee *et al.* 1997, Fenoglio *et al.* 2006) as well as reduced hippocampal neurogenesis in both rodents (Lemaire *et al.* 2000, Mandyam *et al.* 2008, Lucassen *et al.* 2009) and non-human primates (Coe *et al.* 2003) later in adult life. Importantly, these changes induced by prenatal stress may depend upon the genetic background (Bosch *et al.* 2006, Lucassen *et al.* 2009), thus highlighting that gene-environment interactions may modulate AHN and as well as susceptibility and resilience to stress. Similarly, adverse experience in early postnatal life, such as maternal separation, can reduce AHN (Mirescu *et al.* 2004, Kikusui *et al.* 2009, Lajud *et al.* 2012), although these effects may be sex-dependent as one study reported decreases in females but increases in male rats (Oomen *et al.* 2009). Maternally separated pups can exhibit decreased hippocampal cell proliferation in adulthood (Mirescu *et al.* 2004) In addition to prenatal and early life stress protocols, exposure to stressors in adult life have also been shown to decrease AHN, including chronic restraint (Luo *et al.* 2005, Rosenbrock *et al.* 2005, Snyder *et al.* 2011), chronic unpredictable mild stress (Jayatissa *et al.* 2006, Jayatissa *et al.* 2009, Surget *et al.* 2011), social defeat stress (Simon *et al.* 2005, Schloesser *et al.* 2010), and others. Furthermore, chronic corticosterone treatment, which is used as a model

of HPA axis overactivity, has also been shown to decrease AHN (Ekstrand *et al.* 2008, Brummelte and Galea 2010). However, while many studies have investigated the effects of stress on AHN, relatively few have determined whether stress-induced changes in AHN occur specifically in animals that are more resilient or more susceptible to the behavioural and neuroendocrine effects of stress.

While there is a general agreement that chronic stress can decrease AHN (Pham *et al.* 2003, Simon *et al.* 2005, Dranovsky and Hen 2006, Jayatissa *et al.* 2006, Mitra *et al.* 2006, Jayatissa *et al.* 2009, Perera *et al.* 2011, Schoenfeld and Gould 2012, Lehmann *et al.* 2013, Fa *et al.* 2014), it is also important to note that negative findings have also been reported (Lee *et al.* 2009, Lyons *et al.* 2010, Hanson *et al.* 2011, Parihar *et al.* 2011, O'Leary *et al.* 2012). While these negative findings might be stressor, species, sex or strain-dependent (Westenbroek *et al.* 2004, Hanson *et al.* 2011, Lisowski *et al.* 2011, Schoenfeld and Gould 2012), it is also important to consider that interindividual variation in the behavioural susceptibility to stress might contribute to conflicting findings. This also raises the question as to whether changes in AHN may predict resilience or susceptibility to stress-induced changes in behaviour (Alves *et al.* 2017). Alternatively, an individual's behavioural response to stress may be independent of the effects of stress on AHN. One approach to investigating the relationship between stress resilience and AHN is to compare neurogenesis in stress-susceptible and stress-resilient animals, although to our knowledge few studies to date have approached this question in such a manner. Given the evidence that stress decreases AHN in an antidepressant-reversible manner, one might expect stress-induced decreases in neurogenesis to be correlated with increased stress susceptibility. Surprisingly, however, it has been reported that the survival of cells born 24 h after stress was increased four weeks later in mice

that were susceptible to developing social avoidance behaviour following social defeat stress, while similar effects were not observed in resilient mice (Lagace *et al.* 2010). The association of increased AHN with stress susceptibility is also supported by a study in primates that demonstrated increased neurogenesis and improvements in learning in primates housed under stressful conditions (alone or with an unknown male), versus standard conditions (with a familiar male) (Lyons *et al.* 2010). Thus, exposure to some protocols of stress can increase AHN, even in susceptible animals.

Predictability or controllability of the stressor seems to be an important determining factor of whether stress increases or decreases AHN (Van der Borght *et al.* 2005, Parihar *et al.* 2011). While unpredictable chronic stress increased depressive-like behaviour (Lucas *et al.* 2014), predictable stress, which consisted of a daily 5-min session of restraint at the same time each day, decreased anxiety and depressive behaviour and increased AHN (Parihar *et al.* 2011). Similarly, a study reported that controllable stress in the form of chronic exposure to escapable foot shocks, did not change cell proliferation in dentate gyrus of the hippocampus (Van der Borght *et al.* 2005). These data suggest that some types of stress protocols may actually increase AHN (Van der Borght *et al.* 2005, Parihar *et al.* 2011) and that increased survival of newly born cells in the hippocampus might also be associated with increased susceptibility to the negative effects of stress (Van der Borght *et al.* 2005, Lagace *et al.* 2010, Parihar *et al.* 2011).

Most studies report that ablating neurogenesis in rodents either with X-irradiation or with methylazoxymethanol (MAM) does not increase their susceptibility to stress-induced changes in depression-related behaviour when compared with

stressed neurogenesis-intact animals (Jayatissa *et al.* 2009, Schloesser *et al.* 2010, Surget *et al.* 2011, Lehmann *et al.* 2013). For example, chemical ablation of neurogenesis in rats with chronic injection of MAM did not induce anhedonia, a behaviour frequently observed following chronic stress, even though MAM reduced hippocampal cell proliferation to a similar extent as exposure to a stress protocol (Jayatissa *et al.* 2009). Moreover, ablating neurogenesis prevented the ability of social defeat stress to induce social avoidance behaviour, thus suggesting that inhibiting neurogenesis may promote resilience rather than susceptibility to behavioural changes induced by this particular stressor (Lagace *et al.* 2010). Conversely, some studies have reported that neurogenesis ablated animals show a depressive-like phenotype and increased susceptibility to stress-induced depression-like behaviour, including anhedonia and increased immobility in the forced swim test (Snyder *et al.* 2011, Mateus-Pinheiro *et al.* 2013). On the other hand, a recent study showed that increasing AHN is sufficient to prevent chronic stress-induced depressive-like behaviour (Culig *et al.* 2017) in mice. Taken together, although there are some negative reports showing that some types of stressors do not change adult neurogenesis, it seems that at least chronic unpredictable stress does decrease AHN, although more studies are needed to determine the precise role of AHN in behavioural responses to stress. The effects of stress and other factors in the AHN are represented in Fig 1.4.

1.4.4.4. Role of adult hippocampal neurogenesis in antidepressant action

In addition to the chronic stress influence in AHN, chronic treatment with different types of antidepressant treatments such as SSRIs, TCAs, MAOIs, electroconvulsive shock and vagus nerve stimulation have all been shown to increase AHN in rodents (Madsen *et al.* 2000, Malberg *et al.* 2000, Dranovsky and Hen 2006, Biggio *et al.*

2009, Zhou *et al.* 2016). In addition, human post-mortem studies have reported that antidepressant treatment increases hippocampal neural progenitor cell proliferation (Boldrini *et al.* 2009, Boldrini *et al.* 2012). Interestingly, the neurogenic effects of sertraline, an SSRI drug, on human neural progenitor cells occur through activation of the glucocorticoid receptor (Anacker *et al.* 2011). In addition, *in vivo* studies in C57BL/6 mice have shown that fluoxetine increased the proliferation of NPCs, preferentially in the transition between the intermediate and ventral hippocampus (Zhou *et al.* 2016).

There are several studies which show that the behavioural effects of the SSRI antidepressant fluoxetine (Santarelli *et al.* 2003, David *et al.* 2009) and the TCA imipramine (David *et al.* 2009), seem to be, at least in part, neurogenesis-dependent. Indeed, a study showed that X-ray irradiated (i.e. neurogenesis-ablated) animals did not show behavioural recovery from stress after fluoxetine administration, which was observed in neurogenesis-present animals (David *et al.* 2009). Although this result was observed on the novelty-suppressed feeding, a test for anxiety, fluoxetine-induced recovery from stress in the open field and forced swim tests occurred in a neurogenesis-independent manner (David *et al.* 2009). Moreover, neurogenesis-dependent behavioural effects of fluoxetine have also been reported in adult non-human primates under chronic stress conditions (Perera *et al.* 2011). Interestingly, it has also been proposed that normalisation of HPA-axis overactivity by the antidepressant fluoxetine is dependent on intact AHN (Surget *et al.* 2011).

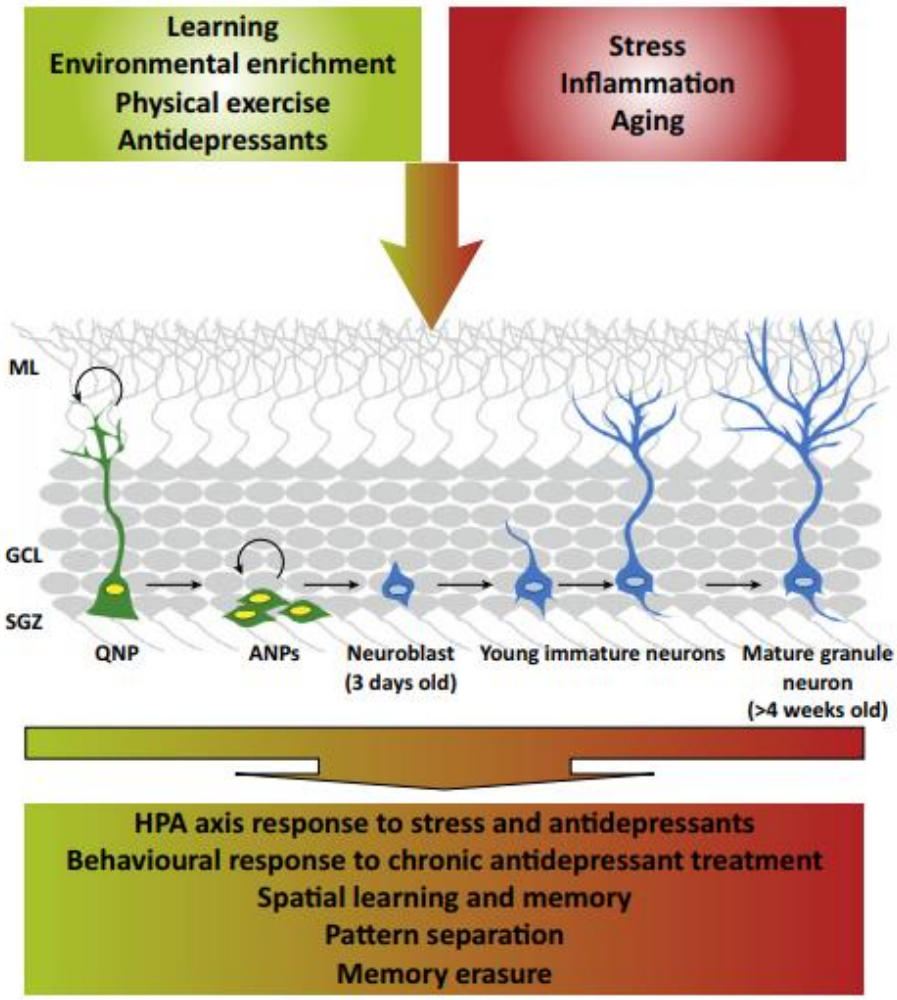

***Figure 1.4**: Learning, environmental enrichment, physical exercise and antidepressant are known to increase adult hippocampal neurogenesis, while stress, inflammation and aging reduce it. Neurogenesis, in turn, has been shown to play a role in the HPA axis response to stress and antidepressants, in the behavioural response to chronic antidepressant, in spatial learning and memory, in pattern separation and in memory erasure. Image from: (O'Leary and Cryan 2014).*

On the other hand the antidepressant-like effects of non-monoaminergic based antidepressant-like drugs, such as CRH1 or V1b antagonists, are not affected by inhibition of AHN (Bessa *et al.* 2009, Surget *et al.* 2011) which is in contrast to many findings with antidepressants that target the monoaminergic system such as fluoxetine and imipramine (Santarelli *et al.* 2003, Perera *et al.* 2011, Surget *et al.* 2011). Taken together, it has been suggested that antidepressant drugs increase AHN, independently of their behavioural effects but that antidepressant-induced

increases in AHN might not be the final process in the recovery from stress-induced depressive-like behaviour (Bessa *et al.* 2009).

1.5. The Hippocampus is Functionally Segregated Along its Longitudinal Axis

Precisely how the hippocampus and AHN play a role in functions as seemingly diverse as spatial learning and memory and the response to stress and antidepressants is currently unclear, but accumulating anatomical, electrophysiological, molecular and lesion studies suggest that the hippocampus is functionally segregated (see Fig 1.5) along its longitudinal axis into dorsal and ventral regions in rodents and anterior and posterior regions in humans.

1.5.1. Anatomical evidence of segregation along the hippocampal longitudinal axis

Previous anatomical studies have shown in rodents that the hippocampus is anatomically segregated along its longitudinal axis (Fanselow and Dong 2010, Strange *et al.* 2014). Indeed, the dHi and vHi in rodents exhibit distinct inputs and outputs to and from other brain regions. While the dHi receives afferents from the retrosplenial cortex and the anterior cingulate cortex (Cenquizca and Swanson 2007), sending projections to structures involved in the processing of visuospatial information, the vHi, connects with the bed nucleus of the stria terminalis, nucleus accumbens, and hypothalamus, structures that regulate neuroendocrine and behavioural responses to stress, anxiety, motivation, (Fanselow and Dong 2010, Poppenk *et al.* 2013). Neuroimaging studies have also shown differences in the anatomical connectivity of the anterior and posterior human hippocampus (ventral and dorsal in rodents, respectively) (Poppenk *et al.* 2013). Taken together, there are

anatomical evidences supporting that the hippocampus might be functionally segregated along its longitudinal axis.

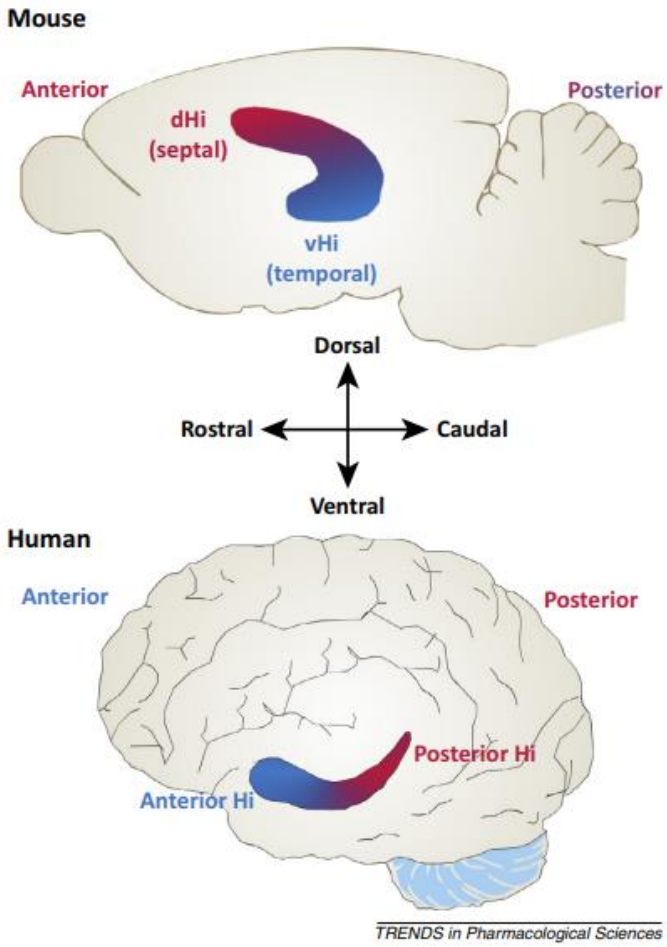

Figure 1.5: *The mouse hippocampus is segregated into dorsal (dHi) and ventral (vHi) sub-regions, while the human hippocampus is segregated into anterior (equivalent to the vHi in rodents) and posterior hippocampus (equivalent to the dHi in rodents). Image from: (O'Leary and Cryan 2014).*

1.5.2. Electrophysiological evidence of segregation along the hippocampal longitudinal axis

Preliminary electrophysiological studies indicated identified dissociable properties in the dorsal versus ventral hippocampus in mammals (Elul 1964, Racine *et al.* 1977). More recent studies have shown that some electrophysiological properties are indeed diverse along the longitudinal axis of the hippocampus, and these sub-regions show diverse neuronal excitability (Dougherty *et al.* 2012), and synaptic

plasticity (Papatheodoropoulos and Kostopoulos 2000, Maggio and Segal 2007) in rodents. Electrophysiological diversity was also observed along the hippocampal axis in non-human primates (Colombo *et al.* 1998) and in humans (Staresina *et al.* 2012). Place cells are cells within the hippocampus, normally in the CA1 or CA3, which fire when an animal walks in a given location and thus they mediate spatial discrimination. These place cells exist in a greater proportion in the dHi versus the vHi (Jung *et al.* 1994). In agreement, the dHi is preferentially involved in spatial navigation. In non-human primates, the preferential role of the posterior hippocampus (dorsal in rodents) in spatial learning and memory is in accordance with an electrophysiological study which demonstrates that a spatial learning task activates a higher proportion of neurons in the posterior hippocampus as opposed to the anterior hippocampus (Colombo *et al.* 1998). A study also showed that corticosterone reduced the frequency of spontaneous inhibitory postsynaptic currents preferentially in the vHi, also suppressing paired-pulse facilitation of evoked inhibitory synaptic currents, in a MR-dependent mechanism, in rat's brain slices (Maggio and Segal 2009). Moreover, a similar magnitude of long-term depression (LTD) was found between dHi and vHi, but stress enhanced LTD only in the dHi, while converted LTD to long-term potentiation (LTP) in the vHi (Maggio and Segal 2009).

1.5.3. Molecular evidence of segregation along the hippocampal longitudinal axis

A growing body of evidence has been supporting the idea that the hippocampus is a gradient and differential gene expression is found along its longitudinal axis (Leonardo *et al.* 2006, Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016, Cembrowski *et al.* 2016). Specifically, several studies have reported

differential gene expression in the dorsal DG versus the ventral DG in rats (Christensen *et al.* 2010) and in mice (Cembrowski *et al.* 2016). Some studies have shown that the vHi has a higher expression of genes involved serotonergic (Tanaka *et al.* 2012), cholinergic and GABAergic neurotransmission (Lee *et al.* 2017). In addition, a recent study also supports the idea that the dHi and vHi have distinct epigenetic responses to acute stressors (Floriou-Servou *et al.* 2018), with the vHi being particularly more sensitive to the stressor. Taken together, these data support that the diverse hippocampal sub-regions display diverse gene expression, which could be one factor for the functional dissociation between dHi, and vHi.

1.5.4. Functional evidence of segregation along the hippocampal longitudinal axis

The hippocampus can be divided along its septotemporal axis into dorsal and ventral regions in rodents and into anterior and posterior regions in primates, based on their distinct afferent and efferent connections (Fanselow and Dong 2010). Lesion, optogenetic and electrophysiological studies in rodents suggest that this anatomical segregation results in a dichotomy in the function of the dorsal hippocampus (dHi) and the ventral hippocampus (vHi) (Bannerman *et al.* 2004, Fanselow and Dong 2010). While the dHi (analogous to the posterior hippocampus in primates) seems to play a preferential role in spatial learning and memory processes, the vHi (analogous to the anterior hippocampus in primates) preferentially regulates anxiety and the response to stress (Moser and Moser 1998, Bannerman *et al.* 2004, Fanselow and Dong 2010).

Specifically, lesion studies have reported a functional dissociation of the hippocampus, whereby lesions of the vHi, but not dHi attenuated anxiety behaviour

in rats, tested in the light-dark box (Bannerman *et al.* 2003), elevated plus maze (Kjelstrup *et al.* 2002), hyponeophagia (Bannerman *et al.* 2002, Bannerman *et al.* 2003) and open field tests (Bannerman *et al.* 2003). On the other hand, dHi lesions but not vHi lesions impaired spatial learning and memory in rats (Moser *et al.* 1995, Bannerman *et al.* 2002, Pothuizen *et al.* 2004).

The precise vHi neural pathway underlying anxiety have yet to elucidated but several optogenetic and pharmacogenetic studies have reported that vHi-mediated pathways have been reported to regulate anxiety behaviour in the open field and elevated plus maze, including projections from the amygdala (Felix-Ortiz *et al.* 2013) or to the prefrontal cortex (Adhikari *et al.* 2011, Padilla-Coreano *et al.* 2016). In addition, a recent paper describes the presence of "anxiety cells" in the ventral hippocampus which project to the lateral hypothalamic area (Jimenez *et al.* 2018), and play a role on the modulation of anxiety. This is in accordance with a previous study that show that the stimulation of the ventral hippocampus causes inhibition of the HPA axis (Casady and Taylor 1976). Moreover, glutamatergic projections from the vHi to the nucleus accumbens have been shown to control stress resilience to chronic social defeat stress, and optogenetic attenuation of the neurotransmission from vHi to accumbens is suggested to induce stress resilience, shown by an increase in the time spent in the interaction zone in the social interaction test (Bagot *et al.* 2015).

1.5.5. Is adult neurogenesis segregated along the longitudinal axis of the hippocampus?

Since AHN has been implicated in processes preferentially regulated by the dHi (spatial learning and memory) and the vHi (stress response), it is possible that adult

neurogenesis might be regulated preferentially in the dHi or the vHi, depending upon the stimulus (see Fig 1.6) (Winocur *et al.* 2006, Tanti and Belzung 2013, O'Leary and Cryan 2014, Wu and Hen 2014, Levone *et al.* 2015, O'Leary *et al.* 2015). Indeed, new neurons in the dHi are preferentially required for contextual discrimination and memory tasks in rats (Winocur *et al.* 2006), while new neurons in the vHi are necessary for anxiolytic effects of the antidepressant fluoxetine in mice (Wu and Hen 2014). A recent study suggests that the mouse dHi has more active neurogenesis than vHi and is enriched with maturing neurons markers, such as NeuroD1 and DCX, while the vHi is enriched of radial glia markers, such as Sox2 and Hes5 (Zhang *et al.* 2018). Accordingly, dHi neural progenitor cells have been shown to mature faster, associated with higher basal network activity (Piatti *et al.* 2011).

Moreover, several studies have reported that stress affects several stages of adult neurogenesis, preferentially in the vHi rather than the dHi (Tanti and Belzung 2013, O'Leary and Cryan 2014). Some (but not all) studies also report that antidepressant-induced increases in cytogenesis and neurogenesis occur preferentially in the vHi but not dHi (Banasr *et al.* 2006, Jayatissa *et al.* 2006, O'Leary *et al.* 2012, Tanti *et al.* 2012, O'Leary and Cryan 2014). Specifically, it has been reported that chronic stress decreases cell proliferation (Jayatissa *et al.* 2006, Tanti *et al.* 2012), neuronal differentiation, maturation and survival (Hawley and Leasure 2012, Lehmann *et al.* 2013), preferentially in the vHi of rodents. Indeed, our laboratory and others have reported that AHN is differentially regulated by stress and antidepressant-like treatments within the sub-regions of the hippocampus in rats and mice (Jayatissa *et al.* 2006, O'Leary *et al.* 2012, Tanti and Belzung 2013, O'Leary and Cryan 2014, Levone *et al.* 2015). Similarly, it has been shown that chronic stress reduced the

number of immature neurons in the anterior hippocampus, but not in the posterior hippocampus of non-human primates (Perera *et al.* 2011). Moreover, some studies have reported that antidepressant-induced increases in cytogenesis and neurogenesis occur preferentially in the vHi under basal conditions (Banasr *et al.* 2006, Soumier *et al.* 2009, Paizanis *et al.* 2010) and under stress conditions in mice (Banasr *et al.* 2006, Jayatissa *et al.* 2006, O'Leary *et al.* 2012, Tanti *et al.* 2012, O'Leary and Cryan 2013). Antidepressant effects on neurogenesis also seem to occur preferentially in the anterior hippocampus (equivalent to vHi in rodents) in humans (Boldrini *et al.* 2009, Boldrini *et al.* 2012).

To date, few studies have investigated whether adult neurogenesis specifically in the vHi correlates with stress resilience or the antidepressant response. Nevertheless, in non-human primates, the number of immature neurons that were at the threshold of complete maturation was reduced by chronic stress in the anterior but not posterior hippocampus, and this effect was correlated with stress-induced anhedonia (Perera *et al.* 2011). Our laboratory recently reported that $GABA_{B(1b)}^{-/-}$ mice, which are resilient to stress-induced anhedonia, exhibit increased proliferation and survival of newly-born cells predominantly in the vHi, and are also resilient to stress-induced decrease in the survival of newly-born cells in the vHi (O'Leary *et al.* 2014). Furthermore, Jayatissa and colleagues reported that rats that exhibit escitalopram-induced behavioural recovery from stress also exhibit increased hippocampal cell proliferation in the vHi, while this selective effect in the vHi was not observed in rats that failed to respond to escitalopram treatment (Jayatissa *et al.* 2006). Moreover, it was recently demonstrated that ablation of neurogenesis in the vHi but not dHi prevents the anxiolytic effects of fluoxetine in animals that had received daily foot shocks for three weeks (Wu and Hen 2014).

Future studies investigating whether the effects of fluoxetine and other antidepressants on recovery from stress-induced changes in behaviour, such as anhedonia, are dependent on neurogenesis in specifically the vHi will be of interest. Ultimately, AHN may be a key factor linking stress to anxiety- and depression-like behaviours (Snyder *et al.* 2011). However, as discussed earlier, studies have shown contradictory results linking stress susceptibility and AHN. In addition to methodological differences, we suggest that such incongruences might also be due to the absence of segregation of the hippocampus into dorsal and ventral regions (O'Leary and Cryan 2014). Therefore, future studies investigating the relationships between AHN and stress-related factors such as stress susceptibility/resilience and the antidepressant response should specify whether changes in AHN occur in the dHi or vHi.

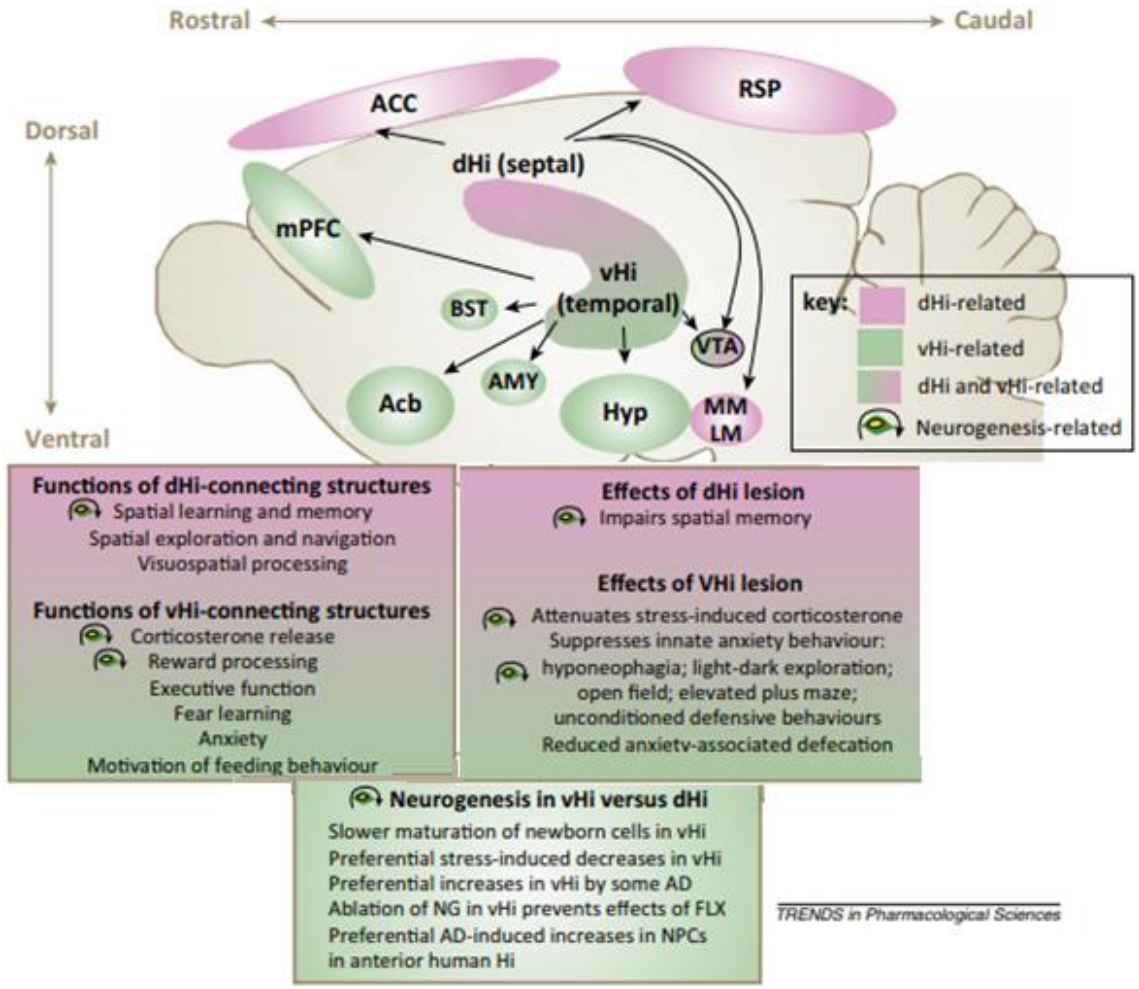

Figure 1.6*: Segregation of the mouse hippocampus along its longitudinal axis and the adult neurogenesis in the dHi versus vHi. The dorsal hippocampus is represented in pink, while the ventral hippocampus is represented in green. The hippocampus has different brain connections along its axis. While the dHi is connected to structures that play a role in spatial learning and memory, the vHi is connected to structures which regulate the stress hormone (corticosterone) release, anxiety, and motivation. While the lesion of dHi impairs spatial learning, the lesion of vHi reduces anxiety and the stress-induced corticosterone release. Adult hippocampal neurogenesis also seems to be differentially regulated along the hippocampal axis. vHi neural progenitor cells mature slower, are more sensitive to the chronic stress and to the antidepressants effects. Abbreviations: ACC, anterior cingulate cortex; Acb, nucleus accumbens; AD, antidepressant; AMY, amygdala; BST, bed nucleus of the stria terminalis; FLX, fluoxetine; Hi, hippocampus; Hyp, hypothalamus; mPFC, medial prefrontal cortex (infralimbic and prelimbic cortex); LM, lateral mammillary nucleus; MM, medial mammillary nucleus; NG, neurogenesis; NPCs, neural progenitor cells; RSP, retrosplenial cortex; VTA, ventral tegmental area. Figure from: (O'Leary and Cryan 2014), adapted.*

Although it is clear that AHN is important for some of the behavioural effects of at least some antidepressants, few studies have investigated whether the rate of neurogenesis in an individual animal directly correlates with its antidepressant-

induced behavioural recovery from stress. Nevertheless, it has been reported that the number of newly-born cells in the hippocampus of stressed rats was restored only in the group of rats that showed a behavioural response to the antidepressant escitalopram, thus suggesting that the restoration of cell proliferation rates at least within a certain area of the dentate gyrus (ventral region) may be important for escitalopram-induced recovery from stress-related behaviours (Jayatissa *et al.* 2006). Similarly, a primate study showed that fluoxetine treatment prevented the onset of depression-like behaviours and increased the number of newly-born neurons that were at the threshold of maturation within a specific region of the dentate gyrus (anterior region), thus leading to the suggestion that AHN may contribute to the recovery promoted by fluoxetine (Perera *et al.* 2011). Together, these data suggest that AHN specifically in the vHi may be an important determinant of the behavioural responses to stress and antidepressant drugs.

1.5.6. The intermediate hippocampus - a functionally distinct hippocampal sub-region?

A growing body of evidence has been supporting the idea that the hippocampus is actually a gradient and differential gene expression is found along its longitudinal axis (Leonardo *et al.* 2006, Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016, Cembrowski *et al.* 2016). Besides demonstrating a clear differential expression of genes between dHi and vHi, studies also support the presence of an intermediate hippocampus (see Fig 1.7) (Leonardo *et al.* 2006, Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016, Cembrowski *et al.* 2016). The intermediate hippocampus does not have clearly defined projections, but it is suggested that it projects to anterior and ventromedial hypothalamic nucleus, which are associated with social behaviour (Strange *et al.* 2014).

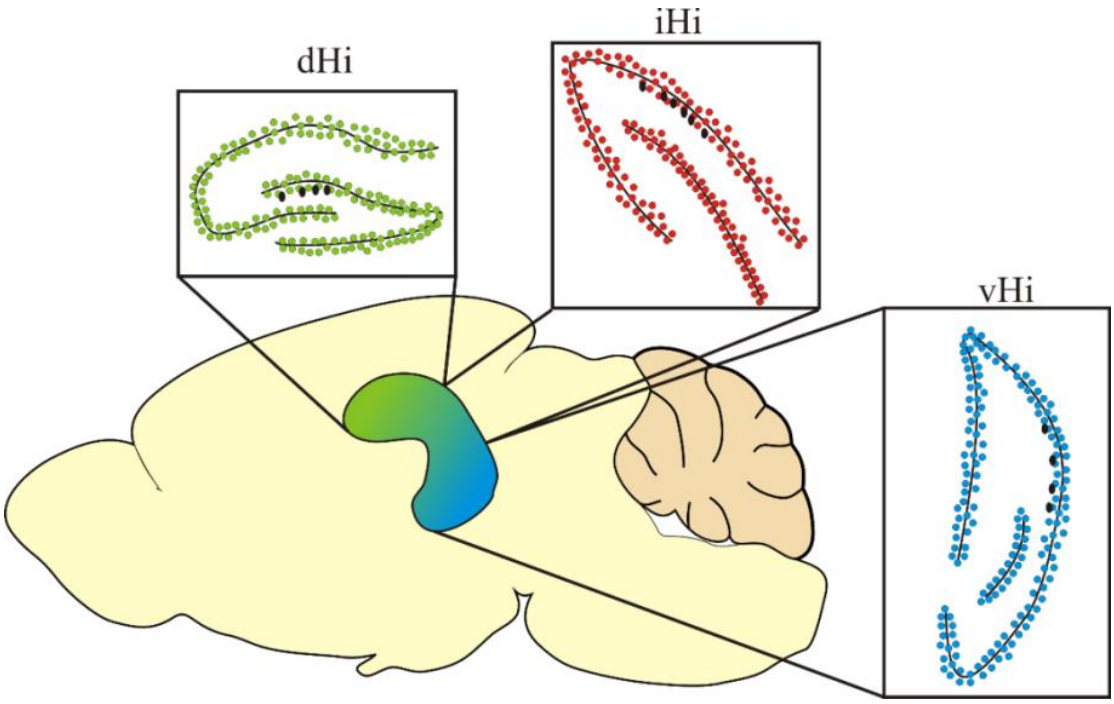

Figure 1.7: *The rodent hippocampus is segregated into dorsal (dHi), intermediate (iHi) and ventral (vHi) sub-regions.*

Most studies that describe the functional roles of the dHi and vHi use coordinates of coronal slices of the hippocampus for the segregation into its sub-regions and many of them disagree on the definition of the boundaries of the dorsal versus the ventral sub-regions of the hippocampus (see Fig 1.8) (Tanti and Belzung 2013). Indeed, some of these definitions of the dHi or vHi sometimes include the intermediate hippocampus (iHi), a sub-region which may in its own right be functionally independent but has been understudied (Bast 2007, Thompson *et al.* 2008, Bast *et al.* 2009, Strange *et al.* 2014). Although the roles of the iHi have yet to be determined, the introduction of this third hippocampal sub-region might somewhat explain some of the conflicting reports of whether AHN is preferentially regulated in the dHi or vHi by antidepressant treatments.

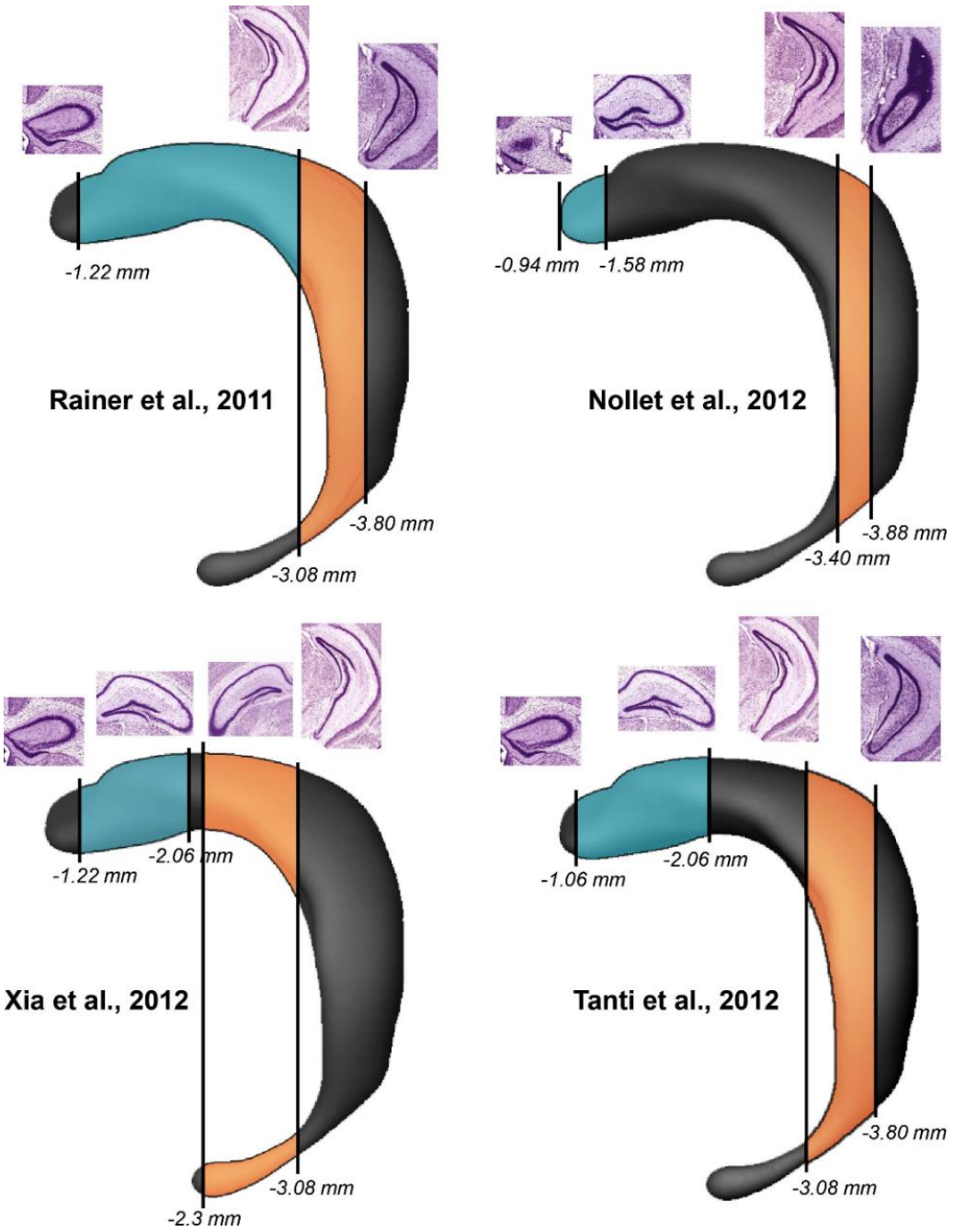

***Figure 1.8**: Different coordinates were used to define the boundaries of dorsal and ventral hippocampal sub-regions. Example of four studies and the coordinates they used in coronal sections in mice. Coordinates are shown in mm from bregma, defining the dHi (blue) and vHi (orange). Figure from: (Tanti and Belzung 2013).*

Very few functional studies have considered this third hippocampal sub-region isolation, but there are reports that it is not only a transitional area between dorsal and ventral hippocampus, but also an integrative region (Bast 2007, Bast *et al.* 2009), which mediates the integration of diverse sensory modalities (Bast *et al.* 2009). The intermediate hippocampus is also involved in cognitive flexibility,

engaging in rapid place encoding (Bast *et al.* 2009). In addition, a study showed that intermediate hippocampus engages in the processing of spatial information differently than the dorsal hippocampus in rats; specifically, the exposure to novel spatial cues facilitate long-term depression in the dorsal hippocampus, while this phenomenon is not observed in the intermediate hippocampus under the same conditions (Kenney and Manahan-Vaughan 2013). Besides playing a role in cognition and place encoding, the intermediate hippocampus has also been shown to play a functional role in anticipatory neuronal firing prior to the release of a reward in trained rats (Burton *et al.* 2009). Finally, a study has also shown that fluoxetine-induced increases on proliferation of NPCs *in vivo* occur preferentially in the area that transitions from the intermediate to the ventral hippocampus (Zhou *et al.* 2016). Taken together, there is enough evidence to suggest that the intermediate hippocampus may indeed be an independent hippocampal sub-region, but precisely which functional roles it plays are yet to be fully understood. Indeed, the specific roles of the dorsal, intermediate and ventral hippocampus in the behavioural and neurogenic responses to chronic stress or antidepressant treatment have yet to be interrogated.

1.6. Methodological Approaches to Interrogate the Role of Hippocampal Sub-Regions in Behaviours Related to Stress, Anxiety, Depression and Antidepressant Action

1.6.1. Methodological approaches to interfering with hippocampal function

In the present days, many techniques can be used to interfere with hippocampal function and thus assess its functional role. The most widely used technique is the

lesion approach. In this approach, the hippocampus or its sub-regions are stereotaxically injected with a chemical - usually ibotenic acid - which is a potent glutamatergic agonist and causes excitotoxicity, followed by cell death in the region in contact. On the other hand, the injection of muscimol, a GABAergic agonist, allows the transient inactivation of specific areas of the hippocampus, without causing death (Zhang *et al.* 2014). Lidocaine, a fast voltage-gated Na^+ channel blocker, has also been injected into the hippocampus for its short, transient inactivation (Pierard *et al.* 2017).

More recently, the use of optogenetics and chemogenetics have opened new possibilities for the study of the hippocampal function. Optogenetics consists in the injection of proteins that modify the activity of the cell in which they are expressed when that cell is exposed to light. One example of these proteins is the Channelrhodopsins (ChRs), which are ion channels discovered in a unicellular green alga. Interestingly, when illuminated with blue light ChR2, the light-gated nonspecific cation channel opens and allows the passage of cations, depolarising the cell. Moreover, halorhodopsin (NpHR) was identified in an archaeon, and pumps chloride ions into the cell upon light activation, resulting in hyperpolarization, being an interesting technique to study the inhibition of specifically tagged cells. Chemogenetics consists of engineered G-protein coupled receptors, specifically activated by small molecules that act like drugs, but would otherwise be inert. Specifically, the use of designer receptors exclusively activated by designer drugs (DREADDs), initially developed by Bryan Roth, has been the most used chemogenetics technique (Armbruster *et al.* 2007). Viral constructs encoding DREADDs, most notably Cre-dependent adeno-associated viruses (AAVs), can restrict DREADD expression to cells that selectively express Cre, and

thus, the activation of the DREADDs receptors by the specific drug would change the function only on those specific cells.

1.6.2. Animal models and tests of stress, anxiety, depression and antidepressant-like behaviour

Animal models are powerful tools to determine the impact of stress and antidepressant treatment on brain and behaviour can be used to determine the neurobiology underlying the negative consequences of chronic stress and the mechanisms of antidepressant action. Several animal models of stress have been used to induce depression and anxiety-like behaviour, for example chronic social defeat stress, maternal separation, chronic unpredictable mild stress, and these are summarised in Table 1.1.

***Table 1.1**: Animal models of stress*

Animal models of stress	Description	Reference
Chronic psychosocial defeat	Animals are exposed to an aggressive unfamiliar animal and physical contact is allowed, followed by sensory contact through a perforated plexiglass. Protocol can also include overcrowding, which consists in placing 10 animals that are usually singly-housed into the same cage. This stress protocol is known to induce anxiety and some studies also report depressive-like behaviour, which are reversed by antidepressants	(Berton *et al.* 2006, Kinsey *et al.* 2007, Savignac *et al.* 2011)
Chronic unpredictable mild stress	Animals are submitted to diverse unpredictable stressors, which alternate each day. This stress protocol induces anxiety and depressive-like behaviour which are reversed by antidepressants	(Willner 1997, Mineur *et al.* 2006, Bergstrom *et al.* 2007)
Maternal separation	On the first postnatal days, animals are daily separated from the mother for some time and this can cause depressive-like behaviour later in life	(Pryce *et al.* 2012, O'Leary and Cryan 2013)

In addition, as summarised in Table 1.2, several tests can be used for assessment of depression-like and antidepressant-like behaviour, including the novelty-induced hypophagia, elevated plus maze, marble burying, light-dark box, open field, forced swim test, tail suspension test, learned helplessness, saccharin preference test and female urine sniffing test.

***Table 1.2**: Animal models of anxiety*

Animal models and anxiety behavioural tests	**Description**	**Reference**
Novelty-induced hypophagia (NIH)	Animals are trained for 3 days (30 min/day) to drink a palatable sweet milk solution. The latency to drink is measured on its own homecage and then measured in a novel highly anxiogenic environment. The latency difference between homecage and novelty is a measure of anxiety.	(Santarelli *et al.* 2003, Dulawa and Hen 2005, Cryan and Sweeney 2011)
Elevated plus maze	Animal are tested in a plus maze, elevated from the ground, which consists of two open and two closed arms. Normally the animals avoid the open arms and an increase of the exploration of the open arms is an anxiolytic effect.	(File *et al.* 2004, Braun *et al.* 2011, Cryan and Sweeney 2011)
Marble burying	Animals are placed in a cage containing 20 marbles, which tends to be aversive to them. The number of marbles buried (2/3 buried) in a period of 30 min is a measure of anxiety.	(Deacon 2006, Nicolas *et al.* 2006, Thomas *et al.* 2009)
Light/dark box	It consists of an apparatus with a bright and a dark box. Normally, animals tend to avoid the bright box, and an increase in the exploration of the light box is an anxiolytic effect.	(Bourin *et al.* 1996, Bourin and Hascoet 2003)
Open field	Animals are placed in an open, bright arena. The animals tend to spend more time near the walls, avoiding the centre of the open field. An increase on the exploration of the central quarter of the arena is an anxiolytic effect	(Lucki *et al.* 1989, Cryan and Sweeney 2011)
Forced swim test	Animals are place in a glass cylinder with water, and display escape-oriented swimming. An increase in the immobility is a measure of depressive-like behaviour and antidepressants are known to reduce this immobility time	(Porsolt *et al.* 1977, O'Leary and Cryan 2013)
Tail suspension test	Mice are hung by the tail and display escape-oriented movements. An increase in the immobility is a measure of depressive-like behaviour and antidepressants are known to reduce this immobility time	(Steru *et al.* 1985, O'Leary and Cryan 2013)

Learned helplessness	Animals are submitted to inescapable footshocks and fail to escape when are allowed to. Antidepressants are thought to increase the number of escapes	(Seligman *et al.* 1975, O'Leary and Cryan 2013)
Saccharin preference test	In this anhedonia test, animals are allowed to drink either water or a saccharin solution. Animals tend to drink the palatable solution, but chronic stress has been shown to decrease it, which is reversed by antidepressants	(Harkin *et al.* 2002)
Female urine sniffing test	In this anhedonia test, animals are allowed to sniff a cotton bud with urine from females in oestrus cycle. A reduction on the time spent sniffing the urine is caused by chronic stress.	(Malkesman *et al.* 2010, Finger *et al.* 2011)

Such animal models and behavioural tests could be used to determine the contribution of the dHi, iHi and vHi to the behavioural responses to chronic stress and antidepressant treatment.

1.7. Aims of This Thesis

The overall goal of this thesis is to test the hypothesis that the dorsal, intermediate and ventral hippocampus have differential roles in modulation of anxiety and depression-like behaviour as well as differential roles in the neurogenic and behavioural responses to chronic stress and antidepressant treatment. This hypothesis was tested via the following experimental aims:

Aim 1: To determine the roles of each hippocampal sub-region, dHi, iHi and vHi, *in vivo* in sociability, anxiety, anhedonia and antidepressant-like behaviours (Chapter 2).

Aim 2: To determine the roles of each hippocampal sub-region, dHi, iHi and vHi, *in vivo* in the anxiolytic, antidepressant and neuroendocrine effects of acute and chronic treatment with the antidepressant fluoxetine (Chapter 2).

Aim 2: To determine the roles of each hippocampal sub-region, dHi, iHi and vHi, in the *in vivo* behavioural and neuroendocrine responses to chronic psychosocial stress (Chapter 3).

Aim 1: To determine *in vitro* whether NPCs isolated from the dHi, iHi and vHi exhibit differential intrinsic sensitivity to the effects of the stress hormone, corticosterone, on cell proliferation, neuronal differentiation and maturation, cell viability and glucocorticoid nuclear translocation (Chapter 4).

Aim 5: To determine *in vitro* whether NPCs isolated from the dHi, iHi and vHi exhibit differential intrinsic sensitivity to the effects of fluoxetine on cell proliferation and neuronal differentiation (Chapter 5).

Aim 6: To determine *in vitro* whether NPCs isolated from the dHi, iHi and vHi exhibit differential intrinsic sensitivity to the effects of fluoxetine on corticosterone-induced reductions in neuronal differentiation and maturation (Chapter 5).

CHAPTER 2

Intermediate and Ventral but not Dorsal Hippocampus are Essential for the Behavioural Effects of Fluoxetine

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Abstract

Depression is one of the leading causes of disability worldwide and yet, antidepressant treatments remain suboptimal. This is due in part to our incomplete understanding of the neurobiology underlying the pathophysiology and successful treatment of depression. However, clinical neuroimaging and preclinical studies suggest that the hippocampus is a key area of the brain in depression. Accumulating studies suggest that in rodents the hippocampus may be functionally segregated along its longitudinal axis into a dorsal region (dHi) which is predominantly involved in spatial learning and memory, and a ventral region (vHi) which regulates anxiety (a behaviour often co-morbid with depression) and the response to stress (a risk factor for depression). However, gene expression studies suggest that the area between them, the intermediate hippocampus (iHi), might also be functionally independent but few studies have interrogated its function. Similarly, little is known about the roles of these specific hippocampal sub-regions in the antidepressant response. Thus, the present study investigated the impact of ibotenic acid-induced lesions of the dHi, iHi or vHi on the regulation of social, anxiety and depressive-like behaviour in the absence or presence of the antidepressant fluoxetine. In the absence of fluoxetine, vHi lesions reduced anxiety, while none of the lesions affected sociability or depressive-like behaviour under these conditions. On the other hand, only vHi lesions prevented the acute antidepressant effects of fluoxetine in the tail suspension test and the anxiolytic effects of chronic fluoxetine treatment in the novelty-induced hypophagia test. Interestingly, iHi lesions prevented the antidepressant effect of chronic fluoxetine treatment in the forced swim test. dHi lesions did not impact behaviour either in the absence or presence of fluoxetine. Taken together, the present data demonstrate that the vHi plays a key role in anxiety

and its modulation by chronic fluoxetine treatment, and that both the iHi and vHi play distinct roles in fluoxetine-induced antidepressant-like behaviour.

2.1. Introduction

Depression is currently one of the leading causes of disability worldwide (WHO 2017). Up to half of depressed patients treated with first-line antidepressant treatment fail to achieve remission and a third of these patients remain treatment-resistant (Fava 2003, Trivedi *et al.* 2006). Antidepressant drug development has been hampered by the fact that we do not yet have a complete understanding of the neurobiology underlying the pathophysiology of depression and its successful treatment by current antidepressants. However, clinical neuroimaging studies report volumetric reductions in the hippocampus in depression (Sheline *et al.* 1996, Bremner *et al.* 2000, Frodl *et al.* 2002, Videbech and Ravnkilde 2004). In parallel, studies in animals report that chronic stress, which is a risk factor for depression, reduces hippocampal volume (Lee *et al.* 2009, Tse *et al.* 2014), causing dendritic atrophy (Lee *et al.* 2009, McEwen 2016) and reducing adult hippocampal neurogenesis (Gould *et al.* 1997, Simon *et al.* 2005, Dranovsky and Hen 2006, Schoenfeld and Gould 2012, Levone *et al.* 2015). These depression-related changes are reversed by chronic treatment with antidepressants including fluoxetine in animals (Malberg *et al.* 2000, Malberg and Duman 2003, Nollet *et al.* 2012, Rainer *et al.* 2012, Tanti *et al.* 2012) and humans (Sheline *et al.* 2003).

It is now well established that the hippocampus plays a critical role in spatial learning and memory in rodents (Morris *et al.* 1982, Moser *et al.* 1995, Moser and Moser 1998) and in humans (Maguire *et al.* 2000, Maguire *et al.* 2006). In addition, it also plays a role in the modulation of anxiety and the stress response (Bannerman *et al.* 2002, Bannerman *et al.* 2003, Bannerman *et al.* 2004, Fanselow and Dong 2010, Levone *et al.* 2015). Precisely how this brain region can modulate such diverse functions is unknown, however accumulating evidence suggests that the

hippocampus is functionally segregated into dorsal and ventral regions in rodents whereby the dorsal hippocampus plays a predominant role in spatial learning and memory and the vHi is predominantly involved in anxiety and response to stress (Bannerman *et al.* 2004, Fanselow and Dong 2010, O'Leary and Cryan 2014). Indeed, some studies have reported a greater density of place cells in the dHi when compared with the vHi (Jung *et al.* 1994), while the presence of anxiety cells, cells that are involved in the modulation of anxiety, is enriched in the vHi when compared to the dHi (Jimenez *et al.* 2018). This hypothesis is further supported by lesion studies which have also shown a functional dissociation of the hippocampus. Indeed, lesions of the dHi but not the vHi have been shown to impair spatial learning and memory (Moser *et al.* 1995, Bannerman *et al.* 2002, Pothuizen *et al.* 2004). On the other hand, it has been reported that the vHi plays a role in the response to stress (Fanselow and Dong 2010, Tanti and Belzung 2013, O'Leary and Cryan 2014, Levone *et al.* 2015), as well as a role in anxiety (Bannerman *et al.* 2002, Bannerman *et al.* 2003, Bannerman *et al.* 2004) and social behaviours (Bannerman *et al.* 2002, McHugh *et al.* 2004, Felix-Ortiz and Tye 2014, Okuyama *et al.* 2016). Recently, it has been proposed that the hippocampus exhibits a gradient of gene expression along its longitudinal axis and an intermediate sub-region (iHi) has been proposed (Leonardo *et al.* 2006, Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016, Cembrowski *et al.* 2016) although some studies have suggested that this area has overlapping characteristics with both the dHi and vHi (Cenquizca and Swanson 2007). Nevertheless, the specific roles of the iHi have yet to be determined although it has been reported that it may mediate the integration of diverse sensory modalities (Bast *et al.* 2009).

Together, such studies suggest that anxiety and response to stress are mediated predominantly by the vHi. Although chronic stress is a risk factor for depression and anxiety is often co-morbid in depression, to date no studies have interrogated whether the beneficial effects of antidepressants are also mediated specifically by discrete sub-regions areas along longitudinal axis of the hippocampus. Thus, the aim of this study was to determine whether social, anxiety and depressive-like behaviour in the absence or presence of antidepressant treatment is modulated by distinct sub-regions of the longitudinal axis of the hippocampus. To this end, we induced excitotoxic lesions of the mouse dHi, iHi or vHi and interrogated their impact on a battery of behavioural and neuroendocrine tests in the presence or absence of the antidepressant, fluoxetine.

2.2. Materials and Methods

2.2.1. Experimental design

Ibotenic acid-induced lesions of the dHi, iHi and vHi were performed and the impact of these on the regulation of social, anxiety and depression-like behaviour with or without chronic treatment with the antidepressant, fluoxetine was examined in two separate experiments as summarised in Fig 2.1A and Fig 2.1B.

We first examined the roles of the dHi, iHi and vHi in social behaviour, anxiety, anhedonia and antidepressant-like behaviour (*Experiment 1*; Fig 2.1A). Mice underwent either sham surgery or ibotenic-induced lesions of the dHi, iHi, or vHi. Seven days following surgery, animals underwent a battery of behavioural tests as shown in Fig 2.1A. The experimental groups, sample number (after surgery corrections), surgery success rate and number of misplaced (excluded) animals were: (1) dHi-sham, n = 10 (83.3% success, 2 excluded); (2) dHi-lesion, n = 8 (66.7% success, 4 excluded); (3) iHi-sham, n = 9 (75% success, 3 excluded); (4) iHi-lesion, n = 9 (57.1% success, 6 excluded); (5) vHi-sham, n = 10 (55.6% success, 8 excluded) and (6) vHi-lesion, n = 9 (52.9% success, 8 excluded).

We then examined the roles of the roles of the dHi, iHi and vHi in the antidepressant and anxiolytic effects of fluoxetine treatment (*Experiment 2*; Fig 2.1B). Ibotenic-induced lesions of the dHi, iHi and vHi of the mouse hippocampus were performed, while some animals underwent a sham surgery. Seven days following surgery, animals were injected with either saline (sham-saline group) or fluoxetine (all other groups; 10mg/kg i.p. per day, PHR1394, Sigma-Aldrich,) daily for 21 days and then underwent a battery of behavioural tests while also receiving daily fluoxetine or

saline injections as shown in Fig. 2.1B. 30 min after the first injection with fluoxetine, animals underwent the tail suspension test, to assess the effects of acute fluoxetine. After 21 days of injections, the anxiolytic and antidepressant effects of chronic fluoxetine were tested. The impact of lesions and chronic fluoxetine treatment on acute swim stress-induced increases in plasma corticosterone concentrations was also investigated. The experimental groups, sample number (after surgery corrections), surgery success rate and number of misplaced (excluded) animals were: (1) sham-saline, n = 10 (100% success, 0 excluded); (2) dHi sham-fluoxetine, n = 10 (83.3% success, 2 excluded); (3) dHi lesion-fluoxetine, n = 10 (83.3% success, 2 excluded); (4) iHi sham-fluoxetine, n = 9 (81.8% success, 2 excluded); (5) iHi lesion-fluoxetine, n = 10 (83.3% success, 2 excluded); (6) vHi sham-fluoxetine, n = 8 (66.7% success, 4 excluded) and (7) vHi lesion-fluoxetine, n = 8 (72.7% success, 3 excluded).

2.2.2 Animals

Male C57BL/6 mice (Envigo, UK) aged 8-weeks old, were housed in groups of 3-4 at arrival in a temperature controlled (21±2°C) room and allowed to acclimatise to the holding room for 7-days prior to initiation of experiments. Laboratory chow and water were provided ad libitum on a 12/12 h light/dark cycle (lights on at 7:30 am). All procedures were conducted with approval from the Animal Experimentation Ethics Committee (AEEC) at University College Cork, under Individual Authorisations and a Project Authorisation approved the Health Products Regulatory Authority (HPRA) Ireland and in accordance with the recommendations of the European Parliament and the Council of the EU Directive (2010/63/EU).

2.2.3. Stereotaxic brain surgery and excitotoxic lesion of the dorsal, intermediate of ventral hippocampus

Mice (20-26g) were anaesthetised with isoflurane (5% induction, 1.7-2.5% maintenance) and received analgesia (5mg/kg, s.c., Carprofen, Norbrook,) prior to being placed in a stereotaxic frame. Fig 2.1C shows the definition of dHi, iHi and vHi in coordinates. As shown in Fig 2.1D, small volumes (0.15-0.2 µl) of ibotenic acid (10 mg/ml, I2765, Sigma-Aldrich) diluted in PBS (pH 7.4) were injected bilaterally into either the dHi, iHi or vHi using coordinates from the Paxinos and Franklin's atlas for mouse brain (Paxinos 2012). The ibotenic acid causes excitotoxicity, damaging cells without affecting passaging fibres. Sham surgeries involved opening holes at the relevant areas of the skull but not injecting anything. Holes in the skull were covered with Bone-Wax (SMI, Z046) and the skin was sutured (Mersilk Suture 4-0, W329). An analgesic (Carpofen, Norbrook, 20 µg/ml) was added to the drinking water for 2 days post-surgery.

At the end of the experiment, mice were deeply anaesthetised with pentobarbital (250mg/kg, i.p., Euthanal, Merial,) and transcardially perfused with PBS followed by 4% PFA. Brains were removed, post-fixed in 4% PFA overnight, cryoprotected in 30% sucrose and then frozen at -80°C. Brains were sectioned coronally (at 35 µm) through the entire rostro-caudal axis of the hippocampus and collected onto gelatinised slides. Sections were stained with cresyl violet (0.1%, Sigma-Aldrich, C5042). Injection site were identified by light microscopy examination by an experimenter blind to the experimental treatment groups. Only animals with confirmed lesions in the specific sub-region were included (see Fig 2.1E for shams and F-H for lesions).

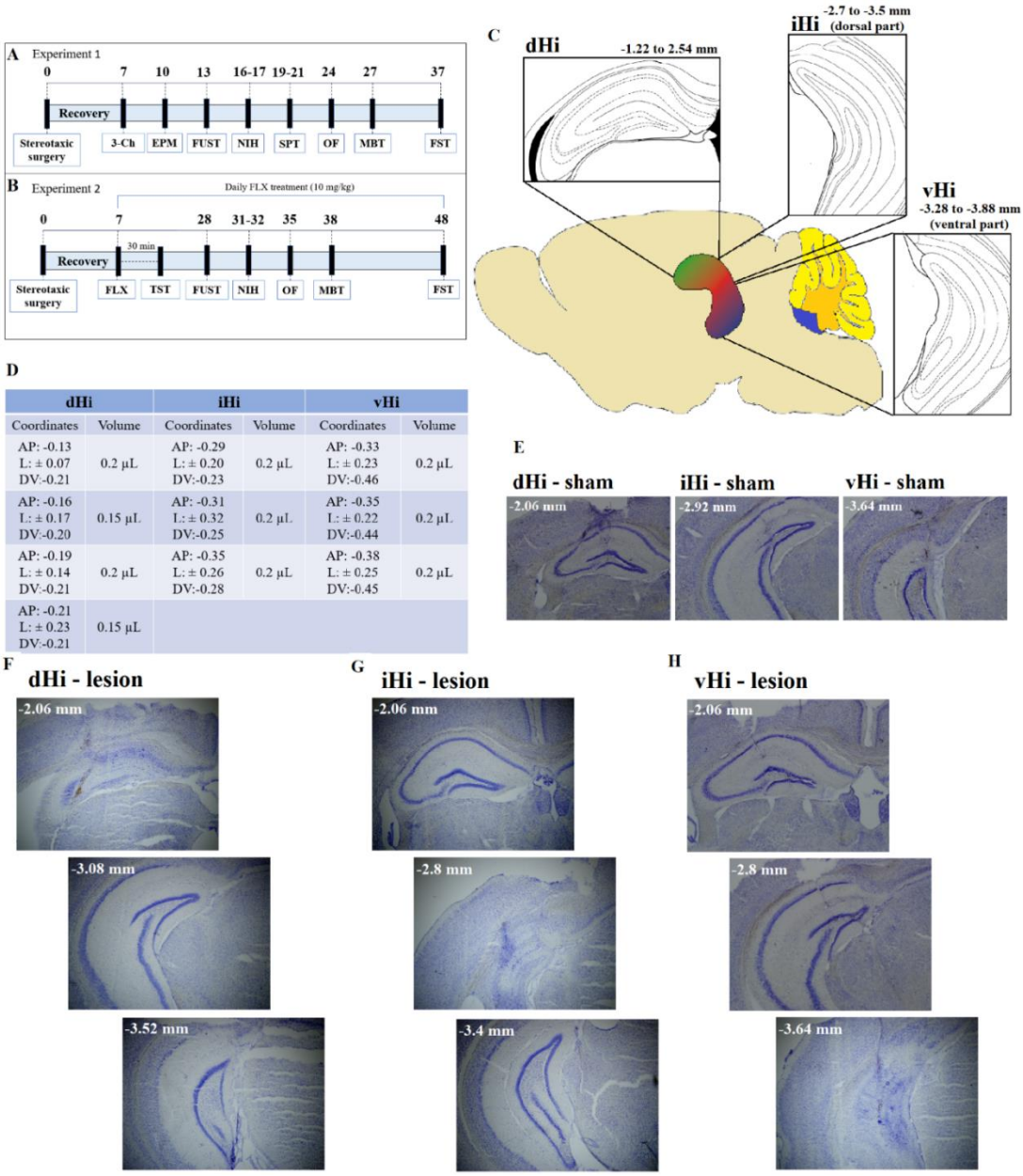

Figure 2.1: *Dorsal (dHi), intermediate (iHi) or ventral (vHi) hippocampus were lesioned and animals followed with a battery of behavioural testing with or without chronic treatment with fluoxetine. A, schematic of the experiment in which animals had their dHi, iHi or vHi lesioned and followed with a battery of behavioural testing under baseline. In B, after surgery, animals followed with a battery of behavioural and neuroendocrine testing under chronic fluoxetine treatment. C, illustration of the definitions and coordinates of dHi, iHi and vHi. D, stereotaxic coordinates used to lesion dHi, iHi and vHi, bilaterally. E-H, representative images of the dHi, iHi and vHi. Lesions were confirmed using cresyl violet staining, E, dHi, iHi and vHi sham groups. F, dHi lesion, showing that iHi and vHi were intact. G, iHi lesion, showing that dHi and iHi were intact. H, vHi lesion, showing that dHi and iHi were intact. Coordinates in the representative images are in relation to bregma. Abbreviations: 3-Ch: three-chambers test, AP: anteroposterior, DV: dorsoventral, EPM: elevated plus maze, FLX: fluoxetine, FST: forced swim test, FUST: female urine sniffing test, L: lateral, MBT: marble burying test, NIH: novelty-induced hypophagia, OF: open field, SPT: saccharin preference test, TST: tail suspension test.*

2.2.4. Three-chambers test of sociability and social novelty preference

Sociability and preference for social novelty were assessed in a three-chamber apparatus (Desbonnet *et al.* 2014). The apparatus consists of a box with three chambers (left and right and a smaller centre chamber) with small circular openings allowing easy free access to all compartments. The test is composed of three sequential 10 min trials or stages. In the first stage, the test animal is allowed to explore the three empty chambers. The second stage tests sociability whereby an unfamiliar animal is placed in an inner mesh wire cage in one chamber and an object is placed in a mesh wire cage in the other chamber. The final stage, tests social novelty preference whereby the object is replaced by a novel animal. All animals were age- and sex-matched and each chamber was cleaned and lined with fresh bedding between trials. In each of the three stages, behaviour was recorded by a video camera mounted above the apparatus and the time spent in each chamber was measured in each individual trial.

2.2.5. Tests of anxiety.

2.2.5.1. Elevated Plus Maze (EPM)

The elevated plus maze is a test used to measure anxiety-like behaviour (File *et al.* 2004, Sweeney *et al.* 2014). Each animal was placed in the centre of the elevated plus maze (a cross shaped maze with 2 open arms and 2 closed arms, elevated from the ground) and their behaviour was monitored and tracked for 5 minutes. Time and percentage of time and number of entries in the open and closed arms were analysed. Mice tend to spend more time in the protected closed arms than the open arms. Thus, an increase in the percentage of time spent in the open versus closed arms is interpreted as reduced anxiety behaviour.

2.2.5.2. Novelty-induced hypophagia (NIH)

The novelty-induced hypophagia test is a test of anxiety sensitive to chronic antidepressant treatment (Dulawa and Hen 2005, O'Leary *et al.* 2013). In this test, mice were trained to drink a diluted solution of sweetened condensed milk (3:1, water to milk) from a 10 ml serological pipette through the lid of the cage for 30 minutes per day for 3 days. On the fourth day, the latency to drink the milk in their homecage was measured. On the fifth day, mice were placed in a novel brightly-lit cage (1200 lux) without bedding, and their latency to drink the milk was recorded. Placement in the novel cage increases the latency to drink the milk which is taken as an index of anxiety. Chronic antidepressant treatment decreases this latency. The data are presented as latency difference (i.e. the latency to drink in the novel cage minus latency to drink in the home cage).

2.2.5.3. Marble Burying Test (MBT)

The marble burying test is a test used to measure phenotypes related to anxiety and obsessive compulsive-like behaviour (Deacon 2006). A cage (35 x 28 cm) was filled with clean corncob bedding overlaid with 20 glass marbles equidistant from each other in a 4×5 arrangement (Savignac, Kiely et al., 2014). Mice were placed in the cage for 30 minutes and the number of marbles that are more than 2/3 buried were counted. An increase in the number of marbles buried is considered to be a proactive response to an anxiogenic stimulus and has also been described as a compulsive behaviour to relieve anxiety (Deacon 2006). Chronic fluoxetine treatment has been shown to decrease the number of marbles buried (Greene-Schloesser *et al.* 2011).

2.2.5.4. Open field test (OF)

The open field test was used to measure locomotor activity as well as anxiety-like behaviour. This test consisted of a rectangle box (perspex sides and base: 40 x 33 cm^2) in a dimly lit room (4 lux). Mice were placed in the centre of the open field and allowed to explore the apparatus for 10 minutes. Locomotor activity was recorded by a camera placed over the open field and distance travelled were automatically analysed using Ethovision 11.5 software. The time spent in the centre quarter (10 x 8.25 cm2) of the chamber was also measured as an index of anxiety behaviour.

2.2.6. Tests of anhedonia

2.2.6.1. Female Urine Sniffing Test (FUST)

The female urine sniffing test assesses anhedonia (a reduction in the ability to experience pleasure) which is a core feature of depression (Malkesman *et al.* 2010, Finger *et al.* 2011, O'Leary *et al.* 2014). This test takes advantage of the fact that rodent males are attracted to pheromonal odours from the opposite sex and thus is used in male mice as an index of sexual interest. Before the test, mice were transferred to a dimly-lit (4 lux) room and habituated to the presence of a cotton tip applicator in their home cage for 1 hour. Following habituation, mice were immediately presented with a cotton tip applicator with 60 µL of sterile water for a period of 3 minutes and the time spent sniffing the water was measured. 45 min later, a cotton tip with 60 µL of fresh urine from female mice in oestrus was presented to the mice for 3 minutes and the time spent sniffing the urine was measured. Data are presented as the percentage of time spent sniffing urine over the total time spent sniffing urine plus water.

2.2.6.2. Saccharin preference test (SP)

The saccharin preference test (Harkin *et al.* 2002) was used as another test for anhedonia. Mice were given access to both a water bottle and a saccharin solution (0.1%) bottle for a total period of 48 h. Cumulative saccharin and water intake were measured every 12 hours (at 7:30 a.m. and 7:30 p.m. each day). Every 12 h, the position of the bottles was reversed to avoid the development of preference for drinking from a particular side of the cage. Mice normally show a preference to consume the saccharin over water and chronic antidepressant treatment counteracts stress-induced decreases in this preference for saccharin (Harkin *et al.* 2002). The data are expressed as the cumulative percentage preference for saccharin over water [(saccharin consumption volume/water consumption volume) * 100].

2.2.7. Tests of antidepressant-like behaviour.

2.2.7.1. Tail Suspension Test (TST)

The tail suspension test is a test of antidepressant-like activity and behavioural despair (Steru *et al.* 1985, O'Leary 2009). In this test, the mouse was suspended by the tail from a horizontal bar for 6 minutes. Initially, the mouse displays escape-oriented behaviours but after several minutes adopts an immobile posture. Antidepressant drugs are known to decrease the time the mouse spends immobile in this test. In the present experiment, a single dose of fluoxetine was given 30 mins prior to the start of the test to examine the role of hippocampal sub-regions in the acute antidepressant-like effects of fluoxetine. The results show the effects of acute fluoxetine and are expressed as time spent immobile.

2.2.7.2. Forced Swim Test (FST)

The forced swim test is the most widely used experimental paradigm to assess antidepressant drug-like activity (Porsolt *et al.* 1977, Cryan and Mombereau 2004). In this test, mice were allowed to swim for 6 min in a glass cylinder (24 × 21 cm) filled with water (23–25 °C) to a depth of 17 cm. The test was videorecorded and the time spent immobile during the last 4 min of the 6-min test was measured. Antidepressant drugs decrease immobility in this test.

2.2.8. Plasma corticosterone measurements

Blood was withdrawn from the tail vein to measure changes in concentrations of the stress hormone corticosterone before and after the acute stress of the forced swim test (FST). Blood was withdrawn 30 min before the FST and 30 and 120 min after the beginning of the test.

Blood was centrifuged at 4000g for 15 min at 4°C. Plasma was removed and kept at -80°C until analysis. Plasma corticosterone was measured using an ELISA kit (Enzo, ADI-901-097) and light absorbance was read with a multi-mode plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm. Samples were analysed in duplicate within a single assay and the minimum threshold of detection was less than 32 pg/mL. The limit for the coefficient of variation used was 20% and the final concentrations are expressed in ng/mL.

2.2.9. Statistical analysis

Data is shown as mean + S.E.M. Statistical analysis was performed using the software IBM SPSS Statistics 23. Data were analysed using two-way ANOVA for the experiment that investigated the roles of dHi, iHi and vHi on emotional behaviours under baseline conditions i.e. in the absence of fluoxetine (*Experiment*

1). Three chambers test in Experiment 1 was analysed using three-way ANOVA. Data from the experiment that investigated the roles of dHi, iHi and vHi on emotional behaviours under acute and chronic fluoxetine treatment were analysed using one-way ANOVA *(Experiment 2*). When appropriate, ANOVA was followed by Fishers LSD post-hoc test for group-wise comparisons. For all comparisons, $p<0.05$ was the criterion used for statistical significance.

2.3. Results

2.3.1. The role of the dHi, iHi and vHi on sociability, anhedonia, anxiety and depressive-like behaviour (Experiment 1)

2.3.1.1. Sociability and social novelty preference are unaffected by dHi, iHi or vHi lesions

The three chambers test was used to assess the effects of hippocampal sub-region lesions on sociability and social novelty preference. The effects of sub-region-specific lesions on sociability are shown in Fig 2.2A. Three-way ANOVA revealed that, animals prefer to explore the chamber containing the unfamiliar animal rather than that containing the object ($F_{1,94}$=179,67, p<0.001). There was no main effect of hippocampal sub-region ($F_{2,94}$=0.04, p=0.96), lesion ($F_{1,94}$=0.05, p=0.83); nor a hippocampal sub-region x lesion interaction ($F_{2,94}$=0.19, p=0.8), nor a lesion x preference for animal interaction ($F_{1,94}$=2.87, p=0.09). However, there was a trend towards an interaction between hippocampal sub-region x preference for animal ($F_{1,94}$=2.87, p=0.054) Finally, there was no interaction between the three factors ($F_{2,94}$=2.03, p=0.14). Specifically, post-hoc analysis revealed that each group preferred to explore the chamber containing the animal (dHi lesion group p=0.037, all other groups p<0.001). Interestingly, dHi-lesioned animals spent less time exploring the chamber with the animal than the dHi sham animals (p=0.046).

The effects of sub-region-specific lesions on the preference for social novelty are shown in Fig 2.2B. Three-way ANOVA demonstrated that animals preferred to explore the chamber containing the novel animal rather than the one containing the familiar animal ($F_{1,94}$=45.62, p<0.001). There was no effect of hippocampal sub-region ($F_{2,94}$=0.02, p=0.98) or lesion ($F_{1,94}$=0.006, p=0.94). There were no

interactions between hippocampal sub-region x lesion ($F_{2,94}$=0.008, p=0.99), hippocampal sub-region x preference for animal ($F_{2,94}$=1.51, p=0.23), lesion x preference for animal ($F_{1,94}$=0.84, p=0.36) or between the three main factors ($F_{2,94}$=0.1, p=0.9). Post-hoc analysis revealed that all groups showed a preference for the novel animal (dHi-sham, p=0.016; dHi-lesion, p=0.018; iHi-sham, p=0.001; iHi-lesion, p<0.001; vHi-sham, p=0.009; vHi-lesion, p=0.005), with no differences between them.

We also calculated the percentage preference for the animal (time spent in the chamber with the animal over the total time spent in chambers), and this result is shown in Fig 2.2C. Two-way ANOVA revealed no effect of lesion ($F_{1,48}$=2.38, p=0.13), hippocampal sub-region ($F_{2,48}$=0.96, p=0.39) nor an interaction between them ($F_{2,48}$=0.64, p=0.53). We then calculated the percentage preference for social novelty (time spent in the chamber with the novel animal over total time spent in chambers), and this result is shown in Fig 2.2D. Two-way ANOVA also showed no effect of lesion ($F_{1,48}$=0.61, p=0.44), hippocampal sub-region ($F_{2,48}$=0.45, p=0.64) nor an interaction between them ($F_{2,48}$=0.21, p=0.81).

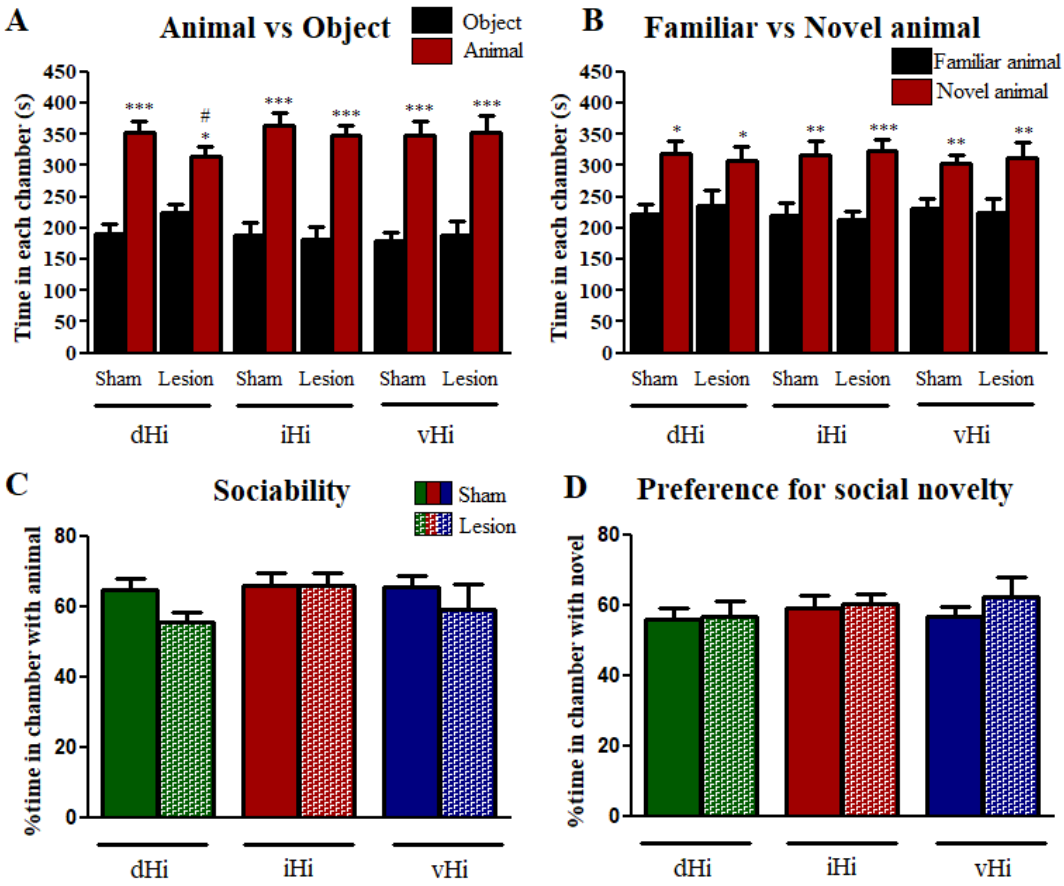

Figure 2.2: *Roles of dHi, iHi and vHi in sociability and preference for social novelty. A, all animals preferred to explore the chamber with the animal rather than the one with the object, however, dHi-lesion reduced the time spent in the chamber with animal in relation to dHi-sham group. B, all animals preferred to explore the chamber with the novel animal rather than the one with the familiar animal. C, the percentage preference for the animal was calculated as an index of sociability and no differences were found between groups. D, the percentage preference for the novel animal was calculated as an index of preference for social novelty and no differences were found between groups. *p<0.05, ***p<0.001, in relation to time spent in the chamber with the object; #p<0.05, in relation to sham group, according to Fishers LSD post-hoc test.*

2.3.1.2. Anhedonia and depressive-like behaviour are unaffected by dHi, iHi or vHi lesions

The effects of hippocampal sub-region-specific lesions in the saccharin preference test is shown in Fig 2.3A-D. All experimental groups preferred the sweet solution. Two-way ANOVA revealed that there were no significant effects of lesion (12 h, $F_{1,49}$=1.52, p=0.22; 24 h, $F_{1,49}$=1.1, p=0.3; 36 h, $F_{1,49}$=1.61, p=0.21; 48 h, $F_{1,49}$=1.66, p=0.2), hippocampal sub-region (12 h, $F_{2,49}$=2.74, p=0.075; 24 h, $F_{2,49}$=2.61,

p=0.084; 36 h, $F_{2,49}$=1.05, p=0.36; 48 h, $F_{2,49}$=1, p=0.38), nor lesion x hippocampal sub-region interaction (12 h, $F_{2,49}$=0.22, p=0.8; 24 h, $F_{2,49}$=0.11, p=0.9; 36 h, $F_{2,49}$=0.05, p=0.96; 48 h, $F_{2,49}$=0.07, p=0.93) at any of the time points measured.

The effects of hippocampal sub-region-specific lesions in the female urine sniffing test is shown in Fig 2.3E. All experimental groups spent more time sniffing urine than water. Two-way ANOVA revealed a significant main effect of lesion ($F_{1,48}$=5.47, p=0.024) but no main effect of hippocampal sub-region ($F_{2,48}$=0.2, p=0.82) nor a lesion x hippocampal sub-region interaction ($F_{2,48}$=0.29, p=0.75). Although there was an overall effect of lesion, subsequent post hoc analysis did not reveal any statistically significant differences between individual groups.

The effects of hippocampal sub-region-specific lesions on time spent immobile in the forced swim test (FST) is shown in Fig 2.3F. Two-way ANOVA revealed no significant effects of lesion ($F_{1,49}$=0.06, p=0.81), hippocampal sub-region ($F_{2,49}$=2.39, p=0.1), nor a lesion x hippocampal sub-region interaction ($F_{2,49}$=0.34, p=0.71).

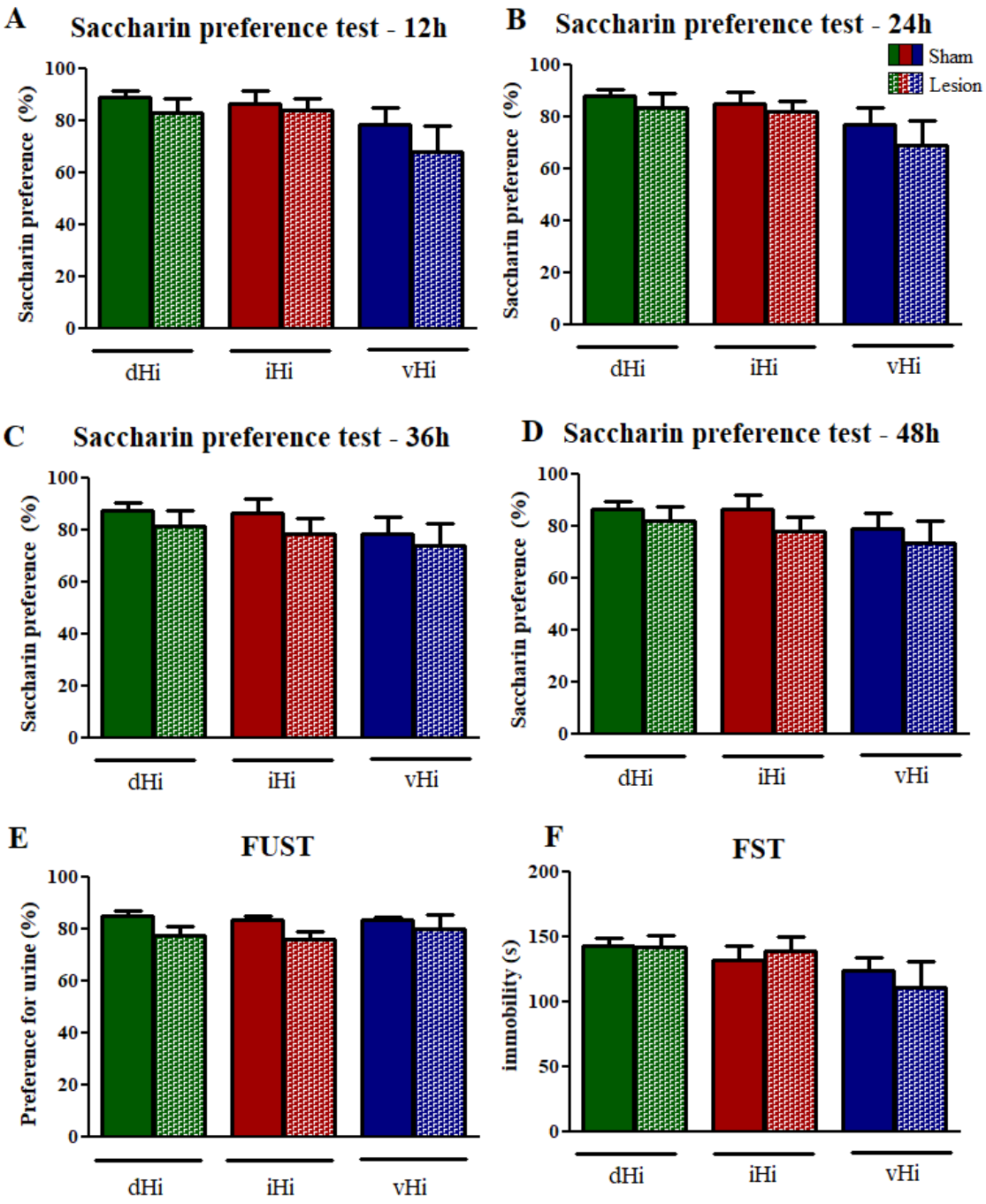

Figure 2.3: *The lesion of any of the hippocampal sub-regions did not alter anhedonia or depressive-like behaviour. A-D, the lesion of the hippocampus did not alter the anhedonia in the saccharin preference test at different timepoints, 12 h (A), 24 h (B), 36 h (C) or 48 h (D). E, the lesion of the hippocampus did not alter anhedonia in the female urine sniffing test (FUST). F, the lesion of the hippocampus did not alter depressive-like behaviour in the forced swim test (FST).*

2.3.1.3. Lesions of the vHi (but not dHi or iHi) reduce anxiety

The effects of hippocampal sub-region-specific lesions on anxiety were measured using several behavioural tests including the novelty-induced hypophagia test (Fig

2.4A), the marble burying test (Fig 2.4B), the elevated plus maze (Fig 2.4C) and the open field (Fig 2.4D).

In the novelty-induced hypophagia test (Fig 2.4A), two-way ANOVA revealed a significant lesion x hippocampal sub-region interaction ($F_{2,47}$=5.73, p=0.006), and a main effect of hippocampal sub-region ($F_{2,47}$=3.65, p=0.034) but no main effect of lesion ($F_{1,47}$=0.5, p=0.48). Specifically, post hoc analysis showed that vHi lesions reduced latency to drink the sweet solution when compared with the vHi sham group (p=0.003).

In the marble burying test (Fig 2.4B), two-way ANOVA revealed a significant main effect of lesion ($F_{1,49}$=5.85, p=0.019), but no effect of hippocampal sub-region ($F_{2,49}$=1.61, p=0.21), and no lesion x hippocampal sub-region interaction ($F_{2,49}$=1.74, p=0.19). Specifically, post hoc analysis revealed that vHi lesions reduced the number of marbles buried when compared with the vHi-sham group (p=0.005).

In the elevated plus maze (Fig 2.4C), two-way ANOVA revealed a significant lesion x hippocampal sub-region interaction ($F_{2,49}$=3.67, p=0.033) and a main effect of hippocampal sub-region ($F_{2,49}$=6.39, p=0.003) but not of lesion ($F_{1,49}$=1.56, p=0.22). Specifically, post hoc analysis revealed that vHi lesions increased the percentage of time spent in the open arms when compared to the vHi-sham group (p=0.005).

In the open field test (Fig 2.4D), two-way ANOVA revealed a trend towards a main effect of lesion ($F_{1,49}$=3.64, p=0.062) and a significant lesion x hippocampal sub-

region interaction ($F_{2,49}$=3.57, p=0.036) but no main effect of hippocampal sub-region ($F_{2,49}$=1.83, p=0.17). Specifically, post hoc analysis revealed that vHi lesions increased the time spent in the centre of the open field when compared to the vHi sham group (p=0.002).

4.3.1.4. Lesions of the dHi, iHi or vHi do not alter locomotor activity

The effects of dHi, iHi and vHi lesions on distance travelled in the open field test are shown in in Fig. 2.4E. Two-way ANOVA revealed no effect of lesion ($F_{1,49}$=0.39, p=0.54), hippocampal sub-region ($F_{2,49}$=0.92, p=0.41), nor a lesion x hippocampal sub-region interaction ($F_{2,49}$=0.36, p=0.7).

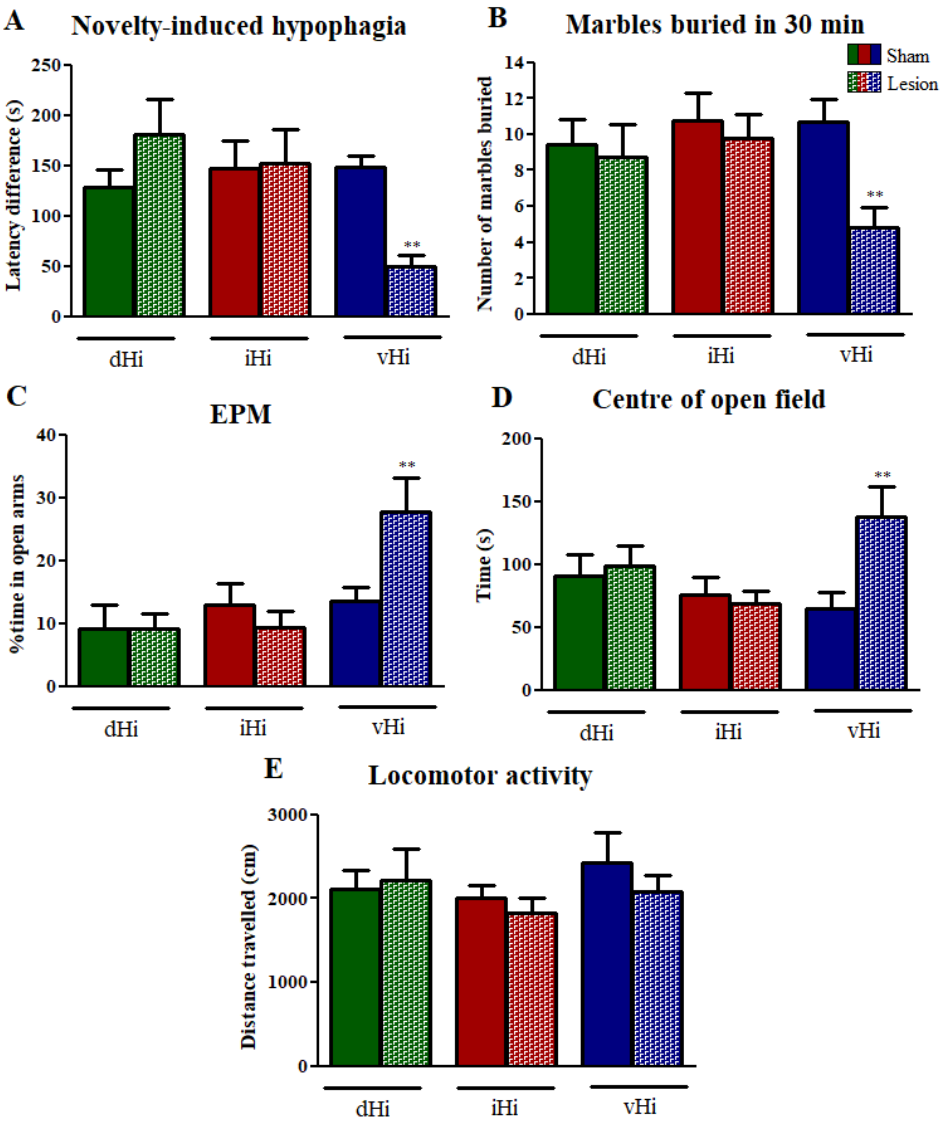

Figure 2.4: *The lesion of the vHi caused a decrease in anxiety in several behavioural tests, including A, the novelty-induced hypophagia test, B, the marble burying test, C, the elevated plus maze (EPM) and D, the time spent in the centre quarter of the open field. E, these results were not due to an increased locomotor activity in the open field. **p<0.01, in relation to its own sham group, according to Fishers LSD post-hoc test.*

2.3.2. The role of the dHi, iHi and vHi in antidepressant regulation of emotional behaviours and neuroendocrine response

When assessing the roles of the dHi, iHi and vHi under basal conditions, we observed that vHi lesions affected anxiolytic behaviours. Our previous studies (Levone et al., unpublished) also found that the vHi but not other sub-regions regulated anxiety behaviour in the open field test as well as antidepressant-like behaviour in the forced swim test. We also found that both the dHi and vHi mediate

stress-induced anhedonia in the saccharin preference test. Given that such behaviours are modulated by chronic antidepressant treatment, we then sought to investigate whether lesions of the vHi, and also dHi or iHi affect antidepressant-induced changes in anxiety and depressive-like behaviour as well as the corticosterone response to acute stress.

2.3.2.1. Only vHi lesions prevent the antidepressant effects of acute fluoxetine treatment in the tail suspension test (TST)

The effects of acute (30 min after the first injection) fluoxetine or saline on immobility in the tail suspension are shown in Fig 2.5. One-way ANOVA revealed a main effect of fluoxetine ($F_{6,57}$=13.02, p<0.001). However, subsequent post hoc analysis revealed that fluoxetine-induced decreases in immobility were prevented in vHi-lesioned animals (p=0.1 vs sham-saline group; all other groups p<0.001 vs sham-saline group). The fluoxetine-treated vHi-lesion group also exhibited higher immobility when compared to the fluoxetine-treated vHi-sham group (p<0.001).

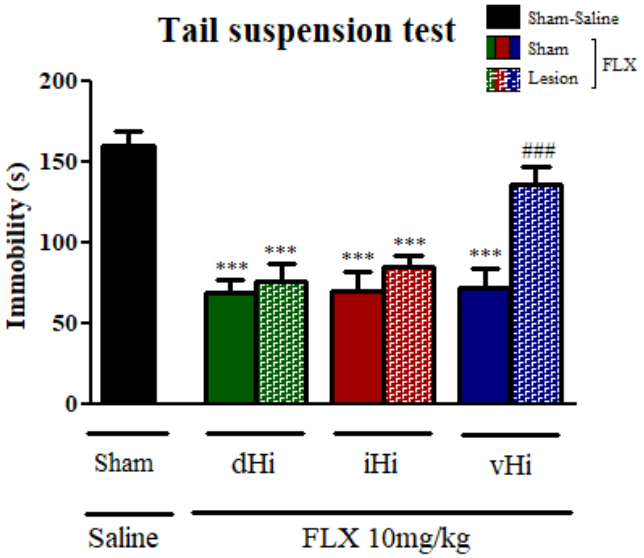

Figure 2.5: *Animals treated with acute fluoxetine (FLX) showed a decrease in the immobility time in the tail suspension test. However, vHi lesion prevented this fluoxetine effect. ***p<0.001, in relation to sham-saline group; ###p<0.001, in relation to vHi-sham group, according to Fishers LSD post-hoc test.*

2.3.2.2. Lesions of the vHi prevent the anxiolytic effects of chronic fluoxetine treatment in the novelty-induced hypophagia test but not in the marble burying test nor the open field test

The effects of chronic fluoxetine treatment and hippocampal sub-region-specific lesions on latency difference to drink milk in the novelty-induced hypophagia test are shown in Fig 2.6A. One-way ANOVA revealed a main effect of fluoxetine ($F_{6,58}$=4.91, p<0.001). Subsequent post hoc analysis revealed that chronic fluoxetine reduced the latency difference in all sham groups when compared to the sham-saline group (dHi sham, p<0.001; iHi sham, p=0.001; vHi sham, p=0.001). This anxiolytic effect was unaffected by dHi lesions (p=0.002 vs sham saline) although it was somewhat attenuated by iHi lesions (p=0.055 vs sham saline). On the other hand, vHi-lesions prevented fluoxetine-induced reduction in anxiety in this test (p=0.78 vs sham saline). The latency in the fluoxetine treated vHi-lesion group was also significantly longer than the fluoxetine-treated vHi-sham group (p=0.004).

The effects of chronic fluoxetine treatment and hippocampal sub-region-specific lesions in the marble burying test are shown in Fig 2.6B. One-way ANOVA revealed a main effect of fluoxetine ($F_{6,57}$=32.73, p<0.001). Subsequent post hoc analysis revealed that fluoxetine significantly reduced the number of marbles buried in all groups when compared to the sham-saline group (all p<0.001).

The time spent exploring the centre of the open field is shown in Fig 2.6C. Fluoxetine had no effect in this measure of anxiety ($F_{6,57}$=0.53, p=0.78). We also measured the distance travelled in the open field as a measure of general locomotor

activity, as shown in Fig 2.6D. There were no alterations on the distance travelled ($F_{6,57}$=1.77, p=0.12).

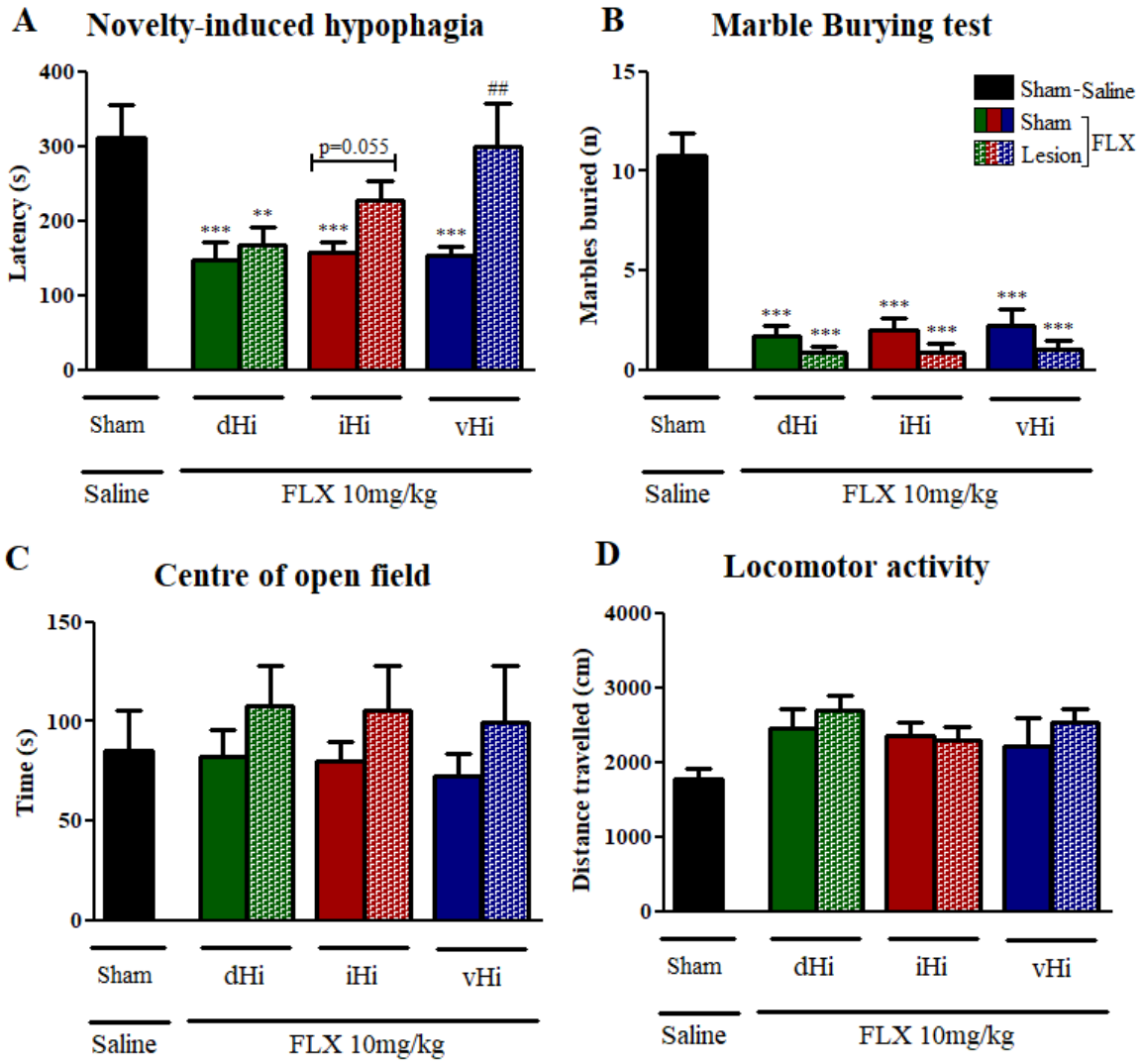

Figure 2.6: *Fluoxetine showed an anxiolytic effect in the novelty-induced hypophagia and marble burying tests. However, vHi lesion prevented these effects in the novelty-induced hypophagia test (A), but not in the marble burying test (B). Fluoxetine did not show an effect on the time spent in the centre of the open field (C) or on the locomotor activity (D). **p<0.01, ***p<0.001, in relation to sham-saline group; ##p<0.01, in relation to vHi-sham group, according to Fishers LSD post-hoc test.*

2.3.2.3. Fluoxetine had no effect in the female urine sniffing test

In the female urine sniffing test, the percentage of preference for urine over water was measured and the results shown in the Fig 2.7A. Fluoxetine did not exert any effect in this test ($F_{6,58}$=1.02, p=0.42).

2.3.2.4. Lesions of the iHi only prevent the antidepressant effects of chronic fluoxetine in the forced swim test

The effects of chronic fluoxetine treatment and hippocampal sub-region-specific lesions on immobility in the forced swim test are shown in Fig 2.7B. There was a significant effect of fluoxetine in this test ($F_{6,57}$=5.67, p<0.001). Specifically, post hoc analysis revealed that, when compared with the sham saline group, fluoxetine decreased immobility in all other sham groups (sham dHi, p=0.003; sham iHi, p=0.001; sham vHi, p<0.001) and that this was (p=0.003, <0.001, 0.001, <0.001, <0.001), and this was unaffected by lesions of the dHi (p<0.001) and the vHi (p<0.001). However, iHi lesions prevented fluoxetine induced reductions in immobility in this this test (p=0.17 vs sham saline). In addition, the fluoxetine-treated iHi-lesion group exhibited higher immobility time when compared to the fluoxetine-treated iHi-sham group (p=0.028).

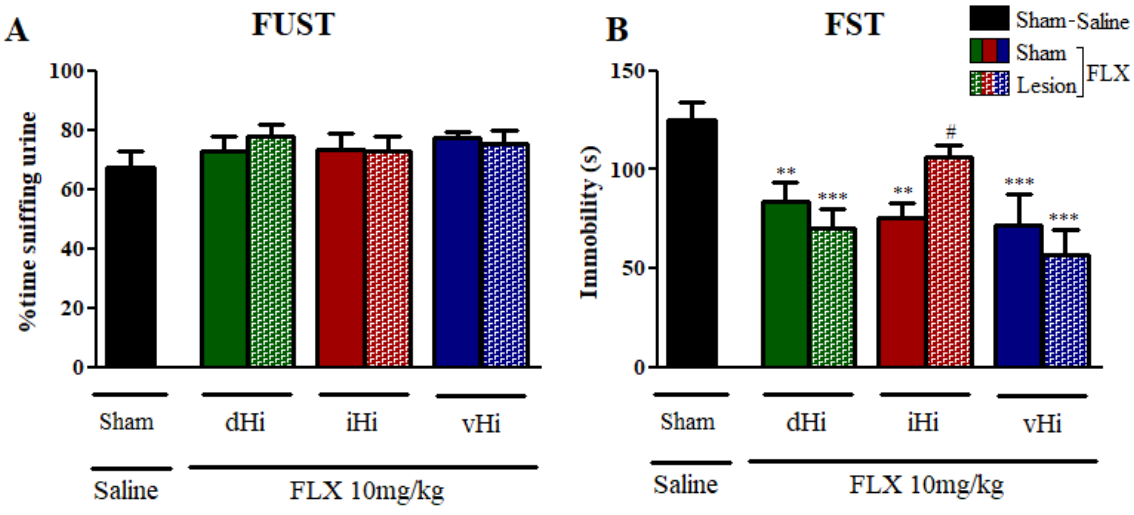

Figure 2.7: *A, fluoxetine showed no effect in the female urine sniffing test (FUST), but showed an antidepressant effect in the forced swim test (FST), which was mediated by the lesion of the iHi (B). **p<0.01, ***p<0.001, in relation to sham-saline group; #p<0.05, in relation to iHi-sham group, according to Fishers LSD post-hoc test.*

2.3.2.5. Lesions of the dHi amplified stress-induced increases in corticosterone 30 min after the forced swim test

The effects of fluoxetine on plasma corticosterone concentrations 30 mins before the FST and 30 min and 120 min after the FST are shown in Fig 2.8. Two-way repeated measures ANOVA revealed an overall effect of timepoint ($F_{2,57}$=415.4, p<0.001), but no effect of treatment ($F_{6,57}$=1.81, p=0.11) and no interaction between them ($F_{12,57}$=1.33, p=0.21). Post hoc analysis at each timepoint revealed that the concentration of corticosterone increased 30 min after the FST in all groups in comparison to baseline (all p<0.05). This swim stress-induced increase in corticosterone at the 30 min timepoint was greater in fluoxetine-treated animals in comparison to sham-saline animal (p<0.05 for dHi-sham, dHi-lesion, iHi-sham and vHi-sham vs sham-saline), except for iHi-lesioned and vHi-lesioned groups.

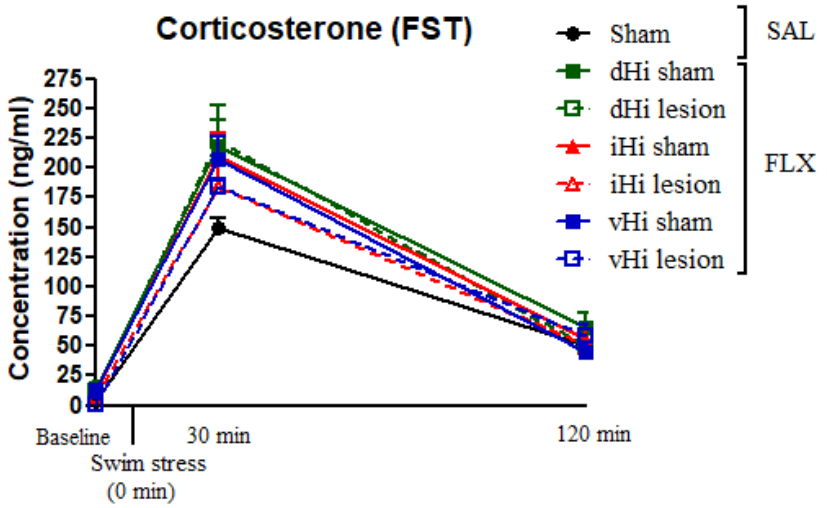

***Figure 2.8**: Fluoxetine-treated animals had an increase in the corticosterone release 30 min after the FST in comparison to sham-saline group, which is prevented by the lesion of iHi and vHi.*

2.4. Discussion

Understanding the circuits underlying antidepressant action may result in novel insights into developing newer therapies for depression. Therefore, this study highlights differential roles for the dHi, iHi or vHi on the regulation of social, anxiety and depressive-like behaviour in the absence or presence of the antidepressant fluoxetine.

Although the hippocampus has been shown to be involved in social behaviour (Montagrin *et al.* 2017) we found that none of the hippocampal sub-region-specific lesions affected sociability or preference for social novelty in the 3-Chamber test. This is in contrast with previous studies, which support a role for the vHi in social behaviour. Indeed, neonatal vHi lesions have been shown to cause deficits in social behaviour in rats in adulthood (Sams-Dodd *et al.* 1997). On the other hand, it has been reported that vHi lesions in adulthood increase social behaviour in the social interaction test in rats (Bannerman *et al.* 2002, McHugh *et al.* 2004). More recently, glutamatergic neuronal projections of the basolateral amygdala to the vHi have also been shown to modulate social behaviour in the social interaction test in C57BL/6 mice while inhibition of this pathway increased social interaction (Felix-Ortiz and Tye 2014). Similarly, it has been reported that vHi CA1 projections to nucleus accumbens store social memory in the social discrimination test in C57BL/6 mice (Okuyama *et al.* 2016). The reasons underlying our conflicting data are unclear but may be due to differences in the methodological approaches used such as the boundary definition of the vHi. In the present study, we defined the vHi as the most ventral third of the hippocampus and the lesion of this area on its own did not affect social behaviour; while the other lesion studies defined the vHi as the most ventral half (Bannerman *et al.* 2002, McHugh *et al.* 2004, Felix-Ortiz and Tye 2014,

Okuyama *et al.* 2016). Alternatively, our findings may be test-specific effect, as none of the published studies used the three-chamber test to assess social behaviour per se.

In the present study, we demonstrated that lesions of the vHi but not of the dHi or iHi robustly reduced anxiety in the elevated plus maze, novelty-induced hypophagia, marble burying and open field tests in mice. In agreement with previous findings in rats, dHi lesions did not alter anxiety-like behaviours (Bannerman *et al.* 2002, Bannerman *et al.* 2003, Bannerman *et al.* 2004). Our findings of vHi modulation of anxiety are in agreement with previous reports, which showed that vHi lesions in rats reduced hyponeophagia (Bannerman *et al.* 2002, Bannerman *et al.* 2003, McHugh *et al.* 2004) and decreased anxiety behaviour in the light-dark box (Bannerman *et al.* 2002, Bannerman *et al.* 2003, McHugh *et al.* 2004), the elevated plus maze (Kjelstrup *et al.* 2002) and in the cat-odour test (Pentkowski *et al.* 2006). Many of these studies defined the ventral hippocampus as the most ventral half of the hippocampus, and the dorsal hippocampus as the most dorsal half (Bannerman *et al.* 2002, Kjelstrup *et al.* 2002, Bannerman *et al.* 2003, McHugh *et al.* 2004). Given the increasing evidence supporting the hypothesis that the iHi may have discrete roles, it was important to determine its potential contribution to anxiety behaviour. Here, we show for what is to our knowledge the first time, that the modulation of anxiety behaviour is restricted to the most ventral third of the hippocampus, while the iHi and dHi lesions did not affect anxiety-like behaviour. Recent optogenetic studies have provided insight into the neural pathways that may underlie the role of the vHi in modulating anxiety. Optogenetic inhibition of glutamatergic projections from the basolateral amygdala to the vHi in C57BL/6 mice reduced in anxiety in the elevated plus maze and open

field test while stimulation of this pathway increases anxiety in this test (Felix-Ortiz *et al.* 2013). In addition, others have shown that the vHi and the medial prefrontal cortex (mPFC) exhibit synchronised activity in response to anxiogenic stimuli (Adhikari *et al.* 2010, Adhikari *et al.* 2011), and that inhibition of the vHi to mPFC pathway in 129SvevTac mice decreases anxiety in the open field (Padilla-Coreano *et al.* 2016). Another study has demonstrated that vHi projections to lateral septum and medial prefrontal cortex mediate anxiety in the elevated plus maze, novelty suppressed feeding and open field tests in C57BL/6 mice (Parfitt *et al.* 2017). In fact, they found that the activation of the vHi to lateral septum pathway reduced anxiety and its inhibition increased anxiety; interestingly, manipulation of vHi to medial prefrontal cortex resulted in opposite effects (Parfitt *et al.* 2017). Taken together, we hypothesise that in the present study, the removal of vHi influences to or from these brain regions may have caused the observed anxiolytic profile.

In the present study, we found that none of the hippocampal sub-region lesions affected anhedonia (in the saccharin preference and female urine sniffing tests), or depressive-like behaviour in the forced swim test, or locomotor activity in the open field. Few studies have described the roles of sub-regions of the longitudinal axis of the hippocampus in anhedonia or depressive-like behaviour (Carreno *et al.* 2016). However, we have previously found (Levone et al., unpublished; Chapter 3) that chronic stress induced-anhedonia was prevented by the lesion of the dHi and vHi, but not iHi, in the saccharine preference test and reduced anxiety novelty-induced hypophagia, which are test sensitive to chronic antidepressant treatment. Interestingly, optogenetic and chemogenetic activation of the vHi to medial prefrontal cortex pathway mimicked the antidepressant effects of ketamine in the forced swim in rats (Carreno *et al.* 2016). Taken together with the fact that lesions

alter behaviour in tests of anxiety which are sensitive to chronic antidepressant treatment, we next investigated the roles of each hippocampal sub-region in the antidepressant and anxiolytic effects of fluoxetine.

In agreement with previous studies, we found that in sham mice, chronic fluoxetine treatment had anxiolytic effects in the novelty-induced hypophagia test (Santarelli *et al.* 2003, Dulawa *et al.* 2004, Dulawa and Hen 2005), and in the marble burying test (Nicolas *et al.* 2006, Greene-Schloesser *et al.* 2011). However, we did not observe an anxiolytic effect of fluoxetine in the open field. vHi lesions prevented the anxiolytic effects of chronic fluoxetine treatment in the novelty-induced hypophagia test but not in the marble burying test. The reasons underlying this differential effect are currently unclear but may indicate that fluoxetine-induced anxiolytic effects occur through different mechanisms or different pathways in these two tests.

Considering that anxiety is modulated by the vHi, under influence of diverse brain regions including the amygdala (Felix-Ortiz *et al.* 2013) and the medial prefrontal cortex (Adhikari *et al.* 2010, Adhikari *et al.* 2011, Padilla-Coreano *et al.* 2016), the diverse effects observed in different behavioural tests in the present study may be due to the recruitment of differential neural pathways for different tests. Indeed, these tests may measure different aspects of anxiety, for example, the marble burying test is also used as a test for compulsive obsessive behaviour (Thomas *et al.* 2009). Moreover, vHi pathways have been shown to modulate other behaviours, e.g. the vHi to lateral septum pathway was shown to regulate feeding behaviour (Sweeney and Yang 2015).

We found that vHi lesions prevented the antidepressant effects of acute fluoxetine treatment in the tail suspension test (TST). In agreement, acute fluoxetine treatment has been shown to reduce immobility time in the TST (Cryan *et al.* 2005, O'Leary *et al.* 2007, O'Leary 2009, Liu *et al.* 2010). To the best of our knowledge, this is the first time that the vHi is shown to mediate the antidepressant effects of acute fluoxetine treatment.

Chronic fluoxetine treatment decreased immobility in the FST in all sham animals, which is in agreement with previous studies in rats (Contreras *et al.* 2001, Vazquez-Palacios *et al.* 2004), and in BALB/c, but not C57BL/6 mice (Cryan *et al.* 2002, Dulawa *et al.* 2004). Here, we showed that iHi lesions prevented the antidepressant effects of chronic fluoxetine in the forced swim test. This is to our knowledge the first time that the iHi has been shown to play a role in the antidepressant effects of fluoxetine. Others have reported that chemogenetic activation of the vHi to medial prefrontal cortex pathway is required for the sustained antidepressant-like effects of a single dose of ketamine in this test in rats (Carreno *et al.* 2016). In contrast, we did not observe an effect of vHi lesions on fluoxetine antidepressant effects in the FST. The mechanisms underlying the effects of iHi lesion on antidepressant effects of chronic fluoxetine treatment are unknown. We then investigated whether there may be a possible relationship with FST-induced modulation of corticosterone. We found that, similarly to other studies in female mice (Machado *et al.* 2012), chronic fluoxetine significantly increased stress-induced increase in plasma corticosterone in most groups. However, this effect was not significant in both the iHi and vHi lesioned groups. Thus, changes in FST-induced increases in plasma corticosterone do not correlate with the behavioural phenotype observed in the FST. An alternative mechanism underlying the differential effects of hippocampal lesion on the

behavioural effects of fluoxetine may include role of adult hippocampal neurogenesis. Interestingly, a recent study that divided the hippocampus into 12 individual sub-regions found that the effects of fluoxetine on the proliferation of hippocampal stem cells occurred specifically in the area in which the iHi transits to the vHi, thus including both iHi and vHi (Zhou *et al.* 2016). This raised the possibility that fluoxetine increases neurogenesis in the iHi to produce its antidepressant effects in the FST. However, such effects of the iHi were not apparent in the NIH, a test known to be dependent on adult hippocampal neurogenesis. In addition, it has been reported that the antidepressant effects of fluoxetine in the forced swim test are neurogenesis-independent in C57BL/6 mice (David *et al.* 2009), although in this study neurogenesis was inhibited in the whole hippocampus, and the specific roles of discrete hippocampal sub-region were not assessed. Interestingly, we also speculate that the effects of iHi lesion could be due to a disconnection between the dHi and vHi and the disruption of the coherence between these other two sub-regions.

In summary, we have shown in the present study for what is to our knowledge the very first time that the iHi is involved in the antidepressant and anxiolytic effects of chronic fluoxetine. Moreover, we illuminate a novel role for the ventral third of the hippocampus in anxiety behaviour, while dHi and iHi seem not to be involved. For the very first time, we show that the vHi mediates the antidepressant effects of acute fluoxetine and the anxiolytic effects of chronic fluoxetine treatment. Here, we suggest that the iHi and vHi sub-regions of the hippocampus are promising targets for studies aimed at identifying novel biochemical or molecular targets for antidepressant and anxiolytic drug development and that such future studies should focus on these sub-regions, rather than in the hippocampus as a whole. Moreover,

future studies using a greater sample number should consider grouping the animals into responders and non-responders to fluoxetine, relating to the possible sub-regional functions.

CHAPTER 3

Sub-Regions of the Longitudinal Axis of the Hippocampus Mediate Specific Behavioural Responses to Chronic Psychosocial Stress

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Abstract

Accumulating evidence suggests that the hippocampus is functionally segregated along its longitudinal axis into a dorsal region (dHi) and a ventral region (vHi). Some studies now also suggest that the intermediate hippocampus (iHi) might also be functionally independent. However, the roles of these three sub-regions in the response to chronic stress, a risk factor for depression and anxiety disorders, has not yet been investigated. First, we examined if there was heterogeneity in the expression of stress-related genes along the longitudinal axis of the hippocampus. Next excitotoxic lesions of the dHi, iHi or vHi in male C57BL/6 mice were used to investigate the roles of these sub-regions in the behavioural (anxiety, anhedonia, depression) and corticosterone responses to chronic psychosocial stress. RNAseq analysis revealed sub-regional differences in the expression of stress-related genes. iHi lesions increased stress-induced social avoidance. Stress-induced increases in anxiety in the novelty-induced hypophagia and marble burying tests were prevented by all lesions however, only vHi lesions attenuated stress-induced anxiety in the open field. Stress-induced anhedonia was reduced in dHi- and vHi- but not iHi-lesioned mice. Only vHi lesions induced an antidepressant effect in the forced swim test and reduced agonistic behavioural responses during social defeat. Although, all hippocampal sub-regions are involved in the anxiogenic effects of chronic stress, here we describe what is to our knowledge for the first time a role for the iHi in stress-induced social avoidance and the vHi in antidepressant-like behaviour under chronic stress conditions.

3.1. Introduction

The hippocampus is known to play a role in spatial learning and memory (O'Keefe and Dostrovsky 1971, O'Keefe 1976, Morris *et al.* 1982, O'Keefe and Speakman 1987, Moser *et al.* 1995, O'Keefe *et al.* 1998, Moser *et al.* 2008), anxiety (Moser and Moser 1998, Bannerman *et al.* 2002, Bannerman *et al.* 2003, Bannerman *et al.* 2004, Mineur *et al.* 2006, Allsop *et al.* 2014, Jimenez *et al.* 2018) and the stress response (McEwen *et al.* 1992, Herman and Cullinan 1997, Buchanan *et al.* 2009, Ulrich-Lai and Herman 2009, Herman *et al.* 2016, Sousa 2016). Precisely how the hippocampus plays such diverse functions is not completely understood. However, accumulating evidence suggests that the hippocampus may be segregated along its longitudinal axis into dorsal and ventral regions in rodents and corresponding posterior and anterior regions in primates (Moser and Moser 1998, Fanselow and Dong 2010).

Anatomical studies in rats have shown that the afferent and efferent connections of the hippocampus change from dorsal to ventral poles (Swanson and Cowan 1977). In rodents, the vHi is more predominantly anatomically connected with brain regions involved with anxiety and response to stress (e.g. amygdala, hypothalamus and medial prefrontal cortex), while the dHi is predominantly anatomically connected to brain regions associated with spatial learning and memory such as the retrosplenial cortex (Fanselow and Dong 2010, Strange *et al.* 2014). Such anatomical evidence for potential functional segregation is further supported by electrophysiological studies which have reported that there is a greater density of place fields in the dHi than in the vHi, and that the place fields in the vHi are functional, but less selective for specific locations than those in the dHi (Jung *et al.* 1994). In addition, gene expression studies of the longitudinal axis of the

hippocampus have suggested that there is heterogeneity in gene expression between dHi and vHi (Leonardo *et al.* 2006, Thompson *et al.* 2008, Dong *et al.* 2009, Christensen *et al.* 2010, Strange *et al.* 2014). Moreover, lesion studies have reported a functional dissociation of the hippocampus, whereby lesions of the vHi, but not dHi attenuated anxiety behaviour in rats, tested in the light-dark box (Bannerman *et al.* 2003), elevated plus maze (Kjelstrup *et al.* 2002), hyponeophagia (Bannerman *et al.* 2002, Bannerman *et al.* 2003) and open field tests (Bannerman *et al.* 2003). On the other hand, dHi lesions but not vHi lesions impaired spatial learning and memory in rats (Moser *et al.* 1995, Bannerman *et al.* 2002, Pothuizen *et al.* 2004). Such preclinical studies are also supported by neuroimaging studies in humans (Maguire *et al.* 1997, Maguire *et al.* 2000, Burgess *et al.* 2002) and non-human primates (Colombo *et al.* 1998) and suggest that the posterior hippocampus (corresponding to the dorsal in rodents) is implicated in spatial learning and memory (Colombo *et al.* 1998, Maguire *et al.* 2000, Poppenk *et al.* 2013), whereas the anterior hippocampus (ventral in rodents) is recruited under stressful conditions (Weerda *et al.* 2010).

While there is now a growing body of evidence of a functional segregation of the hippocampus into the dHi and the vHi, genomic heterogeneity in rodents (Thompson *et al.* 2008, Strange *et al.* 2014), and neurofunctional differences in humans (Robinson *et al.* 2015) have been shown to occur along the longitudinal axis of the hippocampus. It is now also suggested that that the area between the dHi and the vHi, the intermediate hippocampus (iHi), may also be functionally independent although only a few studies have investigated the functional roles of the iHi and this has been in the context of place learning (Bast 2007, Bast *et al.* 2009, Strange *et al.* 2014).

Drug development for stress-related psychiatric disorders has been hampered by our lack of understanding of the processes in the brain that play a critical role in the pathophysiology of depression, anxiety, and the behavioural responses to stress. While much attention has been paid to the potential role of the hippocampus in this regard, relatively few studies have interrogated the potential contribution of specific regions along its longitudinal axis. Nevertheless, there is a growing body of evidence that the dHi and vHi are differentially regulated in response to stress. For example, it has been reported that the dHi and vHi have distinct epigenetic responses to acute stressors (Floriou-Servou *et al.* 2018) and it has been reported that chronic stress causes atrophy of apical dendrites in the CA1 and CA3 of the dHi, while stress increases the density of apical dendrites in the CA3 of the vHi (Pinto *et al.* 2015). In addition, several studies have reported that the impact of stress on adult hippocampal neurogenesis is frequently more apparent in the vHi than the dHi (Tanti and Belzung 2013, O'Leary and Cryan 2014, Levone *et al.* 2015). Moreover, we recently reported that neurons differentiated from neural progenitor cells derived from the vHi are more sensitive to corticosterone-induced reductions in neuronal differentiation and maturation than those derived from the dHi (Levone et al., in preparation; Chapter 4). Taken together, it is clear that stress seems to differentially impact the dHi and vHi however to date no studies have investigated the potential differential roles of the dHi, iHi and vHi in the behavioural responses to chronic stress. Thus, in the present study, we first investigated if there is a heterogeneity in the expression of genes associated with stress, anxiety, depressive-like behaviour or antidepressant action along the longitudinal axis of the hippocampus of mice. We then used ibotenic acid-induced lesions of these hippocampal sub-regions to determine their functional roles in

stress-induced changes in anxiety (novelty-induced hypophagia, marble burying and open field tests), anhedonia and depressive-like behaviour (saccharin preference and female urine sniffing tests) and antidepressant-like behaviour (forced swim test) as well as their potential roles in stress-induced corticosterone release.

3.2. Methods and Materials

3.2.1 Gene expression analysis of "Hipposeq" database

A RNAseq database from the Janelia Research Campus (http://hipposeq.janelia.org) (Cembrowski *et al.* 2016) was used to investigate whether any stress-related genes are differentially expressed along the longitudinal axis of the dentate gyrus and CA1 regions of the mice hippocampi. For the dentate gyrus, data from the dorsal and ventral regions were available and for the CA1, dorsal, intermediate and ventral regions were available. To generate this database, the authors had used transgenic mice to fluorescently label the populations of hippocampal principal cells of interest, euthanised the animals at P25-P32 and dissected the cells from brain slices. Three samples were obtained per dataset and the RNA was isolated, cDNA amplified and sequenced showing the output as FPKM (fragments per kilobase of exon per million reads mapped). The false discovery rate cutoff used was of 5%, for more details, please see Cembrowski et al., 2016 (Cembrowski *et al.* 2016).

We selected 175 genes for analysis based on their previous links to stress and emotional behaviour or their regulation by antidepressants. These genes included neurotransmitter receptors and transporters, growth factors, and genes related to neurogenesis (listed in Table 3.1).

Table 3.1: Mouse gene expression analysed using the database "*Hipposeq*"

Group	All genes/proteins analysed (total: 175)	Genes/ proteins changed in vDG vs dDG	Genes/ proteins changed in iCA1 vs dCA1	Genes/ proteins changed in vCA1 vs dCA1	Genes/ proteins changed in vCA1 vs iCA1
Neurotransmission	All receptors and neurotransmitter transporters of the following systems of neurotransmission: glutamatergic (27), GABAergic (14), serotonergic (17), adrenergic (9), dopaminergic (6), Cholinergic (21) and Purinergic (4)	Purinergic: Adora1 ↓ Adrenergic: Adra1d ↓ Cholinergic: Chrm1 ↓ Chrna3 ↑ Chrna4 ↑ Chrna6 ↑ Chrnb1 ↓ DA: Drd1a ↓ Slc6a3 ↑ GABAergic: Gabra1 ↑ Gabra2 ↑ Slc6a1 ↑ Slc6a11 ↑ Glu: Grin1 ↓ Grin3a ↑ Grm2 ↓ Grm3 ↓ Slc1a4 ↑ 5-HT: Htr1a ↑ Htr5b ↑ Htr6 ↓	Glu: Grin3a ↑ Grm3 ↑ 5-HT: Htr1a ↓	Cholinergic: Chrm3 ↓ Chrna5 ↓ GABAergic: Gabra1 ↓ Gabra3 ↑ Gabra4 ↓ Gabrb1 ↑ Glu: Grin1 ↓ Grin2a ↓ Grin3a ↑ Gria4 ↑ 5-HT: Htr1a ↓ Htr2c ↑ Htr5a ↑	GABAergic: Gabra1 ↓ Glu: Grin2a ↓ Grin3a ↑ 5-HT: Htr2c ↑
GR and MR function	Nr3c1, Nr3c2, Hdac2, Nfkb1, Nfkb2, Fkbp4, Fkbp5, Hsp90, Bag1, Stat5, Sgk1, CEBPα, CEBPß	Nr3c2 ↓ Sgk1 ↓	None	Nr3c2 ↓	None
Growth factors	FGF1, FGF2, EGF, FGFr1, FGFr2, FGFr3, FGFr4, EGFr, VGF, Megf6, Tgfb1,	FGF1 ↑ FGFr1 ↓ FGFr2 ↑	Tgfb2 ↑	FGF2 ↓ FGFr3 ↑ Tgfb2 ↑	Bdnf ↓

	Tgfb2, Tgfbr1, Igf1, Igf2, Bdnf, Gdnf	VGF ↓ Megf6 ↑ Tgfbr1 ↑ Tgfb2 ↓		Bdnf ↓	
Neurogenesis and plasticity	Neural stem cells: ALDOC, APOE, ID4, Hopx, Sox2, Slc1a3, GFAP, Nestin Neural progenitors: Eomes, DCX, Neurod1, Ccnd2 Oligodendrocyte progenitors: Olig1, Olig2, Sox10, Pdgfra, Cspg4 Committed oligodendrocyte precursors: Bmp4, Fyn, Gpr17 Mature oligodendrocytes: Plp1, Mbp, Mal, Mog Microglia: Csf1r, Cx3cr1 Astrocytes: S100b, Fzd2 Cell quiescence: Clu, Apoe, Aldoc, Id3 Cell proliferation: Ccnd2, Mki67, Pcna, Mcm2 Neuronal differentiation: Sox11, Neurog2, Neurod1, Neurod2 Others: Ncam1, Ncam2, Notch1, Notch2	Neural stem cells: ID4 ↑ Neural progenitors: Ccnd2 ↑ Neuronal differentiation: Neurod2 ↓ Others: Ncam1 ↓ Ncam2 ↑ Notch1 ↓ Notch2 ↑	None	Ncam1 ↑ Ncam2 ↑	Ncam2 ↑
Stress-related genes	Hhip, Stx1a, Il1b, Il6, Rgs2	Rgs2 ↓	None	None	None

3.2.2. Experimental design of behavioural studies

Ibotenic-induced lesions of the dHi, iHi and vHi of the mouse hippocampus were performed (Fig 3.1A), while some animals underwent a sham surgery. Seven days

following surgery, some sham animals (sham-stress) and all lesioned animals underwent a daily psychosocial stress paradigm consisting of an unpredictable schedule (Fig 3.1B) of intermittent social defeat/overcrowding for 19 days (Reber *et al.* 2006, Finger *et al.* 2011) prior to the start of behavioural testing and corticosterone measurements (Fig 3.1B). Daily social defeat stress continued throughout the behavioural testing period unless the animal was undergoing a behavioural test on that particular day. Control mice (sham-no stress group) were left undisturbed in their homecage, being handled only three times per week either for cage changing or for weighing. The experimental groups, sample number (after surgery corrections), surgery success rate and number of misplaced (excluded) animals were: (1) sham-no stress, n = 9 (100% success, 0 excluded); (2) sham-stress, n = 10 (100% success, 0 excluded); (3) dHi lesion-stress, n = 9 (75% success, 3 excluded); (4) iHi lesion-stress, n = 8 (80% success, 2 excluded) and (5) vHi lesion-stress, n = 8 (50% success, 8 excluded).

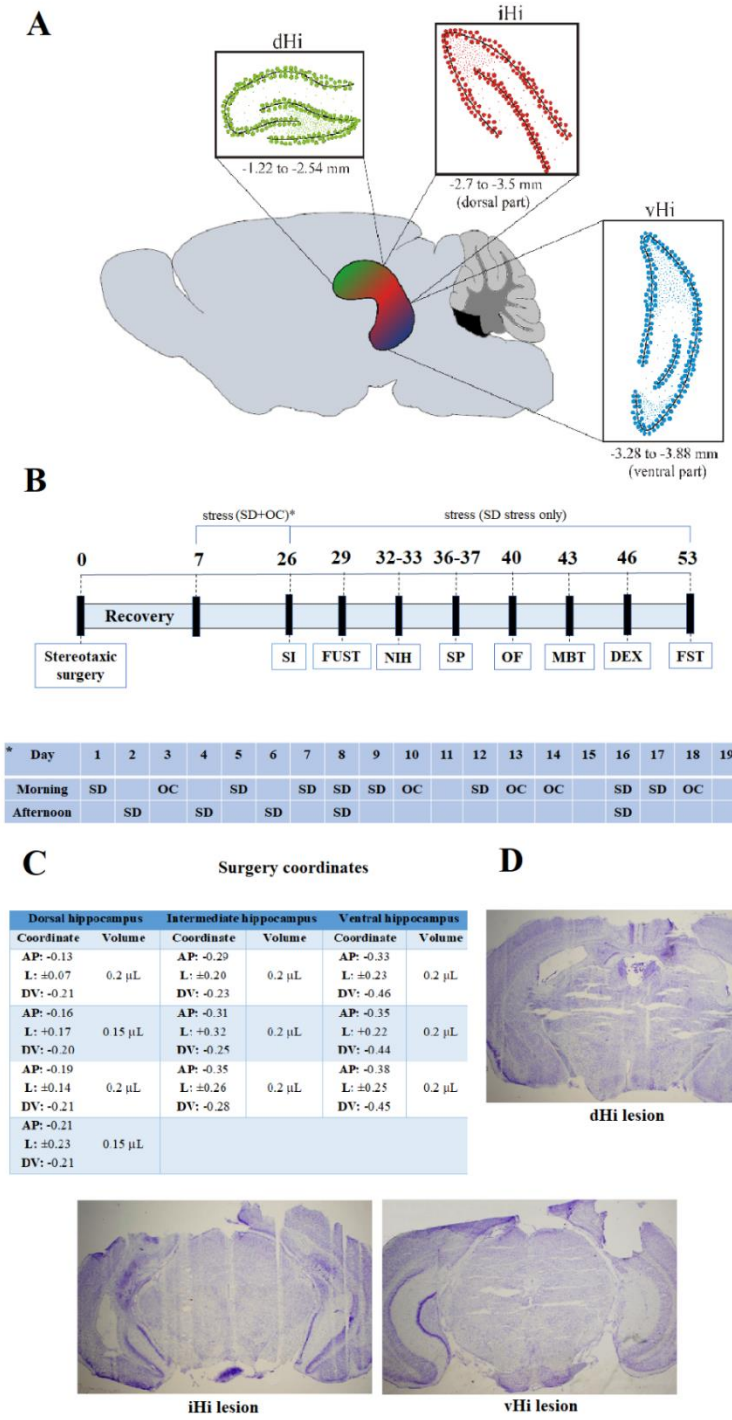
3.2.3 Animals

Male C57BL/6 mice (Envigo, UK) aged 8-weeks old, were housed in groups of 3-4 at arrival in a temperature controlled (21±2°C) room and allowed to acclimatise to the holding room for 7-days prior to surgery, after which they were singly housed. Laboratory chow and water were provided *ad libitum* on a 12/12 h light/dark cycle (lights on at 7:30 am). All procedures were conducted with approval from the Animal Experimentation Ethics Committee (AEEC) at University College Cork, under Individual Authorisations and a Project Authorisation approved the Health Products Regulatory Authority (HPRA) Ireland and in accordance with the recommendations of the European Parliament and the Council of the EU Directive (2010/63/EU).

3.2.4. Stereotaxic brain surgery and excitotoxic lesion of the dorsal, intermediate of ventral hippocampus

Mice (20-26g) were anaesthetised with isoflurane (5% induction, 1.7-2.5% maintenance) and received analgesia (5mg/kg, s.c., Carprofen, Norbrook,) prior to being placed in a stereotaxic frame. As shown in Fig 3.1C, small volumes (0.15-0.2 µl) of ibotenic acid (10 mg/ml, I2765, Sigma-Aldrich) diluted in PBS (pH 7.4) were injected bilaterally into either the dHi, iHi or vHi using coordinates from the Paxinos and Franklin's atlas for mouse brain (Paxinos 2012). Sham surgeries involved opening holes at the relevant areas of the skull but not injecting anything. Holes in the skull were covered with Bone-Wax (SMI, Z046) and the skin was sutured (W329, Mersilk Suture 4-0). An analgesic (20 µg/ml, Carpofen, Norbrook,) was added to the drinking water for 2 days post-surgery. All animals were singly housed after surgery and allowed to recover for 1 week prior to the initiation of the stress paradigm.

At the end of the experiment, mice were deeply anaesthetised with pentobarbital (250mg/kg, i.p., Euthanal, Merial,) and transcardially perfused with PBS followed by 4% PFA. Brains were removed, post-fixed in 4% PFA overnight, cryoprotected in 30% sucrose and then frozen at -80°C. Brains were sectioned coronally (at 35 µm) through the entire rostro-caudal axis of the hippocampus and collected onto gelatinised slides. Sections were stained with cresyl violet (C5042, 0.1%, Sigma-Aldrich). Lesions were identified by light microscopy examination by an experimenter blind to the experimental treatment groups. Only animals with confirmed lesions in the specific sub-region were included (see Fig 3.1D).



* Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Morning	SD		OC		SD		SD	SD	SD	OC		SD	OC	OC		SD	SD	OC	
Afternoon		SD		SD		SD		SD								SD			

C

Surgery coordinates

Dorsal hippocampus		Intermediate hippocampus		Ventral hippocampus	
Coordinate	Volume	Coordinate	Volume	Coordinate	Volume
AP: -0.13 L: ±0.07 DV: -0.21	0.2 µL	AP: -0.29 L: ±0.20 DV: -0.23	0.2 µL	AP: -0.33 L: ±0.23 DV: -0.46	0.2 µL
AP: -0.16 L: +0.17 DV: -0.20	0.15 µL	AP: -0.31 L: +0.32 DV: -0.25	0.2 µL	AP: -0.35 L: +0.22 DV: -0.44	0.2 µL
AP: -0.19 L: ±0.14 DV: -0.21	0.2 µL	AP: -0.35 L: ±0.26 DV: -0.28	0.2 µL	AP: -0.38 L: ±0.25 DV: -0.45	0.2 µL
AP: -0.21 L: ±0.23 DV: -0.21	0.15 µL				

Figure 3.1*: Definition of the hippocampal sub-regions: dorsal (dHi), intermediate (iHi) and ventral (vHi). A, a schematic image of the dHi, iHi and vHi, with the coordinates according to the Paxinos atlas for mice brain (in relation to bregma). B, timeline of the experiment, including the surgery, psychosocial stress and behavioural tests. C, surgery coordinates and volume of ibotenic acid injected (bilaterally). D, images of cresyl violet-stained slices showing the dHi, iHi and vHi lesions. Abbreviations: AP: anteroposterior, DEX: dexamethasone suppression test, DV: dorsoventral, FST: forced swim test, FUST: female urine sniffing test, L: lateral, MBT: marble burying test, NIH: novelty-induced hypophagia, OC: overcrowding, OF: open field, SD: social defeat, SI: social interaction test, SP: saccharin preference test.*

3.2.5. Chronic psychosocial stress paradigm

The chronic psychosocial stress paradigm consisted of 19 days of alternating between social defeat and overcrowding (Fig 3.1B). In the social defeat sessions, male C57BL/6 mice were placed into the homecage of an aggressive male CD1 mouse and their interaction was allowed until the C57BL/6 mouse adopted a defeat posture. Upon the first defeat within each session, mice were immediately physically separated from each other by a perforated plexiglass wall (that allows visual, olfactory and auditory contact) for two hours, after which the separator was removed, and another social defeat session took place until the C57BL/6 mouse assumed a defeat posture. Animals were then separated and returned to their homecage. For the overcrowding sessions, C57BL/6 animals (n=9-10) were housed in one standard holding cage for either 24 or 48 h (Fig 3.1B). Control animals were singly housed and handled three times per week.

3.2.6. Social Interaction Test (SI)

Twenty-four hours after the last stressor (experimental day 26), social avoidance behaviour was assessed using the social interaction test. Previous studies have shown that chronic psychosocial stress paradigms that use social defeat stress decrease social interaction (Berton *et al.* 2006, Finger *et al.* 2011, Golden *et al.* 2011, O'Leary *et al.* 2014). Mice were placed into a plastic box (31×39 cm^3) containing an empty wire mesh cage (9.5×7.5 cm^2) and allowed to explore for 2.5 min. The test was conducted under red light to reduce interference of general anxiety-like behaviour with social interaction behaviour. The mouse was then removed and transferred back to its homecage for 1 minute, while an unfamiliar CD1 mouse (social target) was placed into the wire mesh cage. The test mouse was then returned to the social interaction box and allowed to freely explore for another

2.5 min. At the end of the test, both mice were transferred back to their respective homecages and the arena and mesh cage were cleaned with 70% ethanol. All data were analysed using the software Ethovision 11.5. Results are expressed as the interaction ratio (time spent in the interaction zone) in the presence of a social target divided by time in spent in the interaction zone in the absence of a social target). Corners ratio was also assessed as a measure of social avoidance (time spent in the corners of the box in the presence of a social target divided by time spent in the corners in the absence of a social target), as socially stressed animals tend to spend more time in the corners, the farthest region of the social target. The distance travelled in the arena in the absence of the social target was also analysed as a measure of locomotor activity.

3.2.7. Tests of anxiety.

3.2.7.1. Novelty-induced hypophagia (NIH)

The novelty-induced hypophagia test is a test of anxiety sensitive to chronic antidepressant treatment (Dulawa and Hen 2005, O'Leary *et al.* 2013). In this test, mice were trained to drink a diluted solution of sweetened condensed milk (3:1, water to milk) from a 10 ml serological pipette through the lid of their cage for 30 minutes per day for 3 days. On the fourth day, the latency to drink the milk in their homecage was measured. On the fifth day, mice were placed in a novel brightly-lit cage (1200 lux) without bedding, and their latency to drink the milk was recorded. Placement in the novel cage increases the latency to drink the milk which is taken as an index of anxiety. The data are presented as latency difference (i.e. the latency to drink in the novel cage minus the latency to drink in the home cage).

3.2.7.2. Marble Burying Test (MBT)

The marble burying test is a test used to measure phenotypes related to anxiety and obsessive compulsive-like behaviour (Deacon 2006). A cage (35 x 28 cm) was filled with clean corncob bedding overlaid with 20 glass marbles equidistant from each other in a 4×5 arrangement (Savignac *et al.* 2014). Mice were placed in the cage for 30 minutes and the number of marbles that are more than 2/3 buried were counted. An increase in the number of marbles buried is considered to be a proactive response to an anxiogenic stimulus and has also been described as a compulsive behaviour to relieve anxiety (Deacon 2006).

3.2.7.3. Open field test (OF)

The open field test consists of a rectangle box (perspex sides and base: 40 x 33 cm^2) in a dimly lit room (4 lux). Mice were placed in the centre of the open field and allowed to explore the apparatus for 10 minutes. Locomotor activity was recorded by a camera placed over the open field and distance travelled was automatically analysed using Ethovision 11.5 software. The time spent in the centre quarter (10 x 8.25 cm^2) of the chamber was also measured as an index of anxiety behaviour.

3.2.8. Tests of anhedonia

3.2.8.1. Female Urine Sniffing Test (FUST)

The female urine sniffing test assesses anhedonia (a reduction in the ability to experience pleasure) which is a core feature of depression and has been shown to be induced by chronic stress (Malkesman *et al.* 2010, Finger *et al.* 2011, O'Leary *et al.* 2014). This test takes advantage of the fact that rodent males are attracted to pheromonal odours from the opposite sex and thus is used in male mice as an index of sexual interest. Before the test, mice were transferred to a dimly-lit (4 lux) room and habituated to the presence of a cotton tip applicator in their home cage for 1

hour. Following habituation, mice were immediately presented with a cotton tip applicator with 60 µL of sterile water for a period of 3 minutes and the time spent sniffing the water was measured. 45 min later, a cotton tip with 60 µL of fresh urine from female mice in oestrus was presented to the mice for 3 minutes and the time spent sniffing the urine was measured. Data are presented as the percentage of time spent sniffing urine over the total time spent sniffing urine plus water.

3.2.8.2. Saccharin preference test (SPT)

The saccharin preference test (Harkin *et al.* 2002) was used as another test for anhedonia. Mice were given access to both a water bottle and a saccharin solution (0.1%) bottle for a total period of 48 h. Saccharin and water intake were measured every 12 hours (at 7:30 a.m. and 7:30 p.m. each day). Every 12 h, the position of the bottles was reversed to avoid the development of preference for drinking from a particular side of the cage. Mice normally show a preference to consume the saccharin over water although chronic stress decreases this preference for saccharin (Harkin *et al.* 2002). The data are expressed as the cumulative percentage preference for saccharin over water [(saccharin consumption volume/water consumption volume) * 100].

3.2.9. Test of antidepressant-like behaviour: The Forced Swim Test (FST)

The forced swim test is the most widely used experimental paradigm to assess antidepressant drug-like activity (Porsolt *et al.* 1977, Cryan and Mombereau 2004). In this test, mice were allowed to swim for 6 min in a glass cylinder (24 × 21 cm) filled with water (23–25 °C) to a depth of 17 cm. The test was video-recorded and the time spent immobile during the last 4 min of the 6-min test was measured.

Antidepressant drugs decrease immobility in this test (Porsolt *et al.* 1977, Cryan and Mombereau 2004).

3.2.10. Plasma corticosterone measurements and the dexamethasone test

Blood was withdrawn from the tail vein to measure changes in concentrations of the stress hormone corticosterone before and after the acute stress of the forced swim test (FST) or following a dexamethasone suppression challenge (DEX).

Blood was withdrawn 30 min before and again 30 and 120 min after the beginning of the FST. For the dexamethasone suppression test, mice were injected with dexamethasone (DEX, Sigma-Aldrich) and then two hours later, mice were acutely stressed by placement in a brightly lit cage with no bedding (1200 lux) for 5 min. Blood was withdrawn 15 min after the stressor (2h 15min after DEX) and then again 1h 45min after the stressor (i.e. after four hours after DEX). Corticosterone concentrations of the blood sample taken 30 mins prior to the FST was used as time point 0 in both the FST and DEX datasets.

Blood was centrifuged at 4000g for 15 min at 4°C. Plasma was removed and kept at -80°C until analysis. Plasma corticosterone was measured using an ELISA kit (ADI-901-097, Enzo) and light absorbance was read with a multi-mode plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm. Samples were analysed in duplicate within a single assay and the minimum threshold of detection was less than 32 pg/mL. The limit for the coefficient of variation used was 20% and the final concentrations are expressed in ng/mL.

3.2.10. Statistical analysis

Data is shown as mean + S.E.M. Statistical analysis was performed using the software IBM SPSS Statistics 23. Data were analysed using One-way ANOVA and when appropriate, followed by Fishers LSD post hoc test for group-wise comparisons. Two-way repeated measures ANOVA was used to compare different timepoints of corticosterone concentrations after the FST or dexamethasone challenge. For all comparisons, $p<0.05$ was the criterion used for statistical significance.

3.3. Results

3.3.1. Stress-related genes are differentially expressed along the longitudinal axis of the hippocampus

3.3.1.1. Genes related to neurotransmitter systems, GR and MR function, growth factors and neurogenesis/plasticity were differentially expressed in the ventral dentate gyrus (vDG) versus the dorsal dentate gyrus (dDG)

Of the 175 genes analysed, 38 (21.71%) were differentially expressed in the ventral dentate gyrus (vDG) versus the dorsal dentate gyrus (dDG), as shown in Fig 3.2A and Table 3.1. These included genes related to various neurotransmitter systems. Of those genes, the following were expressed at a lower level in the vDG when compared to the dDG: adenosine receptor A1, adrenergic receptor α1d, muscarinic receptor 1, nicotinic receptor β1, dopamine receptor D1, NMDA receptor 1, metabotropic glutamatergic receptors 2 and 3, serotonergic receptor 6. On the other hand, the following genes were expressed at a higher level in the vDG when compared to the dDG: nicotinic receptors α3, α4 and α6, dopamine transporter, GABA A receptor subunits α1 and α2, GABA transporters 1 and 3, NMDA receptor 3A, glutamate transporter, serotonergic receptors 1a and 5b.

Also, several genes related to glucocorticoid receptor (GR) and mineralocorticoid (MR) receptor function were differentially expressed in the vDG versus dDG. Of those, the mineralocorticoid receptor and serum/glucocorticoid regulated kinase 1 were expressed at a lower level in the vDG when compared to the dDG.

Also, genes related to growth factors were differentially expressed in the vDG versus dDG. Of those, the following were expressed at a lower level in the vDG when compared to the dDG: fibroblast growth factor receptor 1, nerve growth factor and transforming growth factor ß2. On the other hand, the following genes were expressed at a higher level in the vDG when compared to the dDG: fibroblast growth factor 1, fibroblast growth factor receptor 2, multiple EFG-like domains 6 and transforming growth factor ß receptor 1.

Also, genes related to hippocampal neurogenesis were differentially expressed in the vDG versus dDG. Of those, the following were expressed at a lower level in the vDG when compared to the dDG: neuronal differentiation 2, neural cell adhesion molecule 1 and Notch1. On the other hand, the following genes were expressed at a higher level in the vDG when compared to the dDG: inhibitor of DNA binding 4, connective tissue growth factor 2, neural cell adhesion molecule 2 and Notch2. Finally, a gene previously related to chronic stress was differentially expressed in the vDG versus dDG. The Rgs2 gene was expressed at a lower level in the vDG when compared to the dDG.

3.3.1.2. Genes related to neurotransmitter systems, GR and MR function, growth factors and plasticity were differentially expressed in the dorsal CA1 (dCA1) versus intermediate CA1 (iCA1) versus ventral CA1 (vCA1)

Of the 175 genes analysed, 20 (11.43%) were differentially expressed in the vCA1 versus dCA1, as shown in Fig 3.2B and Table 3.1. These included genes related to various neurotransmitters system. Of those genes, the following were expressed at a lower level in the vCA1 when compared to the dCA1: muscarinic receptor 3, nicotinic receptor α5, GABA A receptor subunit α1 and 4, NMDA receptor 1 and

2a and serotonergic receptor 1a. On the other hand, some genes were expressed at a higher level in the vCA1 when compared to the dCA1including: GABA A receptor subunit α3, GABA A receptor subunit β1, NMDA receptor 3a, AMPA receptor 4 and serotonergic receptors 2c and 5a. Also, a gene related to GR and MR function was differentially expressed in the vCA1 versus dCA1. The mineralocorticoid receptor was expressed at a lower level in the vCA1 when compared to the dCA1.

Some genes related to growth factors were differentially expressed in the vCA1 when compared to dCA1. Of those gene, the fibroblast growth factor 2 and BDNF were expressed at a lower level and the fibroblast growth factor receptor 3 and transforming growth factor ß2 were expressed at a higher level in the vCA1 when compared to dCA1. Finally, genes related to plasticity were differentially expressed in the vCA1 when compared to dCA1. Of those genes, neural cell adhesion molecules 1 and 2 were expressed at a higher level in the vCA1 when compared to dCA1.

When we compared the dCA1 with the iCA1, the proportion of genes differentially expressed was much smaller than the dCA1 vs vCA1 comparisons. Of the 175 genes analysed, 4 (2.29%) were differentially expressed in the dCA1 versus the iCA1, as shown in Fig 3.2B and Table 3.1. These included genes related to various neurotransmitters system. Of those genes, the serotonergic receptor 1a was expressed at a lower level in the iCA1 when compared to the dCA1. On the other hand, some genes were expressed at a higher level in the iCA1 when compared to the dCA1, including the NMDA receptor 3A and metabotropic glutamatergic receptor 3. Genes related to GR and MR function were also differentially expressed

and, in this case, transforming growth factor β2 was expressed at a higher level in the iCA1 in comparison to dCA1.

Similar to the dCA1 vs iCA1 comparisons, a much smaller proportion of genes were differentially expressed in the vCA1 vs iCA1 than what we observed in the dCA1 vs vCA1 comparisons. Of the 175 genes analysed, 6 (3.43%) were differentially expressed in the vCA1 versus iCA1, as shown in Fig 3.2B and Table 3.1. These included genes related to various neurotransmitters system. Of those genes, GABA A receptor subunit α1 and NMDA receptor 2a were expressed at a lower level, while NMDA receptor 3a and serotonergic receptor 2c were expressed at a higher level in the vCA1 when compared to iCA1. Also, the BDNF gene was expressed at a lower level and the neural cell adhesion molecule 2 was expressed at a higher level in the vCA1 in comparison to iCA1.

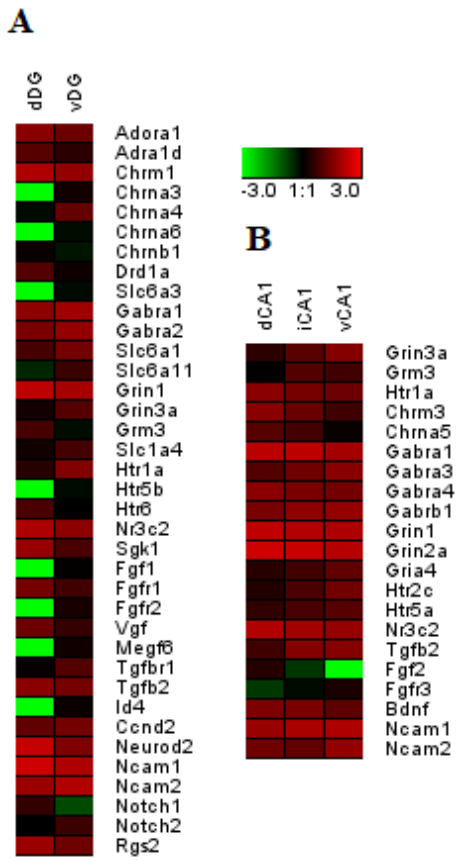


Figure 3.2: *Genes which are differentially expressed in mice, according to the "Hipposeq database". A, between dorsal DG (dDG) and ventral DG (vDG), B, between dorsal CA1 (dCA1), intermediate CA1 (iCA1) and ventral CA1 (vCA1). Gene expression heatmaps are logged, green represents a lower expression while red, a higher expression. Genes names: Adora1: adenosine A1 receptor; Adra1d: adrenergic receptor α1d; Bdnf: brain derived neurotrophic factor; Ccnd2: cyclin D2; Chrm1: muscarinic receptor 1; Chrm3: muscarinic receptor 3; Chrna3: nicotinic receptor α3; Chrna4: nicotinic receptor α4; Chrna5: nicotinic receptor α5; Chrna6: nicotinic receptor α6; Chrnb1: nicotinic receptor ß3; Drd1a: dopaminergic receptor 1a; Fgf1: fibroblast growth factor 1; Fgf2: fibroblast growth factor 2; Fgfr1: fibroblast growth factor receptor 1; Fgfr2: fibroblast growth factor receptor 2; Fgfr3: fibroblast growth factor receptor 3; Gabra1: GABAergic receptor A subunit α1; Gabra2: GABAergic receptor A subunit α2; Gabra3: GABAergic receptor A subunit α3; Gabra4: GABAergic receptor A subunit α4; Gabrb1: GABAergic receptor A subunit ß1; Gria4: AMPA receptor subunit 4; Grin1: NMDA receptor subunit 1; Grin2a: NMDA receptor subunit 2A; Grin3a: NMDA receptor subunit 3A; Grm3: glutamate metabotropic receptor 3; Htr1a: serotonergic receptor 1a; Htr2c: serotonergic receptor 2c; Htr5a: serotonergic receptor 5a; Htr5b: serotonergic receptor 5b; Htr6: serotonergic receptor 6; Id4: inhibitor of DNA binding 4; Megf: multiple epidermal growth factor; Ncam1: neural cell adhesion molecule 1; Ncam2: neural cell adhesion molecule 2; Neurod2: neuronal differentiation 2; Nr3c2: mineralocorticoid receptor; Rgs2: regulator of G protein signalling 2; Sgk1: serum/glucocorticoid regulated kinase 1; Slc1a4: glutamate transporter; Slc6a1: GABA transporter 1; Slc6a11: GABA transporter 3; Slc6a3: dopamine transporter; Tgfb2: transforming growth factor ß2; Tgfbr1: transforming growth factor receptor ß1; Vgf: nerve growth factor inducible.*

3.3.2. Lesions of the iHi exacerbate stress-induced social avoidance, while vHi lesions increase the latency to assume a defeated posture during the first exposure to social defeat

The effects of sub-region-specific lesions on stress-induced reductions in social interaction are shown in Fig 3.3A. One-way ANOVA revealed an overall effect on the social interaction ratio ($F_{4,39}$=12.24, p<0.001). Specifically, post hoc analysis revealed that stress decreased the social interaction ratio in the sham group and in all lesion groups when compared to the non-stressed sham group (all p<0.001). Interestingly however, the stress-induced reductions in the social interaction ratio were exacerbated in the iHi-lesioned (p=0.021) mice when compared with the stressed sham group (Fig 3.3A). Moreover, one-way ANOVA also revealed an overall effect on the corners ratio ($F_{4,39}$=6.83, p<0.001; Fig 3.3B) whereby stress increased the corner ratio in iHi-lesioned mice only when compared with both the non-stressed and stressed sham groups (both p<0.001, Fisher's LSD). Similarly, the corners ratio was also higher in the iHi lesioned group when compared with the dHi (p=0.003) and vHi lesioned groups (p=0.006). Locomotor activity measured in the absence of the social target was not different between treatment groups ($F_{4,39}$=0.9, p=0.48; Fig 3.3C).

As described previously (Krishnan *et al.* 2007), we also classified mice as susceptible or resilient to stress, based on their interaction ratio. Animals were classified as resilient when they had an interaction ratio >1, and as susceptible if the interaction ratio was <1. In the present study, the proportion of animals susceptible to stress was highest in the iHi-lesion-stress and vHi-lesion-stress groups (100% were susceptible), followed by the dHi-lesion-stress group (88.9% were susceptible), followed by the sham-stress group (70% were susceptible).

We also analysed the latency to adopt a defeat posture during the first social defeat of each day during all the social defeat sessions included in the 19-days protocol previous to the start of the behavioural tests (Fig 3.1). We calculated the average latency for the first social defeat, for the second (which took place 2 h after the first) and an average of both. The average latency for the defeated posture on the first social defeat is shown in Fig 3.3D. One-way ANOVA revealed a main effect ($F_{3,31}$=4.11, p=0.015). Post hoc analysis revealed that vHi-lesioned animals took a longer time to assume a defeated posture in relation to sham-no stress group (p=0.031).

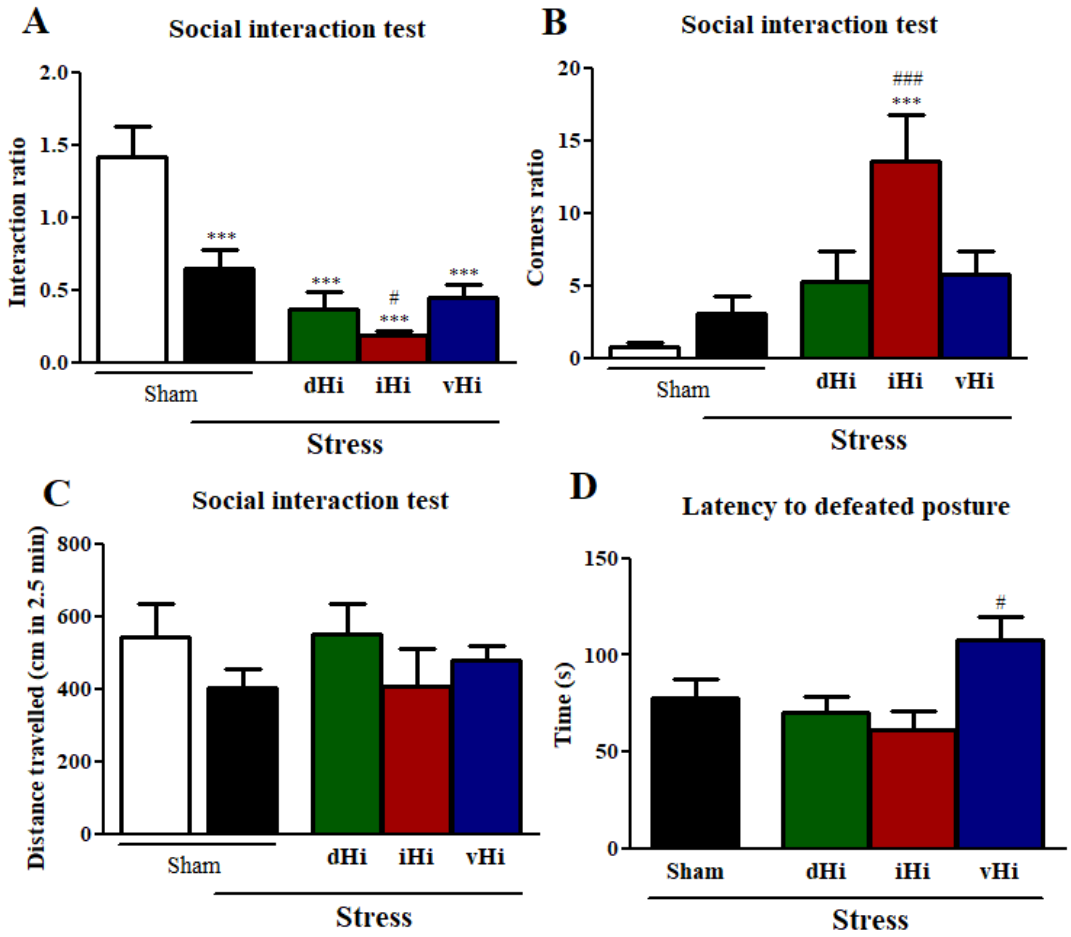


***Figure 3.3**: Social interaction test and latency to assume defeated posture. A, stress induces social avoidance, reducing interaction ratio. B, iHi lesion increases social avoidance, increasing corners ratio. C, stress and/or hippocampal lesions did not alter exploration of the social interaction chamber. D, average latency to assume a defeated posture in the first social defeat session is increased in vHi-lesioned animals. *p<0.05, ***p<0.001, in relation to sham-no stress group; #p<0.05, ###p<0.001, in relation to sham-stress group, according to Fishers LSD post-hoc test.*

3.3.3. All hippocampal sub-regions mediate stress-induced anxiety in the novelty-induced hypophagia test and the marble burying test

The effects of sub-region-specific lesions on stress-induced anxiety in the novelty-induced hypophagia test (NIH) are shown in Fig 3.4A. One-way ANOVA revealed an overall effect on the difference in latency to drink the milk ($F_{4,39}$=13.01, p<0.001). Post hoc comparisons revealed that chronic psychosocial stress increased the latency difference in sham animals (p<0.001) but that this effect was completely

prevented in the dHi- iHi- and vHi-lesioned mice (all $p<0.001$ vs sham-stress group).

Similarly, one-way ANOVA revealed an overall effect in the marble burying test ($F_{4,39}=7.74$, $p<0.001$; Fig 3.4B). Post hoc comparisons revealed that chronic psychosocial stress increased the number of marbles buried in sham animals ($p=0.002$) but that this effect was completely prevented in the dHi- ($p<0.001$), iHi- ($p<0.001$) and vHi-lesioned groups ($p=0.005$).

3.3.4. Only lesions of the vHi attenuate stress-induced anxiety in the open field, and this effect is not due to alterations in locomotor activity

The effects of sub-region-specific lesions on stress-induced decreases on the time spent in the centre of the open field are shown in Fig 3.4C. One-way ANOVA revealed an overall effect on the time spent in the centre of the open field arena ($F_{4,39}=5.26$, $p=0.002$). Specifically, post hoc analysis revealed that chronic psychosocial stress in sham animals increased anxiety as indicated by decreased time spent in the centre of the open field when compared to the non-stressed sham group ($p=0.01$). Chronic psychosocial stress also decreased time spent in the centre of the open field in dHi- and iHi-lesioned mice when compared to the non-stressed sham control group ($p<0.001$; $p=0.001$, respectively). Interestingly, this effect of stress was not observed in vHi-lesioned mice ($p=0.135$ vs. non-stressed sham group).

The absence of significant stress-induced anxiety in vHi-lesioned mice was not due to alterations in general locomotor activity in the open field (Fig 3.4D). While one-way ANOVA revealed an overall effect on the distance travelled in the open field

($F_{4,39}$=6.04, p=0.001). Post hoc analysis revealed that this effect was driven by increased locomotor activity in dHi- and iHi-lesioned groups [(p<0.001 and p=0.003 respectively, vs. sham non-stressed group); (p=0.002 and p=0.016, respectively vs. stressed sham group); (p=0.003 and p = 0.018, respectively vs. vHi-lesioned group)], while vHi lesions had no effect on locomotor activity (p=0.49 vs non-stressed sham; p=0.95 vs stressed sham group).

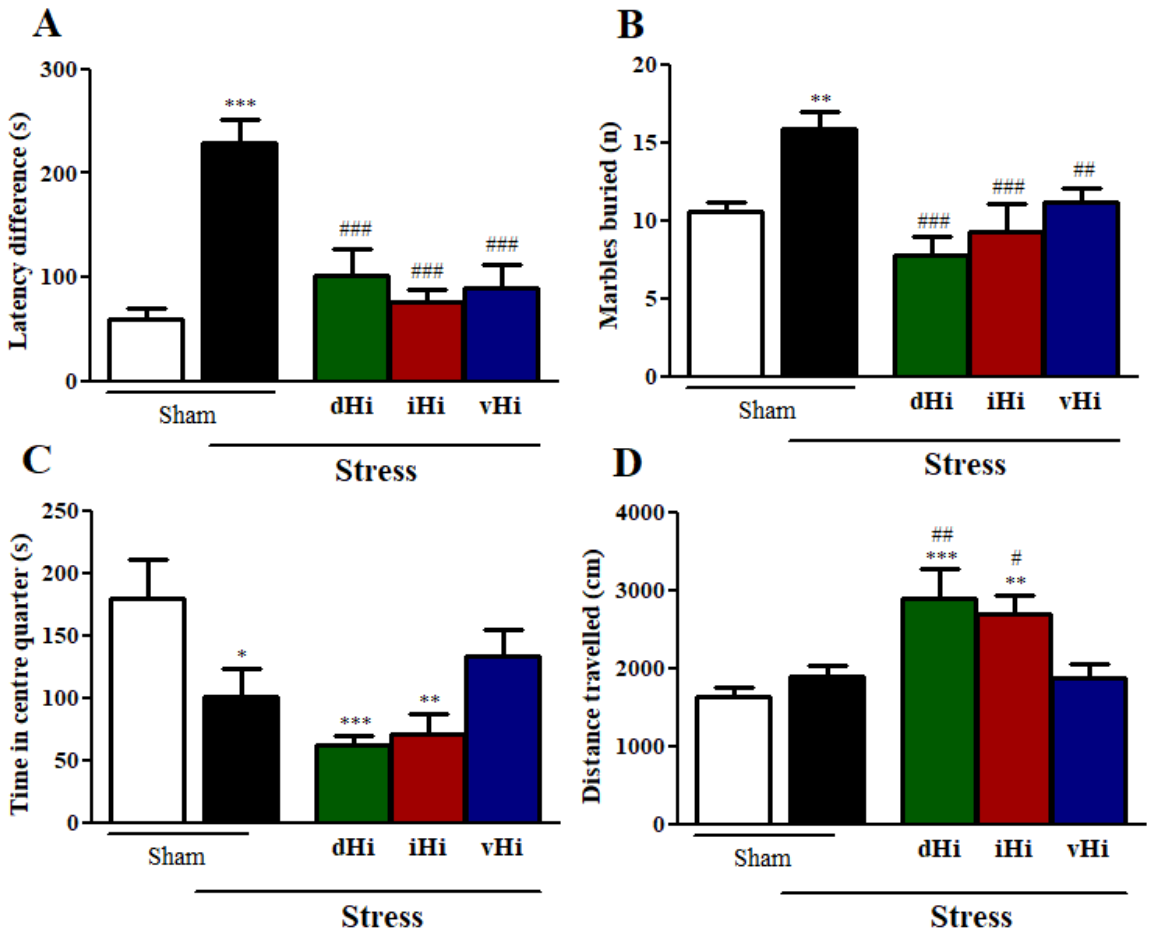


Figure 3.4: *All hippocampal sub-regions are critical for stress-induced anxiety. A, lesions of all hippocampal sub-regions prevent stress-induced anxiety in the novelty-induced hypophagia test. B, lesions of all hippocampal sub-regions prevent stress-induced anxiety in the marble burying test. C, lesions of the vHi attenuate stress-induced anxiety in the time spent in the centre of the open field, and this effect is not due to increased locomotor activity (D). D, lesions of the dHi and iHi increased locomotor activity. *p<0.05, **p<0.01, ***p<0.001, in relation to sham-no stress group; #p<0.05, ##p<0.01, #p<0.001, in relation to sham-stress group, according to Fishers LSD post-hoc test.*

3.3.5. The dHi and vHi mediate stress-induced anhedonia

The effects of sub-region-specific lesions on stress-induced anhedonia in the saccharin preference test are shown in Fig 3.5A-D. One-way ANOVA showed an overall effect on the preference for saccharin after 12 h consumption ($F_{4,39}$=3.41, p=0.018; Fig 3.5A) and after 24 h consumption ($F_{4,39}$=3.9, p=0.009; Fig 3.5B). Specifically, post hoc analysis showed that stress significantly decreased saccharin preference in sham animals after 12 and 24 h consumption (p=0.014, p=0.003, respectively) and in the iHi-lesioned group (p=0.027, p=0.003, respectively), but not in the dHi (p=0.81, p=0.34, respectively) nor vHi-lesioned (p=0.12, p=0.22, respectively) groups. Indeed, the dHi-lesioned group was significantly different from the sham-stressed group after 12 and 24 h consumption (p=0.014, p=0.03, respectively) and the vHi-lesion group showed a similar trend of effect at 12 h consumption (p=0.08), which became more apparent following 36 and 48 h of exposure (Fig 3.5C,D).

A similar pattern of effects on saccharin preference were observed following 36 h ($F_{4,39}$=4.02, p=0.008; Fig 3.5C) and 48 h of exposure ($F_{4,39}$=3.54, p=0.015; Fig 3.5D). Specifically, post hoc analysis revealed that chronic psychosocial stress significantly decreased saccharin preference in sham animals (36h: p=0.004; 48h p=0.008) as well as in the iHi-lesioned group (36h: p=0.024; 48h p=0.03) but not in the dHi-lesioned (36h p=0.85; 48h p=0.91) or the vHi-lesioned groups (36h p=0.74; 48h p=0.8). Indeed, both the dHi-lesioned (36h p=0.006; 48h p=0.01) and vHi-lesioned groups (36h p=0.012, 48h: p=0.019) had a significantly higher preference for saccharin when compared to the sham-stressed group.

The effects of sub-region-specific lesions on stress-induced anhedonia in the female urine sniffing test (FUST) are shown in Fig 3.5E. One-way ANOVA revealed an overall effect on the preference for urine in the FUST ($F_{4,39}$=2.79, p=0.04). Specifically, post hoc analysis revealed chronic psychosocial stress significantly reduced the preference for urine in the sham (p=0.006) and iHi-lesioned (p=0.018) groups but not in the dHi (p=0.089) nor the vHi-lesioned groups (p=0.39) when compared to the non-stressed sham control group.

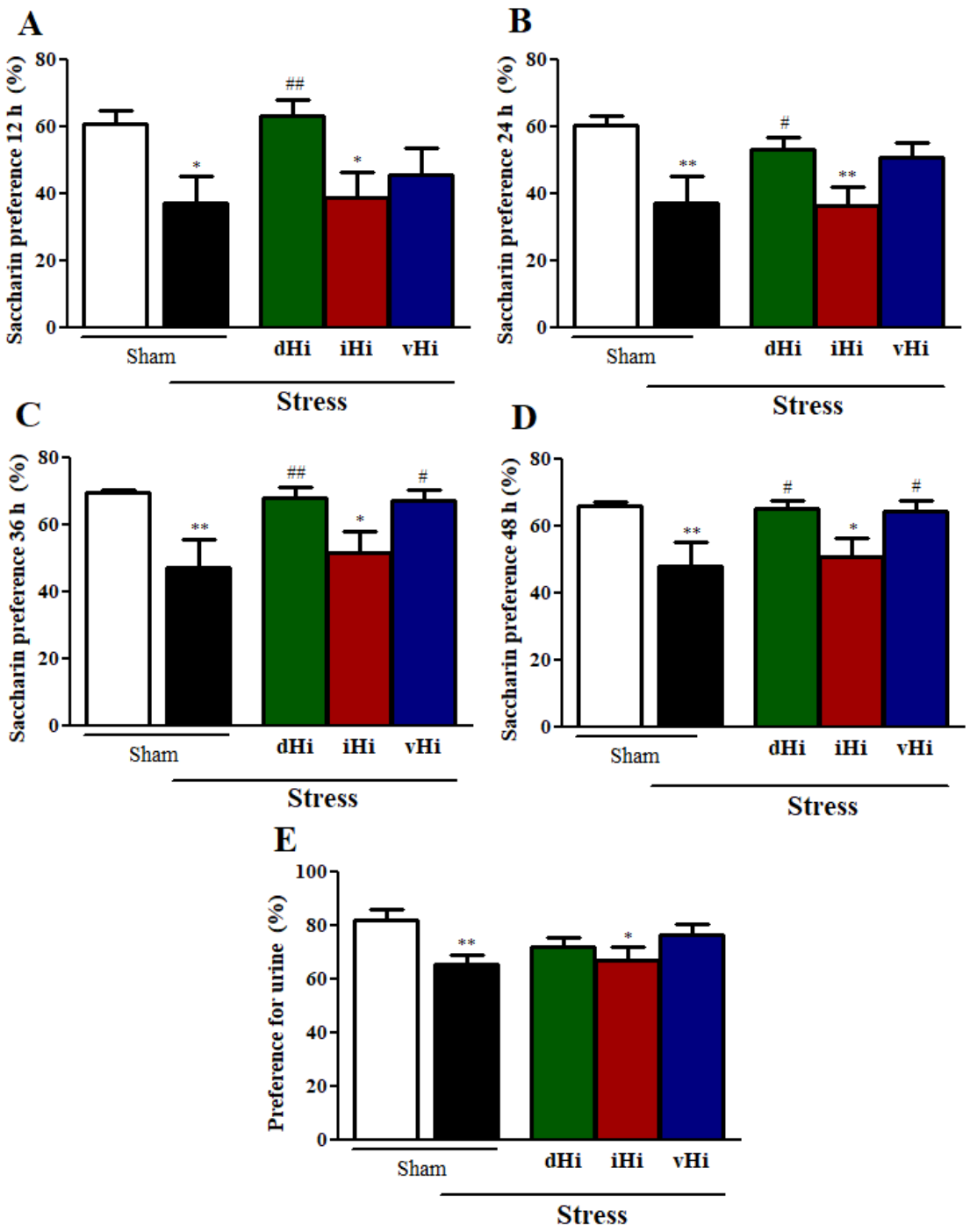


Figure 3.5: *dHi and vHi mediate stress-induced anhedonia. A-B, lesions of the dHi prevents and lesions of the vHi attenuates stress-induced anhedonia after 12 h (A) and 24 h (B) of the saccharin preference test. C-D, lesions of the dHi and vHi prevent stress-induced anhedonia after 36 h (C) and 48 h (D) of the saccharin preference test. E, lesions of both dHi and vHi attenuate stress-induced anhedonia in the female urine sniffing test. *p<0.05, **p<0.01, in relation to sham-no stress group; #p<0.05, ##p<0.01, in relation to sham-stress group, according to Fishers LSD post-hoc test.*

3.3.6. vHi lesions in stressed mice induce antidepressant-like effects in the forced swim test

The effects of sub-region-specific lesions on immobility in the forced swim test (FST) are shown in Fig 3.6. One-way ANOVA revealed an overall effect on immobility time in the FST ($F_{4,39}$=3.74, p=0.011). Specifically, post hoc analysis revealed that while stress alone did not alter immobility in the sham group (p=0.88), vHi lesions in stressed mice decreased immobility when compared to both the non-stressed sham group (p=0.002) and the stressed sham groups (p=0.001). Immobility time of the vHi group was also significantly lower when compared with the dHi- and iHi-lesioned groups (p=0.048 and p=0.025, respectively).

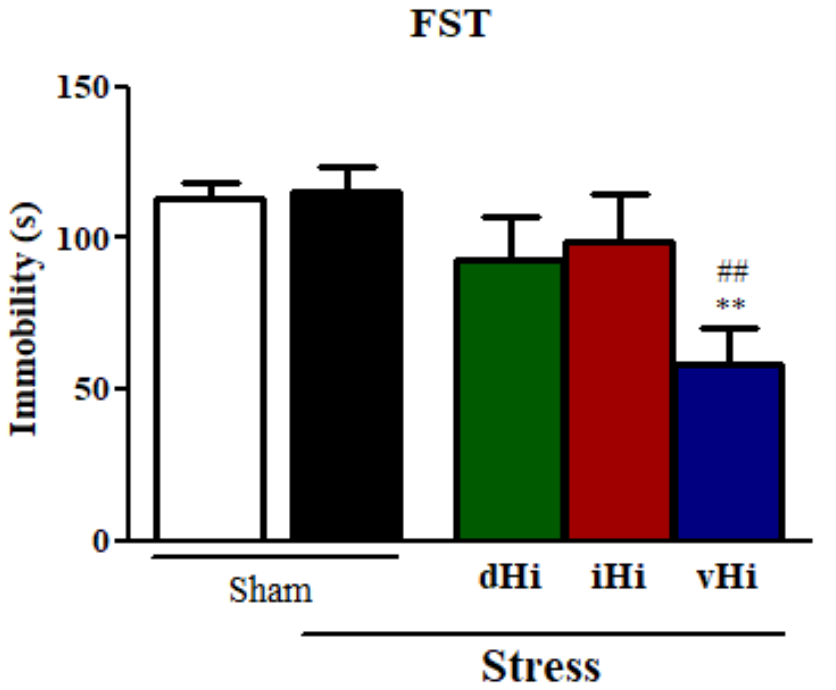


Figure 3.6: *vHi mediates antidepressant-like behaviour in stressed mice in the FST. **p<0.01, in relation to sham-no stress group; ##p<0.01, in relation to sham-stress group, according to Fishers LSD post-hoc test.*

3.3.7. Behavioural effects of hippocampal lesions on chronically stressed mice were largely independent of stress-induced alterations in HPA-axis function

The effects of stress and hippocampal lesion on the CORT release at baseline, 30 min and 120 min after the FST are shown in Fig 3.7A. Two-way repeated measures ANOVA revealed an overall effect of timepoint ($F_{2,39}$=300.8, p<0.001), but no effect of group ($F_{4,39}$=1.92, p=0.13) and no interaction between them ($F_{8,39}$=1.75, p=0.1). Post hoc analysis at each timepoint revealed that neither chronic stress nor hippocampal sub-region lesion affected baseline plasma CORT concentrations (all p>0.05), and that swim stress increased corticosterone 30 min after the start of the FST in all groups (all p<0.001). This swim stress-induced increase in CORT was greater in all hippocampal sub-region lesioned groups when compared to the sham-control group (dHi, iHi and vHi lesion p=0.001, p=0.014 and p=0.008, respectively). However, dHi lesions (but not other sub-region lesions) further amplified stress-induced increases in corticosterone when compared to the sham-stress group (p=0.003). However, such group differences were no longer apparent 120 min after the FST.

The effects of stress and hippocampal lesion on plasma corticosterone concentrations after a dexamethasone (DEX) and a stress challenge are shown in Fig 3.7B. Two-way repeated measures ANOVA revealed an overall effect of timepoint ($F_{2,39}$=39.52, p<0.001), but no effect of group ($F_{4,39}$=1.25, p=0.31) and no interaction between them ($F_{8,39}$=1.71, p=0.11). However, post hoc analysis revealed that the iHi group had higher CORT levels at baseline compared to all other groups (p<0.001 vs. sham-no stress; p=0.005 vs. sham-stress; p=0.005 vs. dHi lesion; p=0.012 vs. vHi lesion. Dexamethasone suppressed acute stress-induced increases in CORT in all groups when measured 15 min after the acute stress (e.g. 2h 15min after DEX injection), sham-stress (p=0.008), dHi lesion (p=0.016), iHi lesion (p<0.001) and vHi lesion (p=0.005), although this did not quite reach

statistical significance in the sham-control group (p=0.083). 2h after stressor (and 4h after DEX injection), all groups still had lower CORT in comparison to baseline (sham-control, p=0.014; sham-stress, p<0.001; dHi lesion, p=0.03; iHi lesion, p<0.001; vHi lesion, p=0.001), with no differences between them.

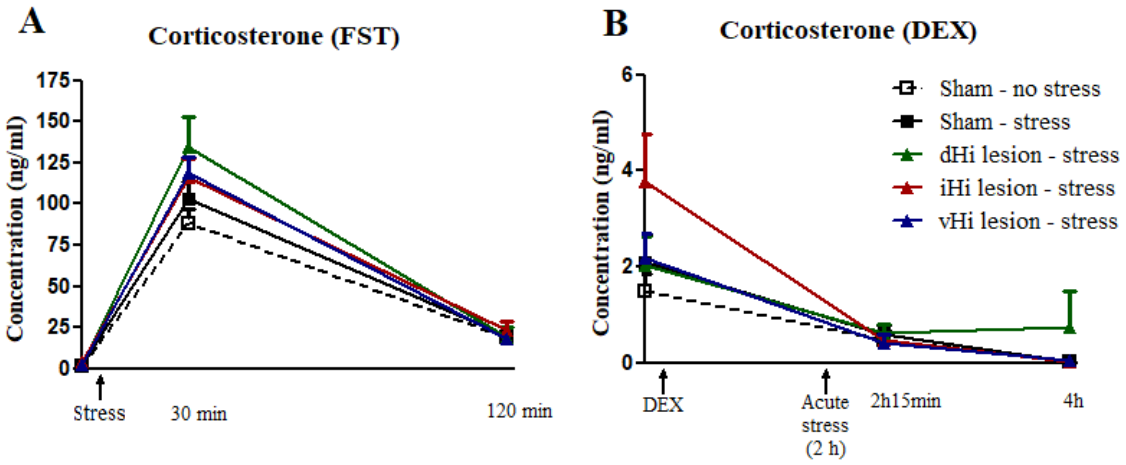


***Figure 3.7**: Chronic stress did not alter the plasma concentration of corticosterone. A, plasma concentration of corticosterone at different timepoints (30 and 120 min) after the forced swim test. B, plasma concentration of corticosterone after different timepoints after a dexamethasone challenge.*

3.4. Discussion

Accumulating evidence suggests that the rodent hippocampus is functionally segregated along its longitudinal axis into dorsal (dHi) and ventral (vHi) regions and most recent studies now also suggest that the area between them, the intermediate hippocampus (iHi) might also be functionally independent. Although the hippocampus is known to play a role in the pathophysiology and treatment of stress-related psychiatric disorders, the functional roles of these three sub-regions in the response to chronic stress have not yet been investigated and thus were the focus of these experiments. We performed a search on the RNAseq database from the Janelia Research Campus (http://hipposeq.janelia.org) (Cembrowski *et al.* 2016) and found a heterogeneity in the expression of stress-related genes along the longitudinal axis of the hippocampus. Subsequently, we found that sub-regions of the longitudinal axis of the hippocampus mediate specific behavioural responses to chronic psychosocial stress. For what is to our knowledge the first time we show a predominant role of iHi in the stress-induced social avoidance and a predominant role of vHi in active coping behaviours and antidepressant effect following chronic stress.

A growing body of evidence has been supporting the idea that the hippocampus operates across a functional neuroanatomical gradient coincident with different gene expression along its longitudinal axis (Moser and Moser 1998, Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016, Cembrowski *et al.* 2016). Using an RNAseq database (Cembrowski *et al.* 2016), we found differences in the expression of stress-related genes in the dentate gyrus of the dHi (dDG) versus vHi (vDG) of the mouse hippocampus. In agreement, several studies have reported differential gene expression in the dDG versus vDG in rats (Christensen *et al.* 2010)

and in mice (Cembrowski *et al.* 2016). Specifically, we found differences in the expression of receptors of several systems of neurotransmissions, growth factors, neurogenesis and glucocorticoid/mineralocorticoid receptors functioning. Genes involved in cholinergic, GABAergic and serotonergic neurotransmission are increased while those relevant to glutamatergic neurotransmission is reduced in the vDG when compared with the dDG. This is in agreement with previous literature which shows that the vHi is enriched with genes involved serotonergic (Tanaka *et al.* 2012), cholinergic and GABAergic neurotransmission (Lee *et al.* 2017). Together with the lower glutamatergic neurotransmission, this might be a reason for the vHi to support a less robust long-term potentiation than the vHi in rats (Maggio and Segal 2007). Moreover, stress has been shown to regulate glutamatergic expression in the hippocampus (Nasca *et al.* 2015), and both stress and corticosterone impair GABAergic neurotransmission in the hippocampus (Maggio and Segal 2009, Hu *et al.* 2010), preferentially in the vHi sub-region (Maggio and Segal 2009). Besides that, we also found that the mineralocorticoid receptor (MR) and serum and glucocorticoid regulated kinase 1 were expressed at lower levels in the vDG when compared with the dDG. Interestingly, MR receptor has also been shown to be reduced in depressed patients, specifically in the anterior hippocampus (vHi in rodents) (Medina *et al.* 2013). Also, differential expression in growth factor genes and genes related to neurogenesis in the vDG compared to dDG support that the vHi expresses more genes that favour cell proliferation, while dHi expresses more genes that favour neuronal differentiation. In agreement, a recent study suggests that the dHi has more active neurogenesis and is enriched of maturing neurons markers, such as NeuroD1 and DCX, while the vHi is enriched of radial glia markers, such as Sox2 and Hes5 (Zhang *et al.* 2018) in mice.

Accordingly, dHi neural progenitor cells have been shown to mature faster, associated with higher basal network activity (Piatti *et al.* 2011).

More recent gene expression studies support however the concept that genes are expressed as a gradient along the hippocampal longitudinal axis in mice (Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016), and as such, the presence of an intermediate hippocampus is suggested. Thus, we also used the database to determine whether the CA1 of the dHi (dCA1), iHi (iCA1) and vHi (vCA1) differentially express stress-related genes. Although less changes were found, here we also found that some of the analysed genes were differentially expressed along the longitudinal axis of the hippocampal CA1, mainly when comparing the in the vCA1 with the dCA1. In agreement, previous studies have shown that genes are differentially expressed along the longitudinal axis of the CA1 (Leonardo *et al.* 2006, Dong *et al.* 2009, Cembrowski *et al.* 2016, Pacheco *et al.* 2017) and CA3 (Thompson *et al.* 2008) areas of the hippocampus. Here, we also found that the vCA1 has a more pronounced serotonergic neurotransmission in comparison to dCA1 and iCA1, while the changes in the other systems of neurotransmission were not as clear as the ones found in the vDG versus dDG. Interestingly, MR was expressed at lower level in the vCA1 versus dCA1, while the iCA1 had an intermediate expression, supporting a gradient expression of the MR along the longitudinal axis of the hippocampus. Also, vCA1 showed an increased expression of FGF2 in comparison with the dCA1, while a lower expression of BDNF in comparison with the dCA1 and iCA1. However most of the studies compared only the dorsal half versus the ventral half of the hippocampus, a study dissected the rat hippocampus into the most dorsal third and the most ventral third, ignoring the intermediate third, and found that some genes are differentially expressed in the vHi

versus the dHi (Lee *et al.* 2017). Interestingly, a study shows that acute social defeat downregulates the fibroblast growth factor and its receptor in the hippocampus of rats (Turner *et al.* 2008), which was also observed in post-mortem study in depressed individuals (Evans *et al.* 2004). In addition, a recent study also supports the idea that the dHi and vHi have distinct epigenetic responses to acute stressors (Floriou-Servou *et al.* 2018), with the vHi being particularly more sensitive to the stressor.

Since we observed differential expression of stress-related genes along the longitudinal axis of the hippocampus, we next used ibotenic lesions of the dHi, iHi, or vHi to investigate whether these hippocampal sub-regions play distinct roles on the behavioural responses to chronic psychosocial stress. In agreement with other studies that incorporate social defeat in psychosocial stress paradigms, we found that all treatment groups, irrespective of lesions, were susceptible to stress-induced social avoidance, as indicated by significant reductions in the social interaction ratio (Krishnan *et al.* 2007, Finger *et al.* 2011, Huang *et al.* 2013, Hammels *et al.* 2015, Burokas *et al.* 2017). However, we did observe that the percentage of susceptible mice was lower in sham animals thus suggesting that the hippocampus likely plays a role in stress resilience. Interestingly, we also observed that iHi lesions exacerbated stress-induced social avoidance as indicated by an increase in the corners ratio in the social interaction test. To the best of our knowledge, this is the first time that a role for the iHi in stress-induced social avoidance has been reported. However, previous studies have implicated the vHi in resilience to social defeat stress (Calfa *et al.* 2007, Kenworthy *et al.* 2014, Laine *et al.* 2017, Hultman *et al.* 2018). In accordance, glutamatergic projections from the vHi to the nucleus accumbens have been shown to control stress resilience to chronic social defeat

stress, and optogenetic attenuation of the neurotransmission from vHi to accumbens is suggested to induce stress resilience, shown by an increase in the time spent in the interaction zone in the social interaction test (Bagot *et al.* 2015). In contrast, in the present study vHi and dHi lesions did not alter stress-induced social avoidance. However, we did observe that vHi lesions increased the latency to adopt a defeat posture suggesting increased active coping behaviour.

Our psychosocial stress paradigm increased anxiety in sham animals in several behavioural tests including marble burying, novelty-induced hypophagia and the open field test. This is in agreement with reports from previous studies, which showed that this particular stress paradigm and the chronic social defeat stress model increased anxiety in the elevated plus maze in mice (Slattery *et al.* 2012, Burokas *et al.* 2017), open field (Kinsey *et al.* 2007, Burokas *et al.* 2017) and in the light-dark box tests (Keeney and Hogg 1999, Kinsey *et al.* 2007, Finger *et al.* 2011, Huang *et al.* 2013). In our study, we also found that lesions of either the dHi, iHi or vHi all prevented stress-induced anxiety in the marble burying and in novelty-induced hypophagia tests. To the best of our knowledge, this is the first time that this is shown in these behavioural tests under stress conditions. While the present results might suggest that all hippocampal sub-regions mediate stress-induced anxiety, this effect may be test-dependent, as a different picture emerged in the open field test, whereby lesions of the vHi had an anxiolytic effect attenuating the stress-induced decrease in time spent in the centre of the open field. This effect was not due to alterations in locomotor activity. In agreement, it has been reported the that vHi, but not dHi, is required for the expression of anxiety behaviours in rats in the hyponeophagia and light-dark box (Bannerman *et al.* 2003) and elevated plus maze (Kjelstrup *et al.* 2002). However, these studies were done in animals that had not

undergone prior chronic stress. Moreover, optogenetic and pharmacogenetic studies have reported that several vHi neural pathways regulate anxiety in the open field and elevated plus maze (Felix-Ortiz *et al.* 2013, Padilla-Coreano *et al.* 2016, Parfitt *et al.* 2017). It is also important to note that a study demonstrated that both dHi and vHi can modulate anxiety behaviour in in open field and elevated plus maze in mice (Kheirbek *et al.* 2013).

Taken together, these data suggest that the anxiolytic effects of hippocampal sub-region lesions in stressed mice are test-dependent. One explanation for differential effects in the open field versus the marble burying test and novelty-induced hypophagia, may be that these tests interrogate different aspects of anxiety behaviour or that different neural circuits may underlie the manifestation of anxiety in these tests. For an example, it has been reported that the open field test increases expression of the immediate early gene c-FOS in the basolateral amygdala, mPFC and CA1 of the ventral hippocampus (Hale *et al.* 2008), while another study revealed that novelty-induced hypophagia increased c-FOS expression in the lateral septal nucleus, cingulate cortex and the CA1 of both the dorsal and ventral hippocampus (Olszewski *et al.* 2014). The precise vHi neural pathway underlying the anxiolytic effect in the open field have yet to elucidated but several vHi-mediated pathways have been reported to regulate anxiety behaviour in the open field and also elevated plus maze, including projections from the amygdala (Felix-Ortiz *et al.* 2013) or to the prefrontal cortex (Adhikari *et al.* 2011, Padilla-Coreano *et al.* 2016). In addition, a recent paper describes the presence of "anxiety cells", cells which are thought to mediate anxiety behaviour, in the ventral hippocampus which project to the lateral hypothalamic area (Jimenez *et al.* 2018). This is in

accordance with a previous study that show that the stimulation of the ventral hippocampus causes inhibition of the HPA axis (Casady and Taylor 1976).

In the present study, chronic psychosocial stress induced an anhedonic effect in sham animals in the saccharin preference and female urine sniffing tests. This is in line with the effects of such a stressor in the female urine sniffing test (Finger *et al.* 2011, Lehmann *et al.* 2013, Burokas *et al.* 2017),and in the saccharin preference test (Liu *et al.* 2018). In our study, we found that lesions of either the dHi or vHi prevented the stress-induced anhedonia in the saccharin preference test, and the lesion of dHi and vHi also attenuated stress-induced anhedonia in the female urine sniffing test. Interestingly, iHi lesions did not affect stress-induced anhedonia in either of these tests. Taken together, we show for the first time that the dHi and vHi, but not the iHi play a role in the stress-induced anhedonia.

When we examined the impact of chronic stress and lesions on antidepressant-like behaviour, we found that chronic psychosocial stress did not alter immobility time in the forced swim test. This is in contrast to some other studies that also used chronic social defeat as the stress paradigm (Finger *et al.* 2011, Huang *et al.* 2013, Burokas *et al.* 2017, Liu *et al.* 2018). However, in accordance with our results, a growing number of studies have reported that chronic psychosocial stress increases anxiety, without altering depressive-like behaviours in the forced swim test (Keeney and Hogg 1999, Kinsey *et al.* 2007, Slattery *et al.* 2012, Hammels *et al.* 2015). Interestingly however, we found that stressed vHi-lesioned animals showed reduced immobility time when compared to both sham groups thus indicating an antidepressant-like phenotype. Intriguingly, this effect is not apparent in non-stressed vHi-lesioned mice (Levone et al., unpublished; Chapter 2). Others have

reported that chemogenetic activation of the vHi to medial prefrontal cortex pathway in non-stressed rats exerts an antidepressant-like effect in the immobility time in the forced swim test and that inactivation of this pathway is also required for the sustained antidepressant-like effects of ketamine in non-stressed rats in this test (Carreno *et al.* 2016). Here, we found that under chronic stress conditions vHi lesions induce antidepressant-like effects thus suggesting that, under chronic stress conditions, other vHi-mediated pathways regulate antidepressant like effects in this test. Taken together, we show what is to our knowledge for the first time that the vHi plays a role in modulating antidepressant-like behaviour in the mouse forced swim test under chronic stress conditions.

Given the region-specific modulation of stress-induced changes in behaviour, we also investigated the corticosterone release after an acute stressor (swim stress, 30 min and 120 min after the test), and after a dexamethasone challenge. In the swim stress experiment, chronic stress alone did not affect plasma corticosterone concentrations at any timepoint. In agreement, it has been previously reported that this chronic psychosocial stress paradigm does not affect plasma corticosterone concentrations during the light-phase (Reber *et al.* 2006, Finger *et al.* 2011, Hammels *et al.* 2015), although other studies showed that mice submitted to chronic psychosocial stress present higher basal corticosterone levels (Keeney *et al.* 2001, Burokas *et al.* 2017), and one study demonstrated that chronic social defeated mice present lower corticosterone levels (Savignac *et al.* 2011). Similarly, neither chronic stress nor lesions of any hippocampal sub-regions altered plasma concentrations of corticosterone after a dexamethasone challenge thus suggesting the GR-mediated negative feedback of the HPA-axis is unaffected by specific lesions of dHi, iHi or vHi.

In summary, the present study shows what is to our knowledge for the first time evidence of a differential role of the dHi, iHi and vHi on emotional behaviours under chronic psychosocial stress. While all hippocampal sub-regions are involved in the anxiogenic effects of chronic stress, only dHi and vHi are involved in the stress-induced anhedonia. Interestingly, here we show that the iHi plays a more predominant role in stress-induced social avoidance, and the vHi is preferentially involved in antidepressant-like behaviour under chronic stress conditions and in active coping behaviour. Thus, we suggest that future studies of the hippocampus which are aimed at the identification of novel drug development targets should consider the differential roles of hippocampal sub-regions in stress-induced changes in behaviour and should focus primarily on the vHi. Moreover, future studies should consider grouping the chronically stressed animals into susceptible and resilient to stress, aiming at identifying possible sub-regional mechanisms of resilience.

CHAPTER 4

Adult-Born Neurons from the Intermediate and Ventral Regions of the Longitudinal Axis of the Hippocampus Exhibit Differential Sensitivity to Glucocorticoids

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Abstract

The hippocampus is one of few brain regions where neurogenesis, the birth of new neurons, occurs throughout life and this process has been shown to play roles in learning, memory and stress responses. These diverse roles may be related to a functional segregation of the hippocampus along its longitudinal axis; indeed, the dorsal hippocampus (dHi) plays a predominant role in spatial learning and memory, while the ventral hippocampus (vHi) is predominantly involved in the regulation of anxiety (a behaviour impacted by stress). Recent studies suggest that the area between them, the intermediate hippocampus (iHi) may also be functionally independent. This study aimed to determine whether stress-induced changes in neurogenesis could be related to differential intrinsic sensitivity of neural progenitor cells (NPCs; isolated from rats at 28PND) from the dHi, iHi, or vHi to the stress hormone, corticosterone (CORT), or the glucocorticoid receptor (GR) agonist, dexamethasone (DEX). Long-term exposure to CORT decreased neuronal differentiation in cultures derived from the vHi but not the dHi, while iHi cultures showed an intermediate response. A similar gradient-like response was observed with dexamethasone on neuronal differentiation and maturation of NPCs. The same trend was also observed on the GR nuclear translocation after CORT or DEX treatment. These data suggest that an intrinsic sensitivity of vHi NPC-derived neurons to chronic glucocorticoid exposure may underlie the increased vulnerability of the vHi to chronic stress-induced reductions in neurogenesis. They also highlight that the iHi deserves more attention as a mediator of stress-induced cellular and behavioural changes.

4.1. Introduction

The hippocampus is one of just a few brain areas where resident stem cells produce new neurons throughout life through a process called adult hippocampal neurogenesis [AHN] (Kempermann *et al.* 2004, Ming and Song 2011). AHN occurs in the subgranular zone of the dentate gyrus of the hippocampus, where the neural progenitor cells (NPCs) reside, and is comprised of several stages including cell proliferation, neuronal differentiation, and survival and maturation of the newly-born neurons (Christie and Cameron 2006). All of these processes can be influenced by several extrinsic factors including stress, antidepressant treatment, environment, physical activity, and learning and memory (Kempermann *et al.* 1997, Gould *et al.* 1999, Malberg *et al.* 2000, Dobrossy *et al.* 2003, Simon *et al.* 2005, Fabel and Kempermann 2008, Schloesser *et al.* 2010, Surget *et al.* 2011, Tanti *et al.* 2012, Hueston *et al.* 2017). Indeed, learning and memory have been shown to increase AHN in rats (Gould *et al.* 1999, Dobrossy *et al.* 2003) and in turn, AHN is required for hippocampal-dependent learning (Marin-Burgin and Schinder 2012). In addition, stress and high levels of glucocorticoids impact the hypothalamus-pituitary-adrenal (HPA) axis, the axis that control the release of glucocorticoids by the adrenal glands, and have been shown to decrease all stages of AHN (Simon *et al.* 2005, Dranovsky and Hen 2006, Jayatissa *et al.* 2006, Mitra *et al.* 2006, Jayatissa *et al.* 2009, Surget *et al.* 2011, Schoenfeld and Gould 2012, Lehmann *et al.* 2013). Specifically, different forms of stress reduce AHN, including predator odour (Galea *et al.* 2001), social stress (Gould *et al.* 1997, Czeh *et al.* 2001), and chronic mild stress (Alonso *et al.* 2004). Moreover, the neurogenic niche in which NPCs reside is located near blood vessels (Wurmser *et al.* 2004), which makes these cells vulnerable to blood-transported molecules and hormones, such as cytokines (Bauer 2009), growth factors (Oliveira *et al.* 2013) and the stress hormone, corticosterone.

Indeed, it has been shown that chronic treatment with corticosterone reduces AHN in mice (David *et al.* 2009) and rats (Ekstrand *et al.* 2008, Brummelte and Galea 2010). Adrenalectomy, and the consequent removal of circulating plasma glucocorticoids, is also known to increase hippocampal cell proliferation (Krugers *et al.* 2007). In turn, AHN facilitates normalization of glucocorticoid levels after stress in mice (Snyder *et al.* 2011, Surget *et al.* 2011, Culig *et al.* 2017).

Precisely how AHN plays a role in functions as seemingly diverse as spatial learning and memory and the response to stress is currently unclear but may be related to functional segregation of the hippocampus along its longitudinal axis into dorsal and ventral regions. Indeed, the dorsal hippocampus (dHi), corresponding to posterior hippocampus in primates, plays a predominant role in spatial learning and memory, while the ventral hippocampus (vHi), corresponding to the anterior hippocampus in primates, plays a predominant role in anxiety (Bannerman *et al.* 2004, Fanselow and Dong 2010), a stress-sensitive behaviour. Emerging evidence suggests that regulation of AHN might also be functionally segregated along the longitudinal axis of the hippocampus (Winocur *et al.* 2006, Tanti and Belzung 2013, O'Leary and Cryan 2014, Wu and Hen 2014, Levone *et al.* 2015, O'Leary *et al.* 2015). New neurons in the dHi are preferentially required for contextual discrimination and memory tasks in rats (Winocur *et al.* 2006), while new neurons in the vHi are necessary for anxiolytic effects of the antidepressant fluoxetine in mice (Wu and Hen 2014). It has also been reported that chronic stress decreases cell proliferation (Jayatissa *et al.* 2006, Tanti *et al.* 2012), neuronal differentiation, maturation and survival (Hawley and Leasure 2012, Lehmann *et al.* 2013), preferentially in the vHi of rodents. Indeed, our laboratory and others have reported that AHN is differentially regulated by stress and antidepressant-like treatments

within the sub-regions of the hippocampus in rats and mice (Jayatissa *et al.* 2006, O'Leary *et al.* 2012, Tanti and Belzung 2013, O'Leary and Cryan 2014, Levone *et al.* 2015). Similarly, it has been shown that chronic stress reduced the number of immature neurons in the anterior hippocampus, but not in the posterior hippocampus of non-human primates (Perera *et al.* 2011).

Some of these conflicting data may be a function of the definition of the boundaries of the dorsal versus the ventral sub-regions (Tanti and Belzung 2013) which sometimes includes the intermediate hippocampus (iHi), a sub-region which may be functionally independent in its own right but has been understudied (Bast 2007, Thompson *et al.* 2008, Bast *et al.* 2009, Strange *et al.* 2014). Very few studies considered this third hippocampal sub-region as its own, but there are reports that it is not only a transitional area between dorsal and ventral hippocampus, but also an integrative region (Bast 2007, Bast *et al.* 2009), which mediates the binding of diverse sensory modalities (Bast *et al.* 2009). A recent paper also showed that the effects of fluoxetine on the proliferation of NPCs *in vivo* are preferentially in the transition between the intermediate and the ventral hippocampus (Zhou *et al.* 2016). The effects of corticosterone on the proliferation and differentiation of NPCs of the intermediate hippocampus, however, has not yet been assessed.

The mechanisms underlying the increased sensitivity of the AHN in the vHi to the stress and corticosterone are currently unknown. Thus, the aim of this study was to determine whether such effects may be a result of differential intrinsic sensitivity of NPCs derived from the dHi, iHi, or vHi to the stress hormone, corticosterone, and through its action on the GR.

4.2. Methods

4.2.1. Experimental design

To isolate the neural progenitor cells (NPCs) from any extrinsic influences they could receive on *in vivo* experiments, we used a protocol of isolation and culture of NPCs from adolescent rats. To assess whether NPCs derived from different sub-regions of the hippocampus respond differently to the stress hormone, corticosterone (CORT), we assessed cell proliferation, neuronal differentiation and maturation in CORT-exposed cells. We allowed NPCs to grow for 4 days *in vitro* as neurospheres, dissociated them into single cells and exposed them to CORT for either 4 h (acute), 4 days (short-term) or 7 days (longer-term) *in vitro*. Cells were then fixed and proceeded for immunocytochemistry.

4.2.2. Preparation of neurosphere cultures

Procedures were performed under individual authorisations issued by the Health Products Regulatory Authority (HPRA) Ireland and in accordance with the European Parliament and the Council of the EU Directive (2010/63/EU). The whole hippocampi (Suppl. Fig 4.1A) or dHi, iHi and vHi (Suppl. Fig 4.1B) were dissected from male Sprague Dawley rats at postnatal day 28 (PND28) (bred at Biological Services Unit, UCC). To obtain a sufficient number of NPCs per independent experiment, whole hippocampi from 3 animals or each sub-region of 6 animals were pooled. NPCs were seeded at a density of 10×10^4 cells per well and allowed to proliferate as neurospheres (Suppl. Fig 4.1C) for 4 days *in vitro* (DIV). A detailed protocol for the isolation of hippocampal NPCs is provided in the Supplementary Material

4.2.3. Dissociation of neurospheres and treatment of NPCs with corticosterone or dexamethasone

After 4 DIV under proliferation conditions, neurospheres were dissociated and seeded at a density of 5 x 10^4 per well in a volume of 40 µl of cell suspension onto Poly-D-Lysine (1mg/ml, Sigma Aldrich, UK) coated coverslips in a 24-well plate. NPCs were allowed to adhere to the coverslips for 1 h prior to the addition of 460 µl of treatment medium.

To determine an appropriate dose range of corticosterone (CORT) to use for our sub-region experiments, we first determined the effect of acute (4 h) CORT exposure on cell proliferation from NPCs from the whole hippocampus. The treatment medium was composed of growth medium (Neurobasal A, 2% B27 minus vitamin A, 0.5mM Glutamax, 50 units/ml Penicillin/Streptomicin, 20 ng/ml FGF and 20 ng/ml EGF) with 0.2 µM of BrdU (Sigma-Aldrich, UK) and different concentrations of corticosterone (CORT, Sigma-Aldrich, UK, 1 µM or 5µM, diluted in 0.1% DMSO). Control cells were treated with vehicle (growth medium with BrdU and 0.1% DMSO). Cells were allowed to proliferate for 4 h *in vitro* prior to fixation.

For acute (4 h) CORT exposure, cells were allowed to proliferate in growth medium with 0.2 µM BrdU and different concentrations of corticosterone (CORT; 0.5 µM, 1 µM or 5 µM diluted in DMSO to a final concentration of 0.1% DMSO) or vehicle (growth medium with 0.2 µM BrdU and 0.1% DMSO). Cells were allowed to proliferate for 4 h *in vitro* prior to fixation.

For short-term (4 DIV) CORT exposure, cells were allowed to differentiate in growth medium in the absence of growth factors or heparin, and in the presence of vehicle (0.1% DMSO) or different concentrations of CORT (0.5 µM, 1 µM or 5 µM diluted in DMSO to a final concentration of 0.1% DMSO). Cells were allowed to differentiate for 4 DIV prior to fixation.

For long-term (7 DIV) CORT or dexamethasone (DEX) exposure, cells were allowed to differentiate in growth medium in the absence of growth factors or heparin and in the presence of vehicle (0.1% DMSO) or different concentrations of CORT (0.5 µM, 1 µM or 5 µM diluted in DMSO to a final concentration of 0.1% DMSO) or DEX (10 nM, 100 nM or 1000 nM diluted in DMSO to a final concentration of 0.1% DMSO). Cells were allowed to differentiate for 7 DIV prior to fixation.

4.2.4. Immunocytochemistry

Cells were washed with warm Hanks' balanced salt solution (HBSS), fixed with ice cold methanol at -20ºC for 10 min followed by 4% paraformaldehyde at room temperature for 10 min. Cells were then washed for 3x5 min with PBS-0.02%TritonX (PBS-T) and blocked overnight at 4ºC with 5% donkey serum (DS; Sigma Aldrich) diluted in PBS-T. 18 h later, cells were washed for 3x5 min with PBS-T and incubated overnight at 4ºC in the appropriate primary antibody diluted in 5% DS PBS-T [(rat monoclonal anti-BrdU (1:100, ab6326, Abcam), goat polyclonal anti-DCX (1:200, sc-8066, Santa Cruz) or mouse monoclonal anti β3-tubulin (1:250 G7121, Promega)]. 18 h later cells were washed for 3x5 min in PBS-T and incubated for 2 h at room temperature in the respective secondary antibody diluted in 5% DS PBS-T [(Alexa Fluor 594 donkey anti-rat (1:1000, A21209,

Invitrogen), Alexa Fluor 488 donkey anti-goat (1:500, A-11055, Invitrogen), Alexa Fluor 488 donkey anti-mouse (1:500, A-21202, Invitrogen)]. For experiments requiring dual labelling immunohistochemistry, cells were then washed 3x5min and incubated overnight at 4ºC in the appropriate primary antibody diluted in 5% DS in PBS-T [(goat polyclonal anti-Nestin (1:200, sc21249, Santa Cruz); rabbit polyclonal anti-GR (1:500 sc-1004, Santa Cruz)]. 18 h later, cells were washed 3x5 min with PBS-T and incubated for 2 h at room temperature in the respective secondary antibody diluted in 5% DS in PBS-T [(Alexa Fluor 488 donkey anti-goat, (1:1000 A-11055, Invitrogen), Alexa Fluor 594 donkey anti-rabbit (1:500, A21207, Invitrogen)]. After 3x5 min washes, cells were incubated in DAPI (1:1000, from a stock of 1ul/ml, Sigma-Aldrich, UK) diluted in 2% DS in PBS-T for 10 min and then washed with PBS. Coverslips were mounted onto slides using DAKO Fluorescence mounting medium.

4.2.5. Fluorescence microscopy and image analysis

Fluorescent images of immunopositive cells were viewed under an Olympus BX53 upright microscope and photomicrographs were captured (all with the same exposure time) at 40x magnification using an Olympus DP71 camera and cellSens Entry camera software™. For each treatment condition, five fields of view were randomly captured per coverslip, there were three coverslips per treatment per plate, and each experiment was repeated three times. Software Image J Version 1.44 was used to count cells, to measure the length of dendritic processes and to measure fluorescence intensity of GR-immunopositive nuclei.

In the proliferation experiments, the percentage of NPCs [(Nestin$^+$ cells/total DAPI$^+$ nuclei) x 100] and the percentage of proliferating NPCs [(BrdU$^+$ cells/Nestin$^+$ cells) x 100] were calculated.

In the experiment examining the effects of short-term (4 DIV) and long-term (7 DIV) corticosterone treatment on neuronal differentiation and maturation, the percentage of neurons [(DCX$^+$ cells/ DAPI$^+$ cells) x 100 for short-term and (β3-tubulin$^+$ cells/DAPI$^+$ cells) x 100 for long-term] was calculated. The percentage of neurons (DCX$^+$ or β3-tubulin$^+$) with a primary [(neurons with primary processes/total number of neurons) x 100] and a secondary process [(neurons with secondary processes/total number of neurons) x 100] were also calculated. As an index of neuronal maturation, the length of these processes was calculated [(sum of the length of all primary or secondary processes/number of neurons with that specific process) x 100]. The total length of processes was also measured [(sum of the length of all processes/number of neurons) x 100]. For long-term corticosterone treatment, the percentage and length of tertiary processes were also calculated.

To determine the effects of long-term (7 DIV) corticosterone or dexamethasone treatment on glucocorticoid receptor (GR) translocation to the nucleus, the fluorescence intensity of all nuclei was assessed. For that end, the software recognised the contour of the nuclei and we measured the GR mean fluorescence intensity inside the nuclear contours. The results for each cell were then averaged and data are expressed as a % from the control group. The software Image J Version 1.44 was used to draw around each nucleus and assess the fluorescence intensity, as a measure of nuclear GR expression.

4.2.6. Cell viability analysis [Thiazolyl Blue Tetrazolium Bromide (MTT) assay]

An MTT assay was used to verify whether the results of long-term exposure to corticosterone or dexamethasone were due to altered cell viability. Briefly, after 7 DIV under differentiation conditions the media was removed and growth media containing 10% MTT solution (5 mg/ml, Sigma Aldrich, UK) was added. Four hours later, the medium was discarded and 100 µl of DMSO was added to each well to dissolve the formazan crystals. 75 µl from each well were plated in a 96-well plate and the absorbance at 570 nm wavelength was measured in an absorption spectrophotometer (Flexstation 3 Multimode Plate Reader, Molecular Devices). For each treatment condition, there were three samples per culture, and the experiment was repeated three times.

4.2.7. Statistical analysis

Data were analysed using IBM SPSS Statistics 23. For the characterisation of the culture, comparisons between medium and vehicle were determined using Student's t test. The effects of corticosterone on NPCs from the whole hippocampus were determined using one-way ANOVA. For all other conditions, two-way ANOVA was used. If a main effect reached statistical significance, group-wise comparisons were made using Fishers post-hoc test. Data are expressed as the mean + SEM. Criteria for statistical significance was set at $p<0.05$.

4.3. Results

4.3.1 Characterisation of NPC cultures

NPCs from whole hippocampus were allowed proliferate as neurospheres for 4 DIV prior to characterisation. We found that approximately 99% of the cells were nestin$^+$ suggesting that the majority of the cells were indeed neural progenitor cells (NPCs) (Suppl. Fig 4.2A). Dissociated NPCs were allowed to proliferate for 4 h in the presence of BrdU. We found that approximately 54% of these cells were BrdU$^+$ suggesting that NPCs were actively proliferating (Suppl. Fig 4.2B). To determine whether the vehicle (0.1% DMSO in culture medium) that would be used to dilute corticosterone and dexamethasone might affect the number of NPCs or cell proliferation, we compared the percentage of nestin$^+$ cells and the percentage of BrdU$^+$ cells in NPC cultures treated with the vehicle with those treated with growth medium. Student's t-test showed that the vehicle did not affect the percentage of NPCs (nestin$^+$ cells; $t(16)=0.08$, $p=0.94$; Suppl. Fig 4.2A) or cell proliferation (BrdU$^+$ cells; $t(16)=0.46$, $p=0.65$; Suppl. Fig 4.2B). Therefore, we used the vehicle as the control group for all further experiments investigating the effects of corticosterone or dexamethasone.

4.3.2 NPCs from the whole hippocampus express the glucocorticoid receptor (GR) and corticosterone decreases their proliferation

We first determined whether the glucocorticoid receptor (GR) was expressed on NPCs. As shown in Suppl. Fig 4.3A, GR was expressed on nestin$^+$ cells. The effects of corticosterone (4 h) on cell proliferation of NPCs from the whole hippocampus is shown in Suppl. Fig 4.3B-F. One-way ANOVA revealed that 4 h exposure to corticosterone decreased cell proliferation as indicated by a reduction in the percentage of BrdU$^+$ cells ($F_{2,24}=4.31$, $p=0.025$; Suppl. Fig 4.3B). Specifically, post

hoc analysis revealed that both concentrations of corticosterone reduced the percentage of $BrdU^+$ cells compared to the vehicle group (1 µM, p=0.011; 5 µM, p=0.031). Thus, for subsequent experiments examining the effects of corticosterone on cell proliferation and neuronal differentiation and maturation, we used these concentrations as well as including a lower concentration of 0.5 µM to investigate potential differences in sensitivity of dHi, iHi and vHi NPCs to CORT.

4.3.3. NPCs derived from the dHi, iHi or vHi do not exhibit differential sensitivity to acute corticosterone-induced reductions in NPC proliferation

The effects of corticosterone (CORT) on cell proliferation in NPC cultures derived from the dHi, iHi and vHi are shown in Fig 4.1A-M. Two-way ANOVA revealed a significant main effect of CORT ($F_{3,96}$=42.26, p<0.001), but not hippocampal sub-region ($F_{2,96}$=1.16, p=0.32) nor a CORT x hippocampal sub-region interaction ($F_{6,96}$=0.388, p=0.89) on NPC proliferation (Fig 4.1A). Specifically, post hoc analysis revealed that when compared to the respective vehicle-treated group, all CORT concentrations (0.5, 1 and 5 µM) reduced cell proliferation to a similar extent irrespective of the hippocampal sub-regions the NPCs were derived from [dHi (p=0.028, p<0.001, p<0.001, respectively); iHi (p=0.013, p<0.001, p<0.001, respectively); vHi (p=0.019, p<0.001, p<0.001, respectively)]. These CORT-induced reductions in NPC proliferation were not due to CORT-induced decreases in NPCs as CORT did not alter the percentage of $nestin^+$ cells (Suppl. Fig 4.4A-M).

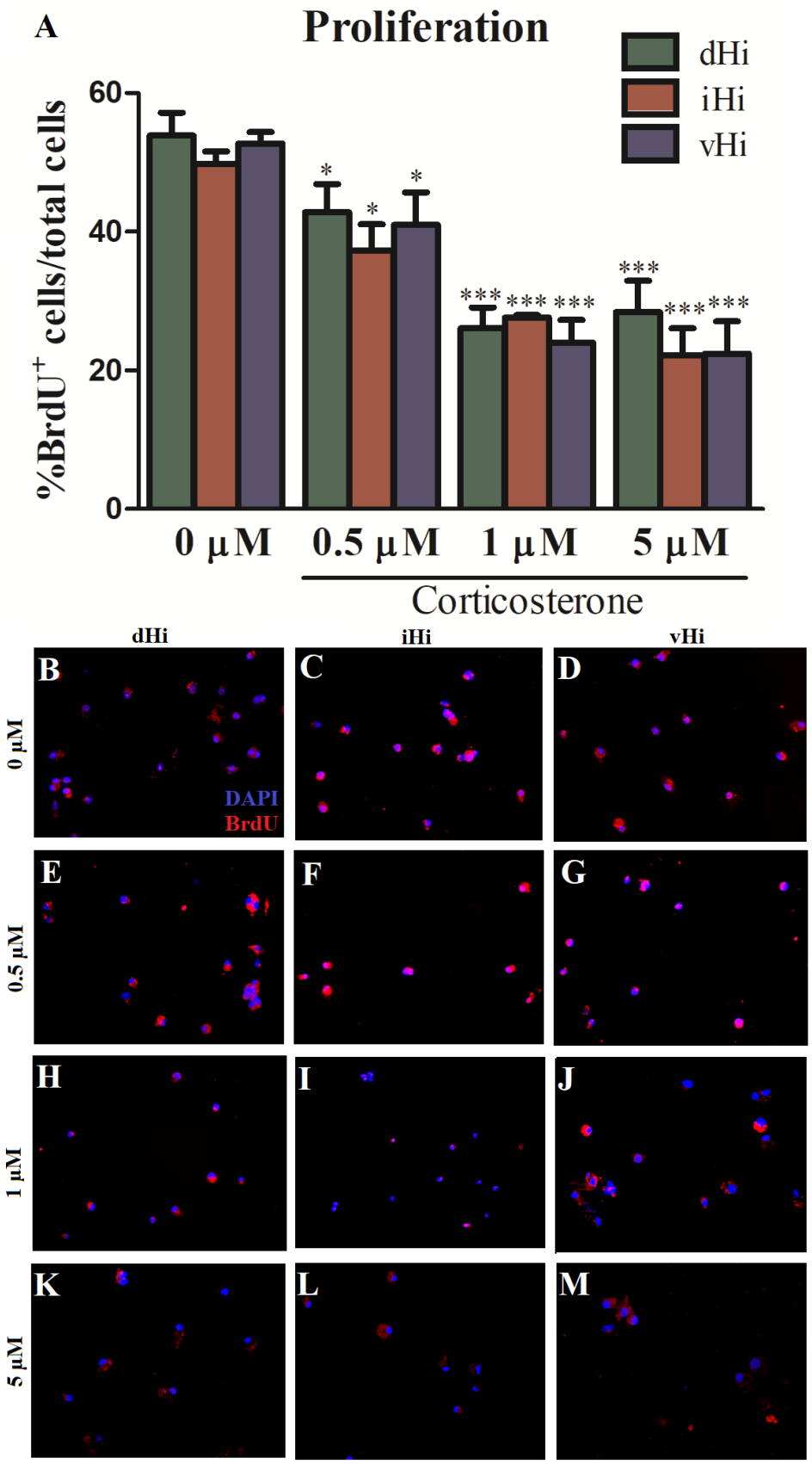


Figure 4.1*: Effects of acute corticosterone exposure on cell proliferation. A, corticosterone reduced cell proliferation and NPCs derived from the dHi, iHi and vHi do not exhibit differential sensitivity to acute corticosterone-induced reductions in cell proliferation; B-M, representative images of cells stained with DAPI (blue) and BrdU (red). *p<0.05 and ***p<0.001, compared with respective 0 µM group, according to Fishers LSD post hoc test.*

4.3.4. Short-term exposure to corticosterone reduced neuronal differentiation and maturation irrespective of the hippocampal sub-region

The effects of short-term exposure (4 DIV) to CORT on neuronal differentiation of NPCs derived from the dHi, iHi and vHi into immature neurons (DCX^+ cells) are shown in Fig 4.2A. Two-way ANOVA revealed a significant main effect of CORT ($F_{3,96}$=23.18, p<0.001), but not hippocampal sub-region ($F_{2,96}$=2.01, p=0.14), nor a CORT x hippocampal sub-region interaction ($F_{6,96}$=0.94, p=0.147) on the percentage of DCX^+ cells. Post hoc analysis revealed that all CORT concentrations (0.5, 1 and 5 µM) reduced neuronal differentiation of NPCs (derived from the dHi (p=0.04, p=0.003, p<0.001, respectively) and iHi (p=0.001, p=0.006, p<0.001, respectively), while only the concentrations of 1 and 5 µM reduced neuronal differentiation of NPCs derived from vHi (p=0.001 and p<0.001, respectively).

As an index of neuronal maturation, we calculated the percentage of DCX^+ cells that had a primary or a secondary process (Fig 4.2B-Q) and we also calculated the average length of these processes (Fig 4.2D,E). Two-way ANOVA revealed a significant main effect of CORT ($F_{3,96}$=5.88, p=0.001), and of hippocampal sub-region ($F_{2,96}$=7.66, p=0.001), but not a CORT x hippocampal sub-region interaction ($F_{6,96}$=0.33, p=0.92) on the percentage of DCX^+ with primary processes (Fig 4.2B). Post hoc analysis revealed that that the percentage of DCX^+ cells with a primary process was significantly reduced by 1 µM and 5 µM of CORT in cells derived from the iHi (p=0.016, p=0.015, respectively), while only 5 µM CORT significantly reduced the percentage of DCX^+ with a primary process in cells derived from the dHi (p=0.02) and vHi (p=0.049).

On the other hand, when we examined the impact of CORT on the percentage of DCX^+ cells with a secondary process, we found that CORT decreased this population of immature neurons to a similar extent in all hippocampal sub-regions. Indeed, two-way ANOVA revealed a significant main effect of CORT ($F_{3,96}$=14.92, p<0.001), but not hippocampal sub-region ($F_{2,96}$=0.43, p=0.65), nor a CORT x hippocampal sub-region interaction ($F_{6,96}$=0.58, p=0.75) on the percentage of DCX^+ cells with secondary processes (Fig 4.2C). Post hoc analysis revealed that the percentage of DCX^+ with a secondary process was reduced by all CORT concentrations (0.5, 1 and 5 µM) in NPC cultures derived from the dHi (p=0.008, p=0.008, p<0.001, respectively), iHi (p=0.003, p<0.001, p=0.002, respectively) and vHi (p=0.012, p=0.04, p<0.001, respectively).

As another index of neuronal maturation, we measured the average length of these primary and secondary dendrites. The effects of CORT on primary processes length are shown in Fig 4.2D. Two-way ANOVA revealed a significant effect of CORT ($F_{3,96}$=5.48, p=0.002) but no effect of hippocampal sub-region ($F_{2,96}$=2.98, p=0.55) nor a CORT x hippocampal sub-region interaction ($F_{6,96}$=0.32, p=0.92). Post hoc analysis showed that the effects of CORT were dose-dependent. 5 µM CORT significantly reduced the primary process length in DCX^+ cells derived from the dHi (p=0.025) and the vHi (p=0.016) while 1 µM CORT significantly reduced primary process length in DCX^+ cells derived from iHi NPC cultures only (p=0.018).

The effects of CORT on secondary processes length are shown in Fig 4.2E. Two-way ANOVA revealed a significant effect of CORT ($F_{3,96}$=16.23, p<0.001), but not hippocampal sub-region ($F_{2,96}$=0.54, p=0.58) nor a CORT x hippocampal sub-

region interaction ($F_{6,96}$=0.35, p=0.91). Post hoc test revealed that all CORT concentrations (0.5, 1 and 5 µM) reduced secondary process length in DCX^+ cells irrespective of the sub-region they were derived from [dHi (p=0.049, p=0.01, p<0.001, respectively); iHi (p=0.002, p=0.049, p<0.001, respectively); vHi (p=0.012, p=0.007, p<0.001)] and there were no sub-regional differences in this effect. The effects of corticosterone on the total average dendritic length was also measured and no clear sub-regional effect was observed (Suppl. Fig 4.5).

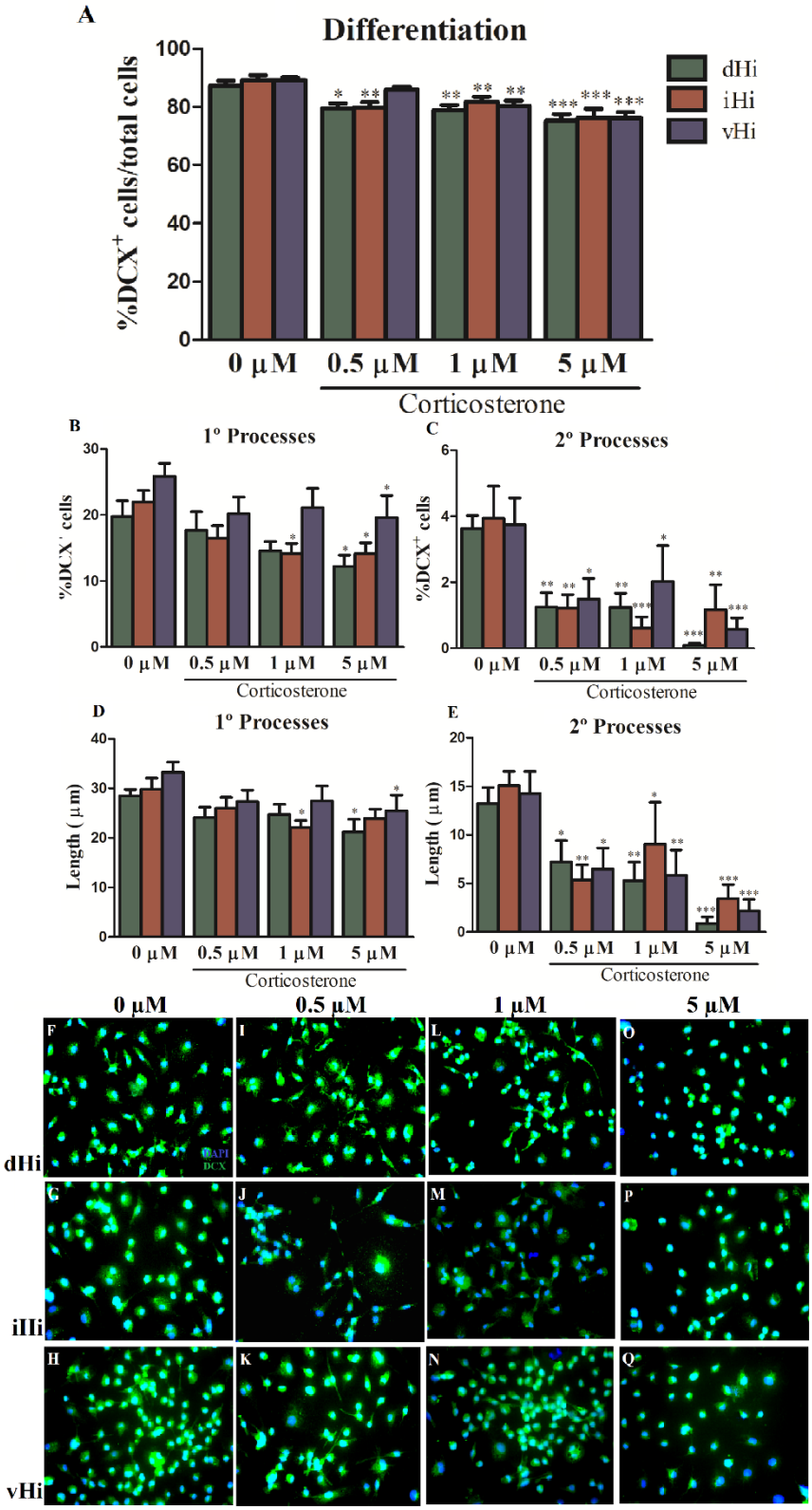


***Figure 4.2**: Effects of short-term corticosterone exposure on NPCs differentiation into immature neurons (DCX$^+$) and their maturation. A, short-term exposure to corticosterone reduces neuronal differentiation irrespective of the hippocampal sub-region; B-E, short-term exposure to corticosterone reduces neuronal maturation in all hippocampal sub-regions to a similar extent, reducing the percentage of DCX cells with primary (B) and secondary processes (C) and the length of these processes (D-E); F-Q, representative images of cells stained with DAPI (blue) and DCX (green). *p<0.05, **p<0.01, ***p<0.001, compared with respective 0 µM group, according to Fishers LSD post hoc test.*

4.3.5. Longer-term exposure to corticosterone decreased neuronal differentiation in NPCs derived from the vHi but not the dHi, while iHi NPCs show an intermediate response.

The effects of longer-term exposure (7 DIV) to CORT on neuronal differentiation (β3-tubulin$^+$ cells) of NPCs derived from the dHi, iHi and vHi and are shown in Fig 4.3A. Two-way ANOVA revealed a significant main effect of CORT ($F_{3,96}$=11.85, p<0.001), hippocampal sub-region ($F_{2,96}$=24.36, p<0.001) and a CORT x hippocampal sub-region interaction ($F_{6,96}$=3.68, p=0.002) on the percentage of β3-tubulin$^+$ cells. Post hoc analysis revealed that none of the concentrations of CORT affected neuronal differentiation in NPCs derived from the dHi (0.5 µM, p=0.82; 1 µM, p=0.77; 5 µM, p=0.9) but all concentrations of CORT (0.5, 1, 5 µM) reduced neuronal differentiation of NPCs derived from the iHi (p=0.015, p=0.003, p=0.005, respectively), and the vHi (all p<0.001). CORT-induced reductions in neuronal differentiation were most pronounced in the NPCs derived from the vHi. Indeed, 0.5, 1 µM and 5 µM CORT induced a greater reduction in β3-tubulin$^+$ cells differentiated from vHi NPCs, but not those from iHi NPCs, compared with those differentiated from dHi NPCs (p<0.001 for all concentrations of CORT), and from the vHi compared to those differentiated from iHi NPCs (0.5 µM, p=0.018; 1 µM, p=0.006; 5 µM, p<0.001).

4.3.6. Neurons derived from vHi NPCs are more sensitive to reductions in neuronal maturation induced by longer-term corticosterone exposure

As an index of neuronal maturation, we calculated the percentage of β3-tubulin$^+$ cells that had primary, secondary, or tertiary processes (Fig 4.3B-S). Two-way ANOVA revealed a significant main effect of CORT ($F_{3,96}$=48,94, p<0.001), a trend

for hippocampal sub-region ($F_{2,96}$=2.56, p=0.082) but no CORT x hippocampal sub-region interaction ($F_{6,96}$=1.47, p=0.2) on the percentage of β3-tubulin$^+$ cells with a primary process (Fig 4.3B). Specifically, post hoc analysis revealed that the percentage of β3-tubulin$^+$ cells with a primary process is reduced by all concentrations of CORT irrespective of the hippocampal sub-regions they were derived from (all p<0.001). However, the number of β3-tubulin$^+$ cells with a primary process treated with 1uM CORT was higher in dHi-derived β3-tubulin$^+$ cells than vHi cells (p=0.03) and the number of β3-tubulin$^+$ cells with a primary process treated with 5 μM CORT was higher in dHi-derived β3-tubulin$^+$ cells than iHi cells treated with the same concentration (p=0.013).

When we measured the number of β3-tubulin$^+$ cells with secondary processes, we observed sub-regional effects of CORT. Two-way ANOVA revealed an overall effect of CORT ($F_{3,96}$=73.45, p<0.001), hippocampal sub-region ($F_{2,96}$=11.66, p<0.001), and a marginal CORT x hippocampal sub-region interaction ($F_{6,96}$=2.17, p=0.053) on the percentage of β3-tubulin$^+$ cells with secondary processes (Fig 4.3C). Post hoc analysis revealed that the percentage of β3-tubulin$^+$ cells with secondary processes is reduced by all concentrations of CORT irrespective of the hippocampal sub-regions they were derived from (all p<0.001). However, fewer β3-tubulin$^+$ cells derived from the vHi NPCs had secondary processes when compared to cells from the dHi [0.5 μM (p=0.006), 1 μM (p=0.001), 5 μM CORT (p=0.001)] or to cells from the iHi [1 μM CORT (p=0.01)]. In addition, when treated with 5 μM CORT, the percentage of β3-tubulin$^+$ cells with secondary processes derived from the iHi NPCs was reduced in comparison to those derived from the dHi NPCs (p=0.008).

When we measured the percentage of β3-tubulin$^+$ cells with a tertiary process, two-way ANOVA revealed an overall effect of CORT ($F_{3,96}$=25.59, p<0.001), hippocampal sub-region ($F_{2,96}$=3.92, p=0.023), but no CORT x hippocampal sub-region interaction ($F_{6,96}$=0.31, p=0.93) (Fig 4.3D). Specifically, post hoc analysis revealed that the percentage of β3-tubulin$^+$ cells with tertiary processes was reduced by all concentrations of CORT (0.5, 1 and 5 μM) in cells differentiated from NPCs irrespective of the hippocampal sub-regions they were derived from (dHi, p=0.002, p<0.001, p<0.001, respectively; iHi, p<0.001 for all; vHi, p<0.001 for all).

We also used dendritic complexity as an index of neuronal maturation by measuring the lengths of primary, secondary and tertiary processes on β3-tubulin$^+$ cells. (Fig 4.3E-G). Two-way ANOVA on the length of primary dendritic processes revealed a significant effect of CORT ($F_{3,96}$=21.16, p<0.001), hippocampal sub-region ($F_{2,96}$=7.54, p=0.001) and a CORT x hippocampal sub-region interaction ($F_{6,96}$=3.35, p=0.005) (Fig 4.3E). Post hoc analysis revealed that the concentration of 0.5 μM of CORT had no effect on primary process length of β3-tubulin$^+$ cells derived from the dHi (p=0.3) but it decreased primary process length in cells derived from the iHi and vHi (p=0.022, p<0.001). Although both of the higher concentrations of CORT (1 and 5 μM) reduced primary process length irrespective of the hippocampal sub-region (dHi: p=0.036, p=0.038, respectively; iHi: p=0.011, p=0.002, respectively; vHi: both p<0.001), these effects were most pronounced in β3-tubulin$^+$ cells derived from the vHi. Indeed, all concentrations of CORT decreased primary dendritic process length to a greater extent in vHi-derived β3-tubulin$^+$ cells compared with those from the dHi (0.5 μM, p=0.003; 1 μM, p=0.003; 5 μM, p=0.006) and those from the iHi (0.5 μM, p=0.025; 1 μM, p=0.003; 5 μM, p=0.022).

Two-way ANOVA on the length of secondary dendritic processes (Fig 4.3F) revealed a significant effect of CORT ($F_{3,96}$=15.11, p<0.001), hippocampal sub-region ($F_{2,96}$=14.68, p<0.001), and a CORT x hippocampal sub-region interaction between them ($F_{6,96}$=5.15, p<0.001). Post hoc analysis revealed that while none of the CORT concentrations (0.5 µM, 1 µM and 5 µM) altered secondary process length of β3-tubulin$^+$ cells derived from the dHi (p=0.62, p=0.14, p=0.37, respectively), all tested concentrations reduced secondary process length in β3-tubulin$^+$ cells derived from the vHi (p<0.001 in all cases). Interestingly, only the highest dose of CORT (5 µM) CORT reduced secondary process length in β3-tubulin$^+$ cells derived from the iHi (p=0.004). In addition, at all CORT concentrations, secondary dendritic process length of β3-tubulin$^+$ cells derived from the vHi were significantly shorter than those derived from the dHi (0.5 µM, p<0.001; 1 µM, p<0.001; 5 µM p<0.001) and those derived from the iHi (0.5 µM, p<0.001; 1 µM, p=0.001; 5 µM, p=0.038). Interestingly, 5 µM CORT-induced reductions in secondary dendritic process length were also greater in β3-tubulin$^+$ cells derived from the iHi than those from the dHi (p<0.001).

Two-way ANOVA on the length of tertiary processes (Fig 4.3G) in β3-tubulin$^+$ cells revealed a main effect of CORT ($F_{3,96}$=14.43, p<0.001), hippocampal sub-region ($F_{2,96}$=8.19, p=0.001), and a CORT X hippocampal sub-region interaction ($F_{6,96}$=3.98, p=0.001). While post hoc analysis revealed that none of the CORT concentrations (0.5 µM, 1 µM and 5 µM) altered tertiary process length of β3-tubulin$^+$ cells derived from the dHi (p=0.47, p=0.17, p=0.49, respectively), all doses reduced tertiary processes length in cells derived from the iHi (p=0.002, p=0.008, p=0.003, respectively) and the vHi (all p<0.001) but that these effects were most

pronounced for the β3-tubulin^{+} cells derived from the vHi. Indeed, at all concentrations of CORT tertiary process length of β3-tubulin^{+} cells derived from the vHi were significantly shorter than those from the dHi (0.5 µM, p=0.003; 1 µM, p=0.01; 5 µM p=0.02). Similarly, at 1µM CORT, these processes of β3-tubulin^{+} cells derived from the vHi were significantly shorter than those derived from the iHi (p=0.035). Corticosterone also reduced the total average dendritic length in a region-dependent manner (Suppl. Fig 4.6A).

An MTT assay revealed that increased sensitivity of vHi NPC cultures to CORT-induced reduction in neuronal differentiation and maturation is not due to reduced cell viability [Suppl. Fig 4.6B; CORT ($F_{3,96}$=2.05, p=0.11); hippocampal sub-region ($F_{2,96}$=0.3, p=0.75), CORT X hippocampal sub-region ($F_{6,96}$=0.85, p=0.54)]

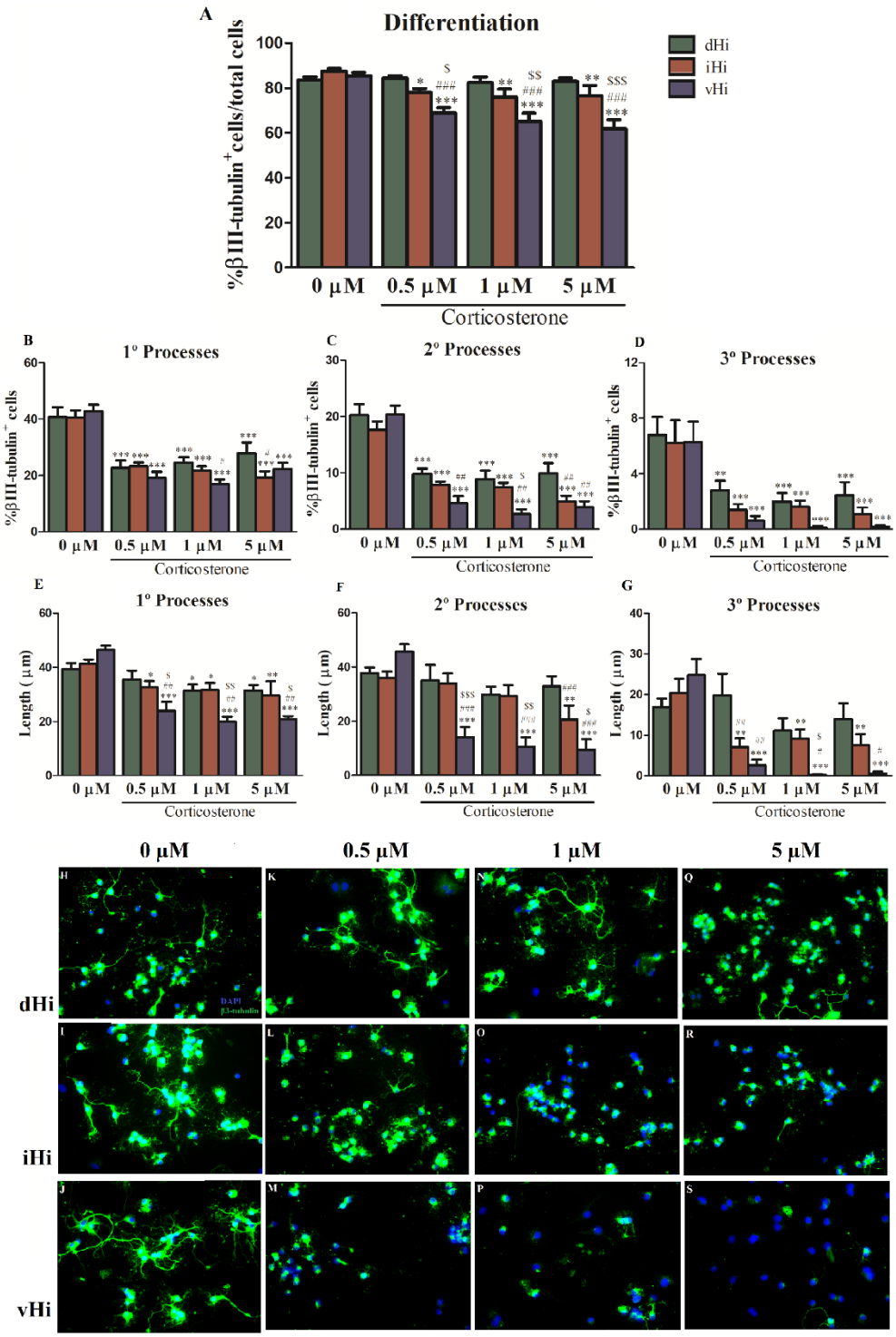


***Figure 4.3**: Effects of longer-term corticosterone exposure on NPCs differentiation into neurons (β3-tubulin$^+$) and their maturation. A, corticosterone reduces neuronal differentiation, especially on cells derived from the vHi; B-G, corticosterone reduces neuronal maturation, especially in cells derived from the vHi, reducing the percentage of β3-tubulin cells with primary (B), secondary (C) and tertiary processes (D) and the length of these processes (E-G); H-S, representative images of cells stained with DAPI (blue) and β3-tubulin (green). *p<0.05, **p<0.01, ***p<0.001, compared with respective 0 µM group, #p<0.05, ##p<0.01, ###p<0.001, compared to cells derived from the dHi treated with the same concentration of corticosterone, $p<0.05, $$p<0.01, $$$p<0.001, compared to cells derived from the iHi treated with the same concentration of corticosterone.*

4.3.7. The long-term corticosterone effects in NPCs, at least in part, are due to changes in GR expression

Glucocorticoids receptors have been shown to regulate hippocampal neurogenesis (Anacker *et al.* 2011, Saaltink and Vreugdenhil 2014) and these receptors are expressed on NPCs derived from the dHi, iHi and vHi (Suppl. Fig 4.8A-C). To assess whether the increased sensitivity of vHi-derived NPC cultures to longer-term corticosterone exposure might be related to differential effects of corticosterone on GR, we measured nuclear GR expression in dHi, iHi and vHi cultures after long-term (7 DIV) exposure to corticosterone (Suppl. Fig 4.8D-P). Two-way ANOVA revealed a significant main effect of CORT ($F_{3,96}$=38.37, p<0.001), hippocampal sub-region ($F_{2,96}$=4.43, p=0.014) but not a CORT x hippocampal sub-region interaction ($F_{6,96}$=0.69, p=0.66) on nuclear GR expression (Suppl. Fig 4.8D). Specifically, post hoc analysis showed that all concentrations of CORT (0.5, 1 and 5 µM) reduced nuclear GR expression when compared to vehicle-treated cells derived from the dHi (p=0.003, p<0.001, p<0.001, respectively); iHi (p<0.001 in all cases); vHi (p<0.001 in all cases). However, vHi cells treated with 5 µM of CORT had lower nuclear GR expression compared to dHi-derived cells treated with the same dose of CORT (p=0.049). In addition, iHi-derived cells treated with either 1 µM or 5 µM of CORT had lower nuclear GR expression compared with dHi-derived cells treated with the same dose (1 µM p=0.035, 5 µM p=0.049, respectively).

4.3.8. The GR agonist dexamethasone decreased neuronal differentiation in NPCs derived from the vHi but not the dHi, while iHi NPCs show an intermediate response

The effects of long-term (7 DIV) DEX exposure on βIII-tubulin$^+$ cells derived from the dHi, iHi and vHi are shown in Fig 4.4. Two-way ANOVA revealed a significant main effect of DEX ($F_{3,96}$=13.02, p<0.001), hippocampal sub-region ($F_{2,96}$=106.48, p<0.001) and a DEX x hippocampal sub-region interaction ($F_{6,96}$=6.36, p<0.001) on the percentage of βIII-tubulin$^+$ cells (Fig 4.4A). Post hoc analysis revealed that none of the concentrations of DEX affected βIII-tubulin$^+$ cells derived from the dHi (10 nM, p=0.94; 100 nM, p=0.87; 1000 nM, p=0.36) but all concentrations of CORT (0.5, 1, 5 μM) reduced the percentage of βIII-tubulin$^+$ cells derived from the iHi (p=0.02, p=0.018, p=0.003, respectively), and the vHi (all p<0.001). Moreover, DEX-induced reductions in βIII-tubulin$^+$ cells were most pronounced in NPCs derived from the vHi. Indeed, 10, 100 and 1000 nM DEX induced a greater reduction in the percentage of β3-tubulin$^+$ cells differentiated from vHi NPCs compared with those differentiated from dHi NPCs (p<0.001 for all concentrations of DEX), and those differentiated from iHi NPCs (p<0.001 for all concentrations of DEX). Also, 10, 100 and 1000 nM DEX induced a greater reduction in β3-tubulin$^+$ cells differentiated from the iHi NPCs compared with those differentiated from dHi (p<0.001 for all concentrations of DEX). It is also important to note that, in vehicle-treated cells, the percentage of differentiated neurons in the vHi was lower than dHi (p=0.037).

4.3.9. Neurons derived from vHi NPCs are more sensitive to reductions in neuronal maturation induced by dexamethasone exposure

As an index of neuronal maturation, we calculated the percentage of β3-tubulin$^+$ cells that had a primary, secondary or a tertiary process (Fig 4.4B-D) and also measured the length of these dendritic processes (Fig 4.4E-G). Primary, secondary and tertiary processes are show in in Fig 2.4H. Two-way ANOVA revealed a

significant main effect of DEX ($F_{3,96}$=68.31, p<0.001), hippocampal sub-region ($F_{2,96}$=17.05, p<0.001) and a DEX x hippocampal sub-region interaction ($F_{6,96}$=4.35, p=0.001) on the percentage of β3-tubulin$^+$ cells with a primary process (Fig 2.4B). Specifically, post hoc analysis shows that although all concentrations of DEX (10, 100 and 1000 nM) decreased the percentage of β3-tubulin$^+$ cells with a primary process irrespective of the hippocampal sub-region they were derived from (dHi: p=0.005, p<0.001, p<0.001, respectively; iHi: p<0.001 in all cases; vHi: p<0.001 in all cases), these DEX-induced reductions were most pronounced in cultures derived from the vHi. Indeed, 10, 100 and 1000 nM DEX induced a greater reduction in the percentage of β3-tubulin$^+$ cells with primary processes in vHi cultures compared with those in dHi cultures (p<0.001, p<0.001, p=0.001, respectively) and iHi cultures (p=0.006, p= 0.001, p=0.002, respectively).

We found a similar pattern of effects by DEX when we measured the percentage of β3-tubulin$^+$ cells that had a secondary process. Two-way ANOVA revealed an overall effect of DEX ($F_{3,96}$=91.99, p<0.001), hippocampal sub-region ($F_{2,96}$=14.77, p<0.001), and a DEX x hippocampal sub-region interaction ($F_{6,96}$=2.9, p=0.012) on the percentage of these cells (Fig 4.4C). Post hoc analysis revealed that although the percentage of β3-tubulin$^+$ cells with secondary processes was reduced by all concentrations of DEX irrespective of the hippocampal sub-regions they were derived from (all p<0.001), these reductions were most pronounced in the vHi-derived cultures. 10, 100 and 1000 nM DEX induced a greater reduction in the percentage of β3-tubulin$^+$ cells with a secondary process in vHi cultures compared with those in dHi cultures (p<0.001, p<0.001, p=0.001, respectively). 10 nM DEX also induced a greater reduction in the percentage of β3-tubulin$^+$ cells with a secondary process in vHi cultures compared with those in iHi cultures (p=0.01). In

addition, iHi-derived cells were more sensitive to these effects than dHi-derived cells when treated with 100 nM DEX ($p=0.01$) thus suggesting a gradient effect of DEX along the dorso-ventral axis.

Although only approximately 8% of β3-tubulin$^+$ cells developed a tertiary process, we also assessed the impact of DEX on these cells. Two-way ANOVA demonstrated an overall effect of DEX ($F_{3,96}=41.02$, $p<0.001$), hippocampal sub-region ($F_{2,96}=5.07$, $p=0.001$), and no DEX x hippocampal sub-region interaction ($F_{6,96}=0.82$, $p=0.55$) on the percentage of β3-tubulin cells with tertiary processes (Fig 4.4D). Specifically, post hoc testing revealed that although the percentage of β3-tubulin$^+$ cells with a tertiary process is reduced by all concentrations of DEX (10, 100 and 1000 nM) in cells differentiated from NPCs irrespective of the hippocampal sub-regions they were derived from (dHi, $p=0.001$, $p<0.001$, $p<0.001$, respectively; iHi, all $p<0.001$; vHi, all $p<0.001$), the effects of the lowest dose (10 nM DEX) was most pronounced in the vHi cultures [vHi 10 nM DEX vs. dHi 10 nM DEX, $p<0.004$)].

We also used dendritic complexity as an index of neuronal maturation by measuring the lengths of primary, secondary and tertiary processes on β3-tubulin$^+$ cells (Fig 4.4E-G). Two-way ANOVA on the length of primary dendritic processes (Fig 4.4E) revealed a significant effect of DEX ($F_{3,96}=99.53$, $p<0.001$), hippocampal sub-region ($F_{2,96}=12.26$, $p<0.001$) and a DEX X hippocampal sub-region interaction ($F_{6,96}=4.5$, $p<0.001$). Although post hoc analysis revealed that all DEX concentrations (10, 100 and 1000 nM) significantly decreased primary process lengths irrespective of the hippocampal sub-regions the cells were derived from (all $p<0.001$), these effects were most pronounced in β3-tubulin$^+$ cells derived from the

vHi. Indeed, all concentrations of DEX decreased primary dendritic process length to a greater extent in vHi-derived β3-tubulin$^+$ cells compared with those from the dHi (10 nM, $p<0.001$; 100 nM, $p=0.001$; 1000 nM, $p<0.001$, respectively), and at the 10 nM concentration the primary dendritic process lengths of vHi-derived β3-tubulin$^+$ cells were shorter than those from the iHi ($p=0.005$). Interestingly, 100 and 1000 nM DEX-induced reductions in primary dendritic process length were also greater in β3-tubulin$^+$ cells derived from the iHi than those from the dHi ($p=0.015$, $p=0.004$, respectively).

Two-way ANOVA on the length of secondary dendritic processes (Fig 4.4F) revealed a significant effect of DEX ($F_{3,96}=50.63$, $p<0.001$), hippocampal sub-region ($F_{2,96}=46.32$, $p<0.001$), and a DEX X hippocampal sub-region interaction ($F_{6,96}=6.75$, $p<0.001$). Post hoc analysis revealed that while all concentrations of DEX (10, 100 and 1000 nM) reduced secondary process length of β3-tubulin$^+$ cells derived from the vHi (all $p<0.001$) and the iHi ($p=0.001$, $p<0.001$, $p<0.001$, respectively) only the higher concentrations of 100 and 1000 nM DEX reduced the secondary dendritic process length of cells derived from the dHi ($p=0.034$, $p=0.005$, respectively). Interestingly, DEX-induced decreases in secondary dendritic process length were more pronounced in cells derived from the iHi compared to cells from dHi (10 nM, $p=0.013$; 100 nM, $p<0.001$; 1000 nM, $p<0.001$). However, DEX-induced decreases in secondary dendritic process length were more pronounced in the cells derived from the vHi when compared to both cells from the dHi (10 nM, $p<0.001$; 100 nM, $p<0.001$; 1000 nM, $p<0.001$) and the iHi (10 nM, $p<0.001$; 1000 nM, $p=0.025$).

We also observed a similar gradient-like effect of DEX when we measured tertiary dendritic process length of β3-tubulin^{+} cells (Fig 4.4G). Two-way ANOVA revealed a main effect of DEX ($F_{3,96}$=19.97, p<0.001) and hippocampal sub-region ($F_{2,96}$=33.19, p<0.001), but no DEX X hippocampal sub-region interaction ($F_{6,96}$=1.54, p=0.17). Post hoc analysis revealed that while all concentrations of DEX (10, 100 and 1000 nM) reduced the tertiary dendritic process length of β3-tubulin^{+} cells derived from the vHi (all p<0.001) and iHi (p=0.002, p<0.001, p<0.001, respectively) only 100 nM and 1000 nM DEX reduced tertiary dendritic process length in dHi-derived cells (p=0.02, p=0.001, respectively). DEX-induced decreases in tertiary dendritic process length were more pronounced in the cells derived from the iHi and the vHi when compared to cells from the dHi but and this effect appeared to be more robust in the vHi-derived cells [(dHi vs. iHi: 10 nM, p<0.001; 100 nM, p=0.01; 1000 nM, p=0.009); dHi vs vHi: 10 nM p<0.001; 100 nM p<0.001; 1000 nM, p=0.002).

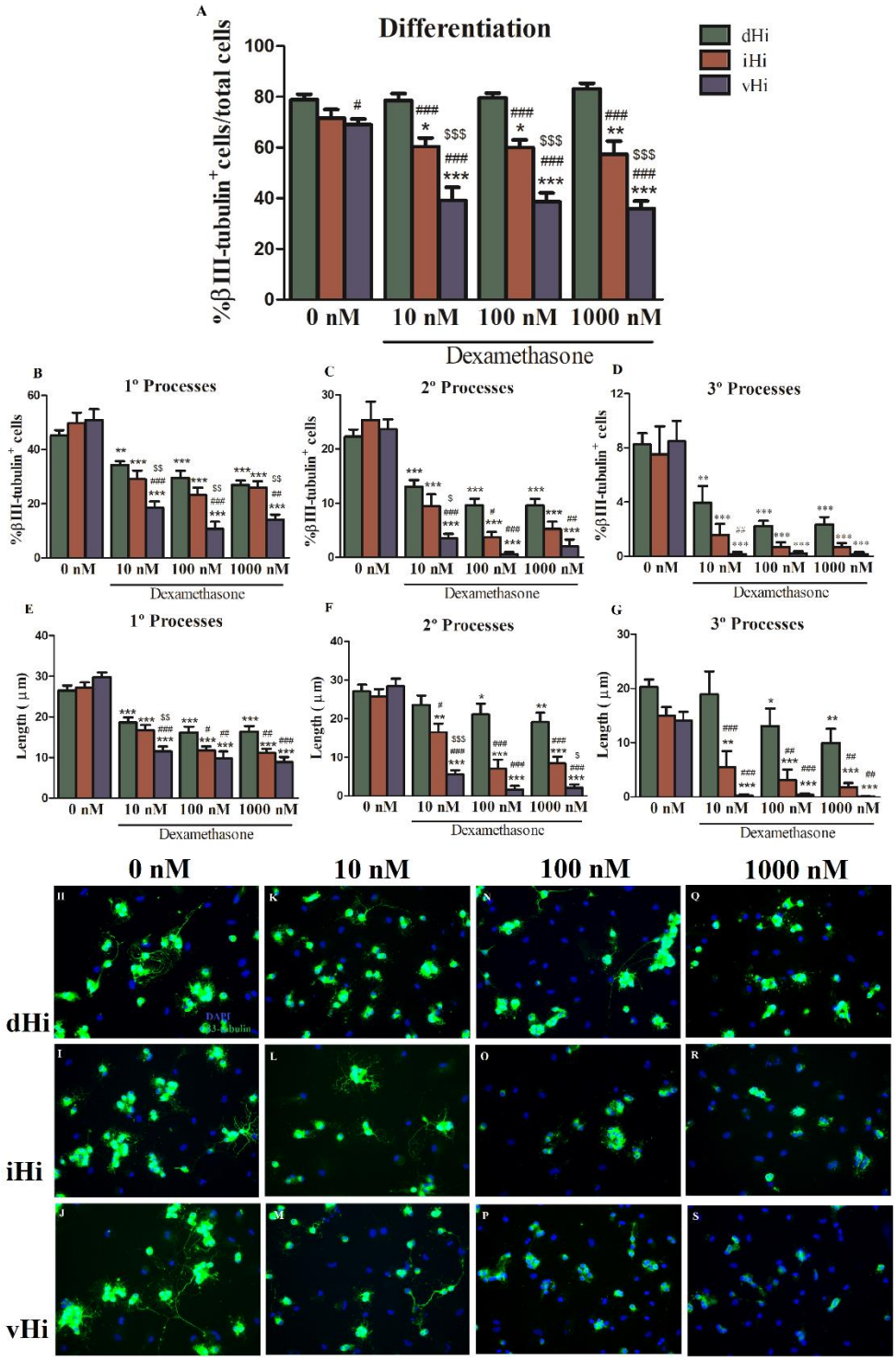


Figure 4.4: *Effects of long-term dexamethasone exposure on NPCs differentiation into neurons (β3-tubulin^{+}) and their maturation. A, dexamethasone reduces neuronal differentiation, specifically on cells derived from the iHi and especially from the vHi; B-G, dexamethasone reduces neuronal maturation, especially in cells derived from the vHi, reducing the percentage of β3-tubulin cells with primary (B), secondary (C) and tertiary processes (D) and the length of these processes (D-F); H-S, representative images of cells stained with DAPI (blue) and β3-tubulin (green). *p<0.05, **p<0.01, ***p<0.001, compared with respective 0 nM group, #p<0.05, ##p<0.01, ###p<0.001, compared to cells derived from the dHi treated with the same concentration of dexamethasone, $p<0.05, $$p<0.01, $$$p<0.001, compared to cells derived from the iHi treated with the same concentration of dexamethasone.*

Dexamethasone also decreased the total average dendritic length on β3-tubulin^{+} cells in a region-dependent manner (Suppl. Fig 4.7).

4.3.10. Dexamethasone-induced reductions in nuclear GR expression are greater in vHi-derived cultures than dHi-derived cultures

To assess whether the increased sensitivity of vHi-derived cultures to dexamethasone-induced reductions in neuronal differentiation and maturation may related to reduced GR nuclear translocation, we measured GR expression in cell nuclei after long-term (7 DIV) dexamethasone (DEX) exposure (Fig 4.5A-M). Two-way ANOVA revealed a significant main effect of DEX ($F_{3,96}$=26.0, p<0.001), hippocampal sub-region ($F_{2,96}$=6.12, p=0.003), but no DEX x hippocampal sub-region interaction ($F_{6,96}$=0.95, p=0.46) (Fig 4.5A). Post hoc analysis revealed that all doses of DEX (10, 100, 100 nM) reduced nuclear GR expression irrespective of the hippocampal sub-region [dHi (p=0.016, p=0.003, p=0.003, respectively); iHi (p=0.002, p<0.001, p<0.001, respectively); vHi (all p<0.001)]. However, the magnitude of effects of 10 nM and 100 nM DEX were more robust in vHi-derived cells than those derived from the dHi (10 nM dHi vs 10 nM vHi p=0.011; 100 nM dHi vs 100 nM vHi p=0.008).

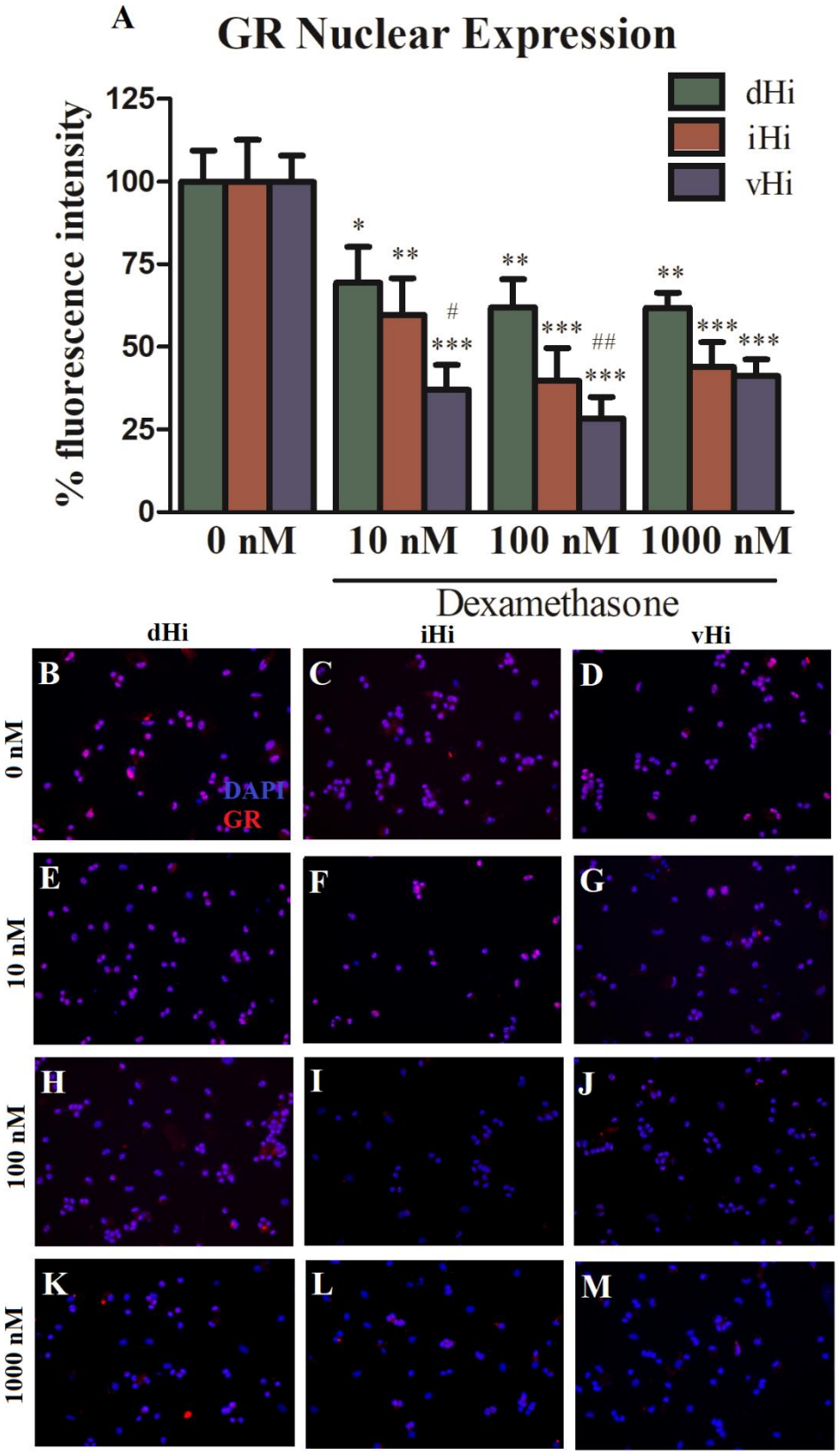


Figure 4.5*: Effects of long-term dexamethasone exposure on nuclear GR expression. A, dexamethasone-induced reductions in nuclear GR expression are greater in vHi-derived cultures; B-M, representative images of cells stained with DAPI (blue) and GR (red). *p<0.05, **p<0.01, ***p<0.001, compared with respective 0 nM group, #p<0.05, ##p<0.01, compared to cells derived from the dHi treated with the same concentration of dexamethasone, according to Fishers LSD post hoc test.*

4.4. Discussion

Stress has been shown to affect AHN preferentially in the ventral sub-region of the longitudinal axis of the hippocampus, but the mechanisms remain unresolved. Here we show differential intrinsic sensitivity of NPCs residing in this hippocampal sub-region to the stress hormone, corticosterone which offers a possible explanation for such effects. We observed that NPCs derived from the dHi, iHi and vHi did not exhibit differential sensitivity to acute (4 h) or short-term (4 DIV) corticosterone-induced reductions in cell proliferation and neuronal differentiation. However, longer-term (7 DIV) treatment with corticosterone or the glucocorticoid receptor (GR) agonist dexamethasone, decreased neuronal differentiation in cultures derived from the vHi but not the dHi, while iHi cultures showed a response intermediate to those of the dHi and vHi. A similar gradient-like response to corticosterone-induced and dexamethasone-induced reductions in both neuronal maturation and in GR nuclear translocation was also observed.

In the present study, acute corticosterone showed detrimental effects on the proliferation of hippocampal NPCs, which was expected and in agreement with *in vivo* experiments that demonstrate that corticosterone and chronic stress reduce cell proliferation in the dentate gyrus of the hippocampus of rats (Jayatissa *et al.* 2006, Brummelte and Galea 2010). Acute stress has also been shown to transiently reduce hippocampal NPCs proliferation (Falconer and Galea 2003, Heine *et al.* 2004) in rats. *In vitro* studies show that short and long-term glucocorticoids exposure reduce hippocampal NPCs proliferation (Boku *et al.* 2009, Anacker *et al.* 2013) in human and rat NPCs. Here we also show that acute corticosterone decreases proliferation of NPCs to a similar extent in cells derived from the three hippocampal sub-regions. Several *in vivo* studies have reported that corticosterone and chronic stress decrease

proliferation of NPCs, preferentially in the vHi rather than the dHi (Tanti and Belzung 2013, O'Leary and Cryan 2014). There is also evidence that acute restraint stress in rats reduces proliferation of hippocampal NPCs, preferentially in the vHi (Kim *et al.* 2005). This may indicate that the acute effects of stress or corticosterone on NPCs proliferation observed *in vivo* are not due to the intrinsic characteristics of the NPCs residing in each hippocampal sub-region, but to other extrinsic factors. It should also be noted that stress-induced reductions in NPCs proliferation are through cell cycle arrest (Heine *et al.* 2005). In rats, chronic stress has also been shown to elevate levels of p27Kip1, an endogenous cell cycle inhibitor, in the DG, which suggests that stress prevents the NPCs from re-entering the cell cycle (Heine *et al.* 2004).

Corticosterone induced detrimental effects on the differentiation of NPCs into neurons as well as their maturation. Short-term CORT exposure reduced the percentage of NPCs differentiated into immature neurons (DCX cells) in all sub-regions, as well as having a detrimental effect on the development of these DCX cells. Interestingly, many *in vivo* studies showed that the ventral part of the hippocampus tend to be more sensitive to corticosterone and to the effects of chronic stress in cell differentiation (Tanti and Belzung 2013, O'Leary and Cryan 2014), but these studies used coronal sections for the segregation of the hippocampus into dHi and vHi only.

Longer-term exposure to corticosterone also reduced the differentiation of NPCs into neurons and their maturation. Here we observed that, in these conditions, corticosterone reduced the percentage of NPCs differentiated into neurons (βIII-tubulin^{+} cells) in cells derived from all hippocampal sub-regions. However, cells

derived from the iHi and especially the vHi are more sensitive to this long-term CORT effect. Raising levels of circulating glucocorticoids are known to inhibit differentiation of hippocampal NPCs *in vivo* (Wong and Herbert 2006). The present results are also in line with *in vivo* studies that show that the vHi is more sensitive to the effects of chronic stress (Zuena *et al.* 2008, Brummelte and Galea 2010, Morley-Fletcher *et al.* 2011, Hawley *et al.* 2012, Tanti *et al.* 2012, Tanti and Belzung 2013). Moreover, here we show for the first time that cells from the iHi are also more sensitive to these CORT-induced effects on hippocampal neurogenesis and thus more attention should be given to this hippocampal sub-region in future studies that assess the effects of stress on AHN. Importantly, vHi is more sensitive to CORT only after long-term, but not short-term exposure. We suggest that the expression of GR may play a role in these changes, as it is known that the expression of the GR is variable during the neuronal differentiation of transient-amplifying cells to neuroblasts and then immature neurons (Garcia *et al.* 2004, Egeland *et al.* 2015).

Besides having detrimental effects in the differentiation of NPCs into neurons, longer-term CORT exposure also blunts the development of neuronal dendrites of the differentiated neurons derived from each hippocampal sub-region, but again preferentially in cells from the iHi and especially the vHi. It has previously been shown that chronic stress reduces hippocampal volume, causing dendritic atrophy (Lee *et al.* 2009) in rats. However, some studies show a specific decrease in hippocampal volume only in the dHi, with a corresponding increase in hippocampal volume in the vHi after chronic stress (Pinto *et al.* 2015). Nevertheless, few studies have reported the effects of stress on the maturation of neurons along the longitudinal axis of the hippocampus. It has been shown that stress reduces neuronal

maturation in rodents, preferentially in the vHi (Tanti *et al.* 2012) or not (Hawley *et al.* 2012). Chronic stress also reduces neuronal maturation in non-human primates (Perera *et al.* 2011), preferentially in the anterior hippocampus.

Here we used high concentrations of corticosterone, which are thought to demonstrate their effects on glucocorticoid receptors (Anacker *et al.* 2013). At low concentrations, corticosterone activates preferentially mineralocorticoid receptors (MR), which have more affinity to the stress hormone (De Kloet *et al.* 1998). Interestingly, previous studies show that GR is expressed only in 50% of the NPCs (Garcia *et al.* 2004, Boku *et al.* 2009, Egeland *et al.* 2015), while the MR is almost undetectable, but expressed once these cells are differentiated. To assess whether the preferential effects of long-term CORT exposure in cells from iHi and vHi are due to changes in GR translocation into the nucleus, we then assessed the expression of these receptors in the nuclei of cells after 7 DIV treatment with CORT. We observed that the longer-term treatment with CORT reduces the nuclear translocation of GR, specifically in cells derived from the iHi and vHi. Interestingly, it has been shown *in vivo* that 5 days stress is able to decrease GR immunoreactivity and that 20 days of stress can increase that, both preferentially in the dHi (Robertson *et al.* 2005). Also, blockade of GR prevents the negative effects of CORT administration on the proliferation of NPCs (Wong and Herbert 2005).

To follow up with these results, we then confirmed that the results were GR-dependent using the GR agonist, dexamethasone (DEX). The exposure of NPCs to DEX for 7 DIV reduced neuronal differentiation and maturation, in a more pronounced way than the CORT did. The susceptibility of vHi NPCs was even more clear, which indicates that the cells within this sub-region have unique intrinsic

characteristics in relation to the response to GR challenge. GR activation has already been shown to affect neurogenesis *in vitro*. Indeed, an *in vitro* study using human NPCs found that high levels of cortisol decreases proliferation and neuronal differentiation (Anacker *et al.* 2013). Importantly, corticosteroids activate glucocorticoid and mineralocorticoid receptors, translocating them from the cytosol into the nucleus. Interestingly, it has also been shown that chronically stressed rats have increased GR in the cytoplasm, but not in the nucleus in the vHi cells, while no changes were observed in the dHi cells (Guidotti *et al.* 2013).

A recent study suggests that the dHi has more active neurogenesis, as it is enriched in maturing neurons markers, such as NeuroD1 and DCX, while the vHi is enriched in radial glia markers, such as Sox2 and Hes5 (Zhang *et al.* 2018). This is in accordance to previous literature that shows that dHi NPCs display faster maturation rate, associated with higher basal network activity (Piatti *et al.* 2011). Interestingly, we did not observe major changes in the maturation rate in vehicle-treated NPCs, which reinforces *in vivo* data that the dHi NPCs faster maturation is connectivity-related, rather than intrinsic properties.

While there is a general agreement that chronic stress decreases AHN, it is important to note that controverse findings have also been reported (Hanson *et al.* 2011, O'Leary *et al.* 2012). These conflicting findings could be explained as a genomic heterogeneity has also been shown to occur along the longitudinal axis of the hippocampus of rodents, primates and humans (Thompson *et al.* 2008, Hawrylycz *et al.* 2012, Strange *et al.* 2014, O'Reilly *et al.* 2015) and recently it has been proposed that the hippocampus is a gradient, and the presence of an intermediate hippocampus (iHi) is suggested (Strange *et al.* 2014). The

discrepancies found in literature are possibly due to the boundaries used to define dorsal versus ventral hippocampus (Zuena *et al.* 2008, Morley-Fletcher *et al.* 2011, Tanti and Belzung 2013). Most studies use coordinates of coronal slices of the hippocampus for the segregation into its sub-regions. It is important to note that these sections are not functionally independent and in the same slice there may be one or more hippocampal sub-region. The tripartite model of the hippocampus is supported by developmental gene expression study in rats, but the precise number of hippocampal domains is currently unknown (O'Reilly *et al.* 2015). Although the roles of the iHi have yet to be determined, the introduction of this third hippocampal sub-region might tackle the finding of confounding factors when assessing the neurogenesis across the dorso-ventral axis of the hippocampus. The few studies on this intermediate hippocampal sub-region show that it seems to be not only a transitional area, but also an integrative region (Bast 2007, Bast *et al.* 2009), mediating the integration of diverse sensory modalities (Bast *et al.* 2009). In the present study, we included this third hippocampal sub-region and we suggest that further research consider this possibility in the future.

In summary, we have shown in the present study for the very first time that NPCs derived from the iHi and vHi but not the dHi are sensitive to the deleterious effects of the stress hormone, corticosterone, in neurogenesis. These effects are correlated with reduced translocation of GR into the nucleus, especially in cells from the iHi and vHi. Therefore, we suggest that the iHi and vHi sub-regions are promising targets for the treatment of stress-related psychiatric disorders and future studies should focus in these sub-regions, rather than in the hippocampus as a whole. Although NPCs culture from embryonic animals are easier to handle, here used a protocol for isolation of NPCs from postnatal rats, once the niche of neurogenic

cells in embryonic animals has been shown to be located in the vHi, and cells migrate along the longitudinal axis of the hippocampus during early postnatal days (Li *et al.* 2013). This could lead to the interpretation that the NPCs from different adult hippocampal sub-regions might have the same intrinsic characteristics, but in the present study we observed for the first time that NPCs isolated from different sub-regions of the hippocampus have unique intrinsic characteristics in response to the stress hormone, corticosterone. This suggests that NPCs probably develop these intrinsic characteristics in response to glucocorticoids after migration, and this is probably due to the input influences they receive and differential gene expressions.

Future studies should focus at the development of these sub-regional differences along the longitudinal axis of the hippocampus. Interestingly, at the time we dissected the hippocampi (at P28), the neurogenic pool from the dHi is younger than the one from the vHi (once the NPCs pool is initially located in the vHi and migrates during the first weeks of life). Thus, we suggest that, besides having functional changes during this migration, the age difference of NPCs from the vHi versus dHi might have had an impact in our research findings. Additionally, a complementary experiment would be to inject corticosterone directly into dHi, iHi and vHi and assess changes in emotional behaviours and neurogenesis. Specifically, the local injection of corticosterone and/or dexamethasone directly into the dHi, iHi and vHi would give us an insight of how the acute activation of MR/GR (in the case of corticosterone) or GR only (in the case of dexamethasone) may change emotional behaviours, including anxiety, sociability, depressive-like behaviours and learning & memory, but also the cell proliferation, neuronal differentiation and neuronal maturation could be assessed.

Chapter 4 - Supplementary Material

S4.1. Methods

S4.1.1. Preparation and dissociation of neurospheres

Hippocampal tissue was collected in ice cold HABG medium [Hibernate A (Invitrogen, UK), B-27 minus Vitamin A (2%, Invitrogen, UK) and Glutamax (0.5 mM, Invirogen, UK). Depending upon the experiment, neurosphere cultures were prepared either from the whole hippocampus (Suppl. Fig 4.1A), or from hippocampal sub-regions: dorsal (dHi), intermediate (iHi) and ventral (vHi) hippocampus (Suppl. Fig 4.1B). To obtain a sufficient number of NPCs per independent experiment, tissue from whole hippocampi or each hippocampal sub-region was pooled from three or six animals, respectively. Tissue was minced and incubated in a warm enzymatic mix (Dispase 1 U/ml, Papain 2.5 U/ml and DNase I 250 U/ml); gently mixed every 3 minutes for a total of 30 min, being triturated after 20 min with a polished glass Pasteur pipette and with regular plastic tips at the end of the 30 min enzymatic digestion. The tissue was then centrifuged at 300g for 5 minutes at room temperature (RT) and the pellet was resuspended in 1 ml HABG solution. After further centrifugation at 300g for 5 min at RT, the pellet was resuspended in 20% Percoll solution diluted in Neurobasal-A. After 10 minutes centrifugation at 800g at RT, the pellet was resuspended in 10 ml HABG solution. After 5 min centrifugation at 300g at RT, the pellet was resuspended in growth medium [Neurobasal A (Invitrogen, UK), B-27 minus Vitamin A (2%, Invitrogen, UK), Glutamax (0.5 mM, Invitrogen, UK), Penicillin/Streptomycin (50 units/ml, Sigma-Aldrich, UK), Heparin (2 µg/ml, Sigma-Aldrich, UK), basic fibroblast growth factor (bFGF, 20 ng/ml, Millipore, UK) and epidermal growth factor (EGF, 20 ng/ml, Sigma-Aldrich, UK). Viable cells were counted with

haemocytometer/Trypan blue (Sigma Aldrich, UK) and seeded at a density of 10 x 10^4 cells per well with 500 µl of medium in ultralow adherence 24-wells plates (Corning Life Sciences, UK), and allowed to proliferate as neurospheres for 4 days *in vitro* (DIV) under a humidified atmosphere containing 5% CO_2 at 37ºC (Suppl. Fig 4.1C). Cells were supplemented with fresh media (replacement with 200 µl) at 2 DIV.

Supplementary Figure 4.1*: Dissection of the hippocampus and growth of NPCs as neurospheres. For some experiments, NPCs were isolated from the whole hippocampus (A), and for most of the experiments, NPCs were isolated from the hippocampus segregated into three equal thirds (B), the dorsal, intermediate and ventral hippocampus. NPCs are isolated and cultured for 4 DIV as neurospheres (C), which are then dissociated and follow for the respective treatment with corticosterone or dexamethasone.*

After 4 DIV, all the content from each well of each sub-region was pooled together and centrifuged at 300g for 5 min at RT. The pellet was resuspended in 3 ml of 0.08% Trypsin diluted in Neurobasal-A. After 5 minutes of enzymatic digestion, 3 ml of trypsin inhibitor (0.125 mg/ml) with DNase I (250 U/ml) were added and the cell suspension was centrifuged at 300g for 5 minutes at RT. The pellet was resuspended in growth medium and then dissociated with a pipette, followed by dissociation with a syringe and needle. Viable cells were counted with haemocytometer/Trypan blue (Sigma Aldrich, UK) and seeded at a density of 5 x 10^4 in a volume of 40 µl per well in a Poly-D-Lysine-coated (1mg/ml, Sigma

Aldrich, UK) coverslip in a 24-wells plate. After allowing 1 h for attachment of the NPCs to the surface, 460 µl of treatment medium was added.

S4.1.2. Immunocytochemistry

Cells were washed with warm Hanks' balanced salt solution (HBSS), fixed with ice cold methanol at -20ºC for 10 min followed by 4% paraformaldehyde at room temperature for 10 min. Cells were then washed 3x5 min with PBS-0.02%TritonX (PBS-T) and blocked overnight at 4ºC with 5% donkey serum (DS; Sigma Aldrich) diluted in PBS-T. 18 h later, cells were washed 3x5 min with PBS-T and incubated overnight at 4ºC in the appropriate primary antibody diluted in 5% DS PBS-T [(rat monoclonal anti-BrdU (1:100, ab6326, Abcam), goat polyclonal anti-DCX (1:200, sc-8066, Santa Cruz) or mouse monoclonal anti β3-tubulin (1:250 G7121, Promega)]. 18 h later, cells were washed 3x5 min in PBS-T and incubated for 2 h at room temperature in the respective secondary antibody diluted in 5% DS PBS-T [(Alexa Fluor 594 donkey anti-rat (1:1000, A21209, Invitrogen), Alexa Fluor 488 donkey anti-goat (1:500, A-11055, Invitrogen), Alexa Fluor 488 donkey anti-mouse (1:500, A-21202, Invitrogen)]. For experiments requiring dual labelling immunohistochemistry, cells were then washed 3x5min and incubated overnight at 4ºC in the next appropriate primary antibody diluted in 5% DS in PBS-T [(goat polyclonal anti-Nestin (1:200, sc21249, Santa Cruz); rabbit polyclonal anti-GR (1:500 sc-1004, Santa Cruz)]. 18 h later, cells were washed 3x5 min with PBS-T and incubated for 2 h at room temperature in the respective secondary antibody diluted in 5% DS in PBS-T [(Alexa Fluor 488 donkey anti-goat, (1:1000 A-11055, Invitrogen), Alexa Fluor 594 donkey anti-rabbit (1:500, A21207, Invitrogen)]. After 3x5 min washes, cells were incubated in DAPI (1:1000, from a stock of 1ul/ml, Sigma-Aldrich, UK) diluted in 2% DS in PBS-T for 10 min and then washed

with PBS. Coverslips were mounted onto slides using DAKO Fluorescence mounting medium.

S4.1.3. Fluorescence microscopy and image analysis

Fluorescent images of immunopositive cells were viewed under an Olympus BX53 upright microscope and photomicrographs were captured (all with the same exposure time) at 40x magnification using an Olympus DP71 camera and cellSens Entry camera software™. For each treatment condition, 5 fields of view were randomly captured per coverslip, there were 3 coverslips per treatment per plate, and each experiment was repeated 3 times. Software Image J Version 1.44 was used to count cells, to measure the length of dendritic processes and to measure fluorescence intensity of GR-immunopositive nuclei.

In the proliferation experiments, the percentage of NPCs [(Nestin$^+$ cells/total DAPI$^+$ nuclei) x 100] and the percentage of proliferating NPCs [(BrdU$^+$ cells/Nestin$^+$ cells) x 100] were calculated.

In the experiment examining the effects of short-term (4 DIV) corticosterone treatment on neuronal differentiation and maturation, the percentage of immature neurons [(DCX$^+$ cells/ DAPI$^+$ cells) x 100] was calculated. The number of DCX$^+$ neurons with a primary [(DCX$^+$ cells with primary processes/total DCX$^+$ cells) x 100] and a secondary process [(DCX$^+$ with secondary processes/total DCX$^+$ cells) x 100] were also calculated. As an index of neuronal maturation, the average total length of all dendrites was calculated [(length of all dendrites/number of DCX$^+$ cells) x 100]. The individual lengths of primary and secondary DCX$^+$ processes

were also measured [(length of all primary or secondary processes/number of DCX^+ cells with primary or secondary processes, respectively) x 100].

In the experiments examining the effects of long-term (7 DIV) corticosterone or dexamethasone treatment on neuronal differentiation, the percentage of neurons [(β3-$tubulin^+$ cells/$DAPI^+$ cells) x 100] was calculated. The percentage of β3-$tubulin^+$ neurons with a primary [(β3-$tubulin^+$ cells with primary processes/total β3-$tubulin^+$ cells) x 100], a secondary [(β3-$tubulin^+$ with secondary processes/total β3-$tubulin^+$ cells) x 100] and a tertiary process [(β3-$tubulin^+$ with tertiary processes/total β3-$tubulin^+$ cells) x 100] were also calculated. As an index of neuronal maturation, the total dendritic length was also calculated [(length of all dendrites/number of β3-$tubulin^+$ cells) x 100]. The individual lengths of primary, secondary and tertiary dendritic processes were also measured [(length of all primary, or secondary, or tertiary processes/number of DCX^+ cells with primary, or secondary, or tertiary processes, respectively) x 100].

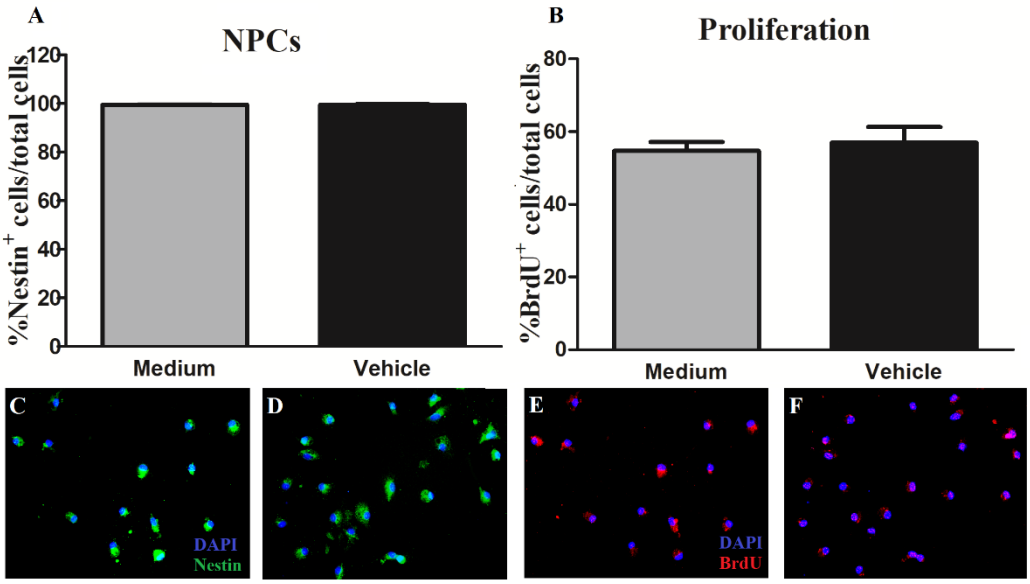
To determine the effects of long-term (7 DIV) corticosterone or dexamethasone treatment on glucocorticoid receptor (GR) translocation to the nucleus, the fluorescence intensity of all nuclei was assessed. The software Image J Version 1.44 was used to draw around each nucleus and assess the fluorescence intensity, as a measure of nuclear GR expression.

S4.1.4. Cell viability analysis [Thiazolyl Blue Tetrazolium Bromide (MTT) assay]

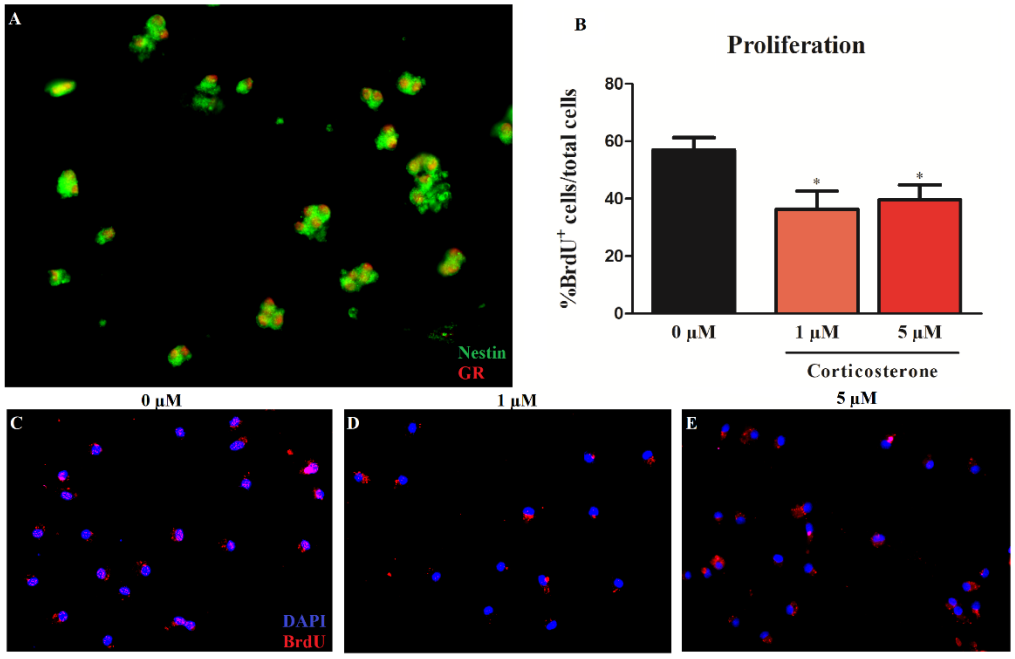
An MTT assay was used to verify whether the results of long-term exposure to corticosterone or dexamethasone were due to altered cell viability. Briefly, after 7 DIV under differentiation conditions the media was removed and growth media

containing 10% MTT solution (5 mg/ml, Sigma Aldrich, UK) was added. 4h later, the medium was discarded and 100 µl of DMSO was added to each well to dissolve the formazan crystals. 75 µl from each well were plated in a 96-well plate and the absorbance at 570 nm wavelength was measured in an absorption spectrophotometer (Flexstation 3 Multimode Plate Reader, Molecular Devices). For each treatment condition, there were 3 samples per culture, and the experiment was repeated 3 times.

S4.2. Results



***Supplementary Figure 4.2**: Characterisation of NPCs cultures, comparing the percentage of NPCs and cell proliferation between cells exposed to growth medium alone and cells exposed to growth medium with the vehicle used to dissolve corticosterone and dexamethasone (0.1% DMSO). The vehicle did not change the percentage of NPCs (A) or the percentage of proliferating cells (B); C-D, representative images of cells stained with DAPI (blue) and nestin (green); E-F, representative images of cells stained with DAPI (blue) and BrdU (red).*



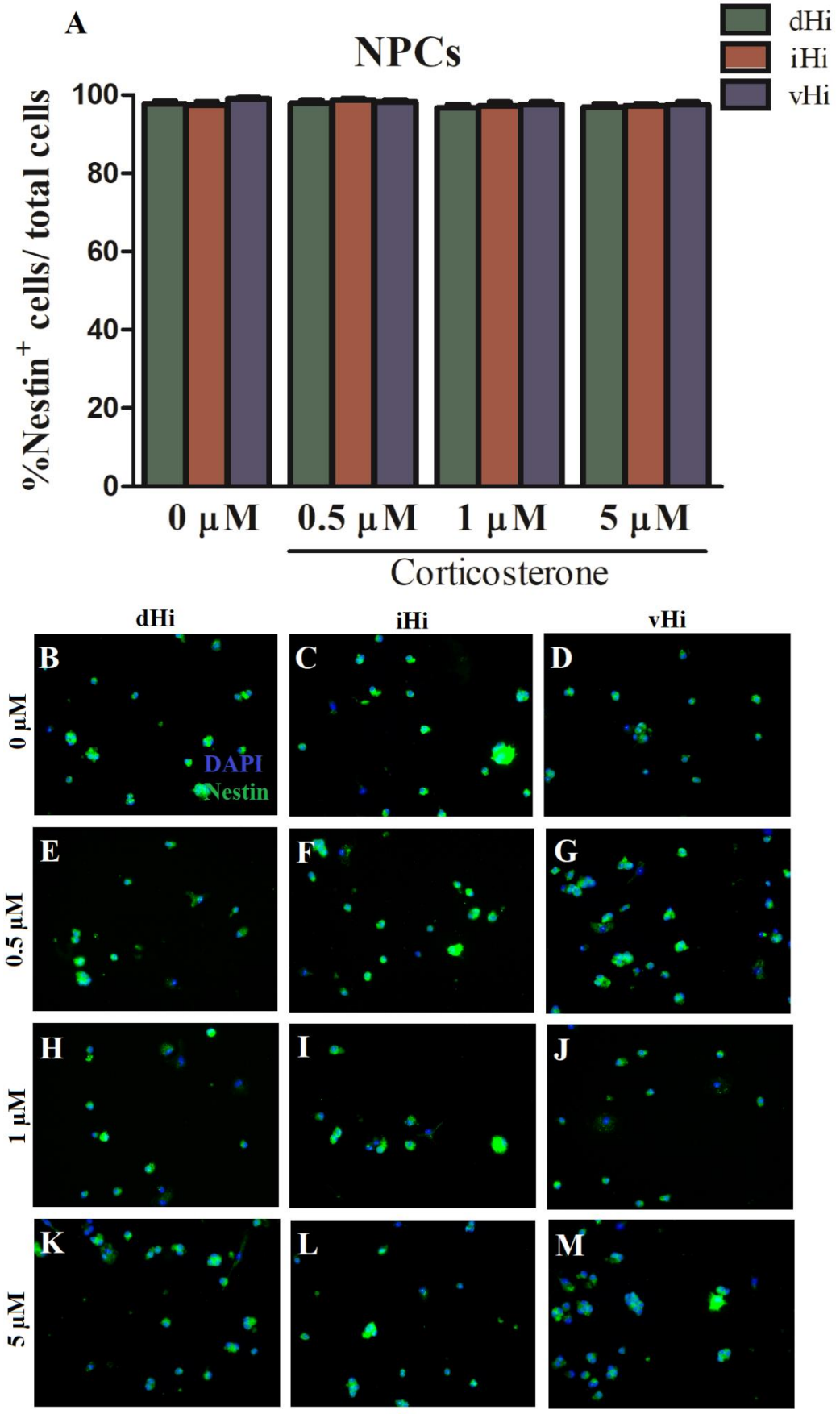
***Supplementary Figure 4.3**: Effects of acute corticosterone exposure (4 h) on cell proliferation of NPCs derived from the whole hippocampus. A, representative image showing that NPCs (green) express the GR (red); B, acute corticosterone exposure reduced cell proliferation on NPCs from the whole hippocampus; C-E, representative images of cells stained with DAPI (blue) and BrdU (red).*

S4.2.1. Acute corticosterone (4 h in vitro) exposure did not alter the percentage of NPCs in cultures derived from the dHi, iHi and vHi

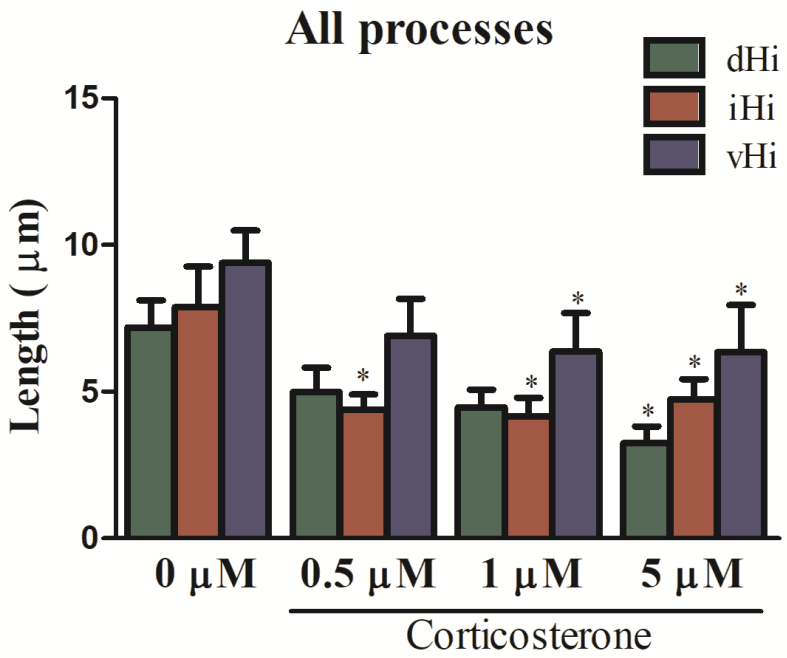
The effects of corticosterone (CORT) on the percentage of nestin$^+$ cells in cultures derived from the dHi, iHi and vHi are shown in Suppl Fig 4.4. Two-way ANOVA revealed no significant effect of CORT ($F_{3,96}$=2.14, p=0.1), hippocampal sub-region ($F_{2,96}$=1.28, p=0.28) nor a CORT x hippocampal sub-region interaction ($F_{6,96}$=0.43, p=0.86) on the percentage of nestin$^+$ cells.

S4.2.2. The effects of short-term (4DIV) corticosterone exposure on total dendritic length in DCX$^+$ cells derived from the dHi, iHi or vHi

As another index of neuronal maturation, we measured the average combined length of these primary and secondary dendrites per DCX$^+$ cell. Two-way ANOVA revealed a significant main effect of CORT ($F_{3,96}$=7.11, p<0.001), hippocampal sub-region ($F_{2,96}$=5.9, p=0.004) but no CORT X hippocampal sub-region interaction ($F_{6,96}$=0.24, p=0.96) on the total length of processes (Fig 4.5). Specifically, Fishers LSD post hoc test revealed that all concentrations of CORT (0.5, 1 and 5 µM) significantly reduced the total process length in cells derived from the iHi (p=0.017, p=0.011, p=0.032, respectively), while 1 and 5 µM of CORT reduced the length in cells derived from the vHi (p=0.038, p=0.037, respectively), and only the concentration of 5 µM reduced the length in cells derived from the dHi (p=0.007).



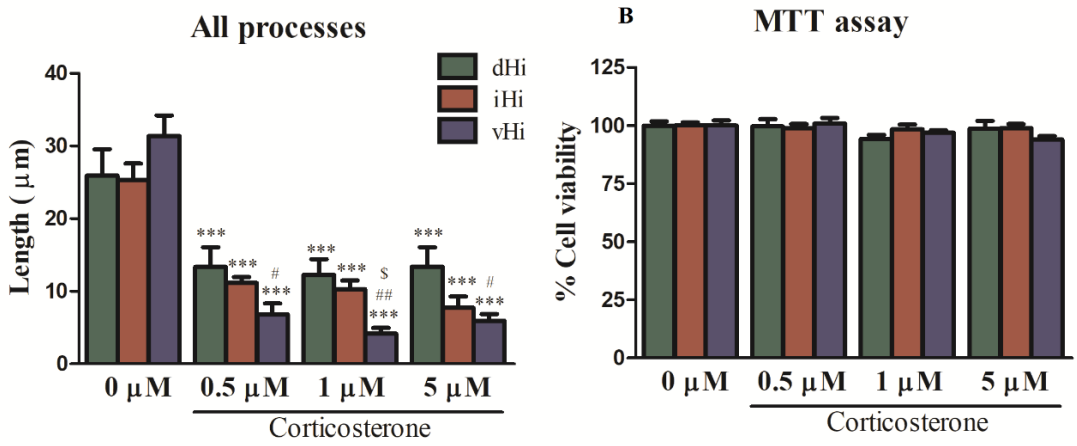
Supplementary Figure 4.4*: Effects of acute corticosterone (4 h) on the percentage of NPCs in cultures derived from the dHi, iHi and vHi. A, acute corticosterone does not alter the percentage of NPCs; B-M, representative images of cells stained with DAPI (blue) and Nestin (green).*



***Supplementary Figure 4.5**: Short-term (4 DIV) exposure to corticosterone reduces the total dendritic length of DCX$^+$ cells. *p<0.05, compared with respective 0 µM group, according to Fishers LSD post hoc test.*

S4.2.3. Neurons derived from vHi NPCs are more sensitive to reductions in neuronal dendritic length induced by longer-term (7 DIV) corticosterone exposure

As another feature of cellular maturation, we assessed the total length of the processes. Two-way ANOVA revealed a significant main effect of CORT treatment ($F_{3,96}$=55.69, p<0.001), by hippocampal sub-region ($F_{2,96}$=3.96, p=0.022), and by a CORT X hippocampal sub-region interaction ($F_{6,96}$=2.82, p=0.014) on the total length of processes (Suppl. Fig 4.6A). Specifically, Fishers LSD post hoc test shows that the length of processes is reduced by all concentrations of CORT (0.5, 1 and 5 µM) in cells differentiated from NPCs irrespective of the hippocampal sub-regions they were derived from (p<0.001 in all cases). Additionally, processes are further reduced in cells from the vHi, when compared to cells from the dHi, when treated with 0.5 µM (p=0.031), 1 µM (p=0.008) and 5 µM (p=0.014), and when compared to cells from the iHi, when treated with 1 µM (p=0.043).

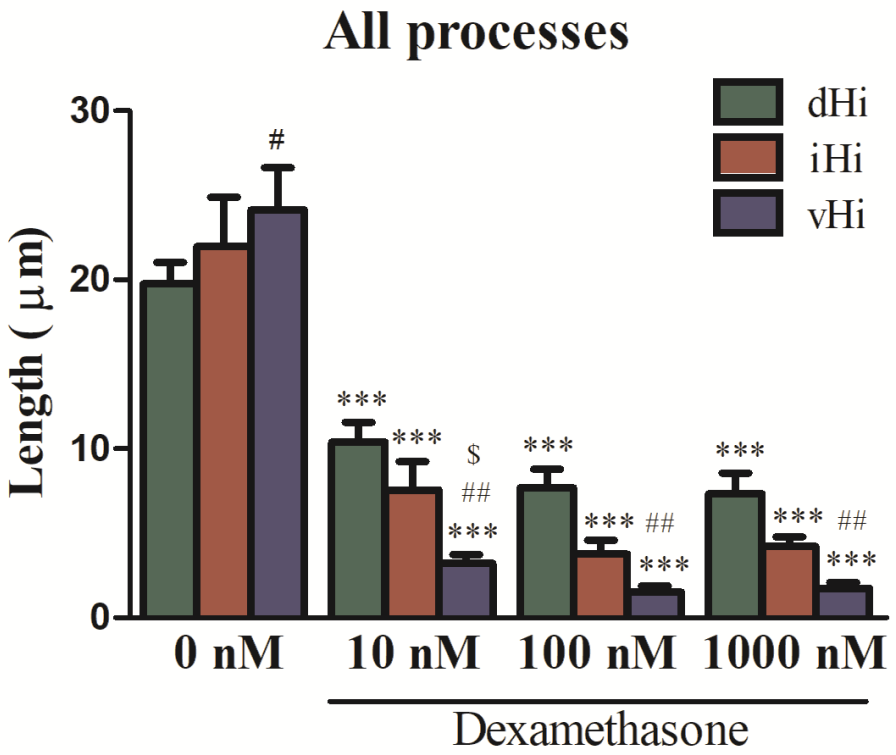


***Supplementary Figure 4.6**: Effects of longer-term (7 DIV) exposure to corticosterone on neuronal maturation and cell viability. A, longer-term exposure to corticosterone reduces the total dendritic length of β3-tubulin$^+$ cells, especially the ones derived from the vHi; B, longer-term exposure to corticosterone does not affect cell viability, as shown by an MTT assay. ***p<0.001, compared with respective 0 µM group; #p<0.05, compared to cells derived from the dHi treated with the same concentration of corticosterone; $p<0.05, compared to cells derived from the iHi treated with the same concentration of corticosterone, according to Fishers LSD post hoc test.*

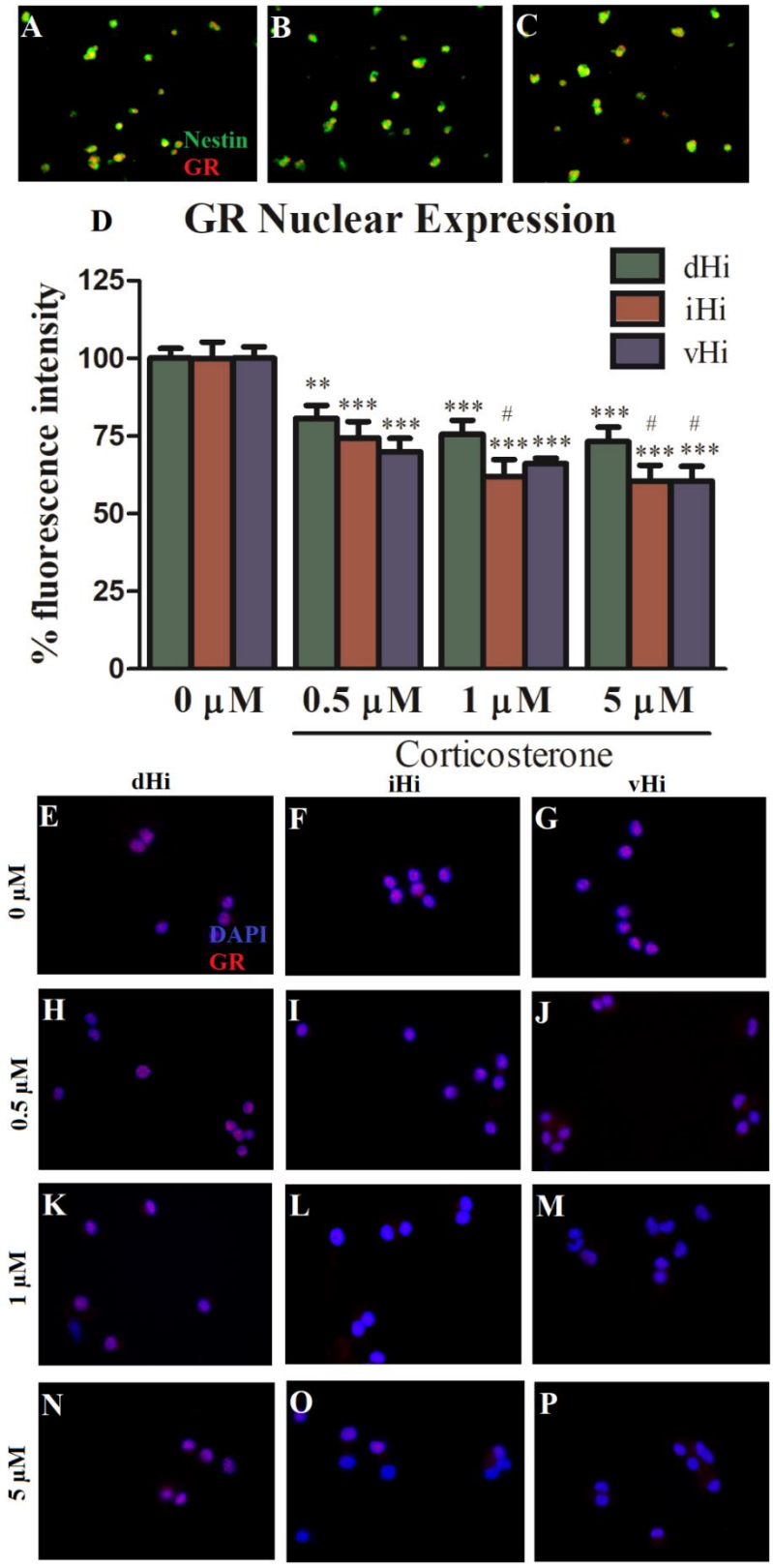
S4.2.4. Neurons derived from vHi NPCs are more sensitive to reductions in neuronal dendritic length induced by dexamethasone

As another feature of cellular maturation, we assessed the total length of the processes. Two-way ANOVA revealed a significant main effect of DEX ($F_{3,96}$=103.65, p<0.001), hippocampal sub-region ($F_{2,96}$=6.41, p=0.002), and a DEX X hippocampal sub-region interaction ($F_{6,96}$=3.58, p=0.003) on the total length of processes (Suppl. Fig 4.7). Specifically, Fishers LSD post hoc test revealed that all concentrations of DEX decreased the average total length of dendritic processes per β3-tubulin+ cell irrespective of the hippocampal sub-regions they were derived from (all p<0.001), however these effects were more pronounced in vHi-derived cultures. Indeed, all concentrations of DEX decreased total dendritic process length to a greater extent in vHi-derived β3-tubulin$^+$ cells compared with those from the dHi (10 nM, p=0.001; 100 nM p=0.003; 1000 nM p=0.007). In addition, 10 nM

DEX-induced reductions in total dendritic process length were larger in vHi-derived β3-tubulin^{+} cells compared with those from the iHi (p=0.035). It is important to note that the total dendritic process length was longer in vehicle-treated cells derived from the vHi than those from the dHi- (p=0.034).



Supplementary Figure 4.7*: Effects of long-term (7 DIV) exposure to dexamethasone on neuronal maturation. Longer-term exposure to dexamethasone reduces the total dendritic length of β3-tubulin^{+} cells, especially the ones derived from the vHi. ***p<0.001, compared with respective 0 nM group; ##p<0.05, compared to cells derived from the dHi treated with the same concentration of dexamethasone; $p<0.05, compared to cells derived from the iHi treated with the same concentration of dexamethasone, according to Fishers LSD post hoc test.*



Supplementary Figure 4.8*: Effects of longer-term (7 DIV) exposure to corticosterone on nuclear GR expression. A-C, GR (red) is expressed in NPCs (green) derived from dHi (A), iHi (B) and vHi (C); D, corticosterone-induced reductions in nuclear GR expression are greater in iHi and vHi-derived cultures; E-P, representative images of cells stained with DAPI (blue) and GR (red). ***p<0.001, compared with respective 0 µM group, #p<0.05, compared to cells derived from the dHi treated with the same concentration of corticosterone, according to Fishers LSD post hoc test.*

CHAPTER 5

The Antidepressant Fluoxetine Prevents Corticosterone-Induced Reductions in Neurogenesis: Differential Responses in Neural progenitor Cells Derived from the Ventral, Intermediate and Dorsal Hippocampus

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Abstract

The hippocampus has been implicated in the pathophysiology of depression and is a target for antidepressant action. Adult hippocampal neurogenesis (AHN), the birth of new neurons throughout adult life, has been shown to play roles in learning, memory, and response to stress and antidepressants. Accumulating evidence suggests that the hippocampus is functionally segregated along its longitudinal axis such that the dorsal hippocampus (dHi) plays a preferential role in spatial learning and memory, while the ventral hippocampus (vHi) plays a preferential role in anxiety and stress response. The area between these two, the intermediate hippocampus (iHi), also seem to be functionally independent. Recent *in vivo* studies suggest that stress and antidepressants preferentially affect neurogenesis in the vHi. The aim of this study was to determine whether NPCs derived from the dHi, iHi, or vHi exhibit differential intrinsic sensitivity to the neurogenesis-promoting effects of the antidepressant fluoxetine (FLX). To that end, we isolated and cultured NPCs from the three hippocampal sub-regions of 28 days old rats, treated these cells with either fluoxetine alone or with fluoxetine in the presence of the stress hormone corticosterone and measure cell proliferation and neuronal differentiation. Fluoxetine alone has no effect on cell proliferation or neuronal differentiation but prevented the effects of corticosterone on neurogenesis. However, NPCs of the iHi and vHi were more sensitive to fluoxetine's effects than those from the dHi. In conclusion, this study highlights the important role for iHi and vHi in mediating the potentially therapeutic effects of fluoxetine under stressful challenges.

5.1. Introduction

The hippocampus has been implicated in the pathophysiology of depression and is a target for antidepressant action (Bremner *et al.* 2000, Malberg *et al.* 2000, Frodl *et al.* 2002, Sheline *et al.* 2003, Morais *et al.* 2017). This brain area is also one of just a few regions of the mammalian brain where neurogenesis, the birth of new neurons, occurs throughout life (Spalding *et al.* 2013, Kempermann *et al.* 2018, Sorrells *et al.* 2018). Adult hippocampal neurogenesis is a complex process encompassing several stages including cell proliferation, neuronal differentiation, maturation and integration onto the neural circuitry of the hippocampus, a process which takes approximately 5-6 weeks to complete. In the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus, quiescent radial glia-like neural progenitor (type 1) cells rarely divide but give rise to highly proliferative intermediate progenitor cells first with a glial (type 2a) and then a neuronal (type 2b) phenotype. Type 2b cells then develop into neuroblasts (type 3) which exit the cell cycle to become post-mitotic and migrate into the granule cell layer. These immature neurons then enter a maturation stage whereby they extend their axon to CA3 and their dendrites into the molecular layer eventually becoming functionally integrated into the neural circuitry of the hippocampus (Christie and Cameron 2006, Kempermann *et al.* 2015).

While many immature neurons are generated, many die by apoptosis and do not survive to become mature hippocampal granule cells. Indeed, each of the stages of hippocampal neurogenesis is under regulation by endogenous intrinsic and extrinsic factors in the neurogenic niche as well non-endogenous extrinsic factors (Gould *et al.* 1999, Malberg *et al.* 2000, Ming and Song 2011, Surget *et al.* 2011, Hueston *et al.* 2017). While some endogenous factors including some nuclear receptors such

the TLX receptor (Shi *et al.* 2004, O'Leime *et al.* 2017), Wnt, Notch and Nuclear factor kappa ß (Mu *et al.* 2010, Aimone *et al.* 2014) have been shown to increase adult hippocampal neurogenesis; others such as corticosterone (Wong and Herbert 2006) and pro-inflammatory cytokines (Bauer 2009) decrease hippocampal neurogenesis. Non-endogenous extrinsic factors also influence adult hippocampal neurogenesis. Indeed, learning and memory have been shown to increase adult hippocampal neurogenesis in both rats and mice (Gould *et al.* 1999, Dobrossy *et al.* 2003, Snyder *et al.* 2005, Tronel *et al.* 2010, Epp *et al.* 2013) and in turn, adult hippocampal neurogenesis is required for some types of hippocampal-dependent learning (Marin-Burgin and Schinder 2012). Voluntary running and environmental enrichment have also been shown to increase adult hippocampal neurogenesis (Kempermann *et al.* 1997, van Praag *et al.* 1999, Kozareva *et al.* 2018). On the other hand, chronic stress has been shown to decrease adult hippocampal neurogenesis (Gould *et al.* 1997, Jayatissa *et al.* 2009, Schloesser *et al.* 2010, Eisch and Petrik 2012, Levone *et al.* 2015). In addition, chronic treatment with antidepressant drugs such as fluoxetine has been shown to increase adult hippocampal neurogenesis in rodents (Malberg *et al.* 2000, Dranovsky and Hen 2006, Zhou *et al.* 2016). Moreover, inhibition of adult hippocampal neurogenesis in mice has been shown to prevent the behavioural effects of chronic treatment with some antidepressants including fluoxetine in the novelty suppressed feeding test (Santarelli *et al.* 2003, David *et al.* 2009).

How adult hippocampal neurogenesis can play a role in processes as diverse as learning and memory and response to stress is not yet fully understood, but accumulating evidence suggests that the hippocampus is functionally divided along its septotemporal axis into ventral and dorsal regions whereby the ventral

hippocampus (vHi) is plays a predominant role in anxiety, stress and antidepressant response and the dorsal hippocampus (dHi) preferentially regulates spatial learning and memory (Bannerman *et al.* 2004, Fanselow and Dong 2010). Recent gene expression studies suggest that the hippocampus is a gradient of gene expression along its longitudinal axis (Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016, Cembrowski *et al.* 2016) and the presence of an intermediate hippocampus (iHi) is suggested, however little is known about the specific functional roles of this hippocampal sub-region (Bast 2007, Bast *et al.* 2009). However, in our recent findings (Levone et al., unpublished; Chapter 2) we found that the iHi lesion prevents the antidepressant effects of chronic fluoxetine, suggesting that along with the vHi, these regions can regulate fluoxetine-induced changes in emotional behaviours. Moreover, iHi lesion attenuated fluoxetine-induced anxiolytic effect in the novelty-induced hypophagia test (Levone et al., unpublished; Chapter 2), which is a neurogenesis-related behaviour (Santarelli *et al.* 2003, David *et al.* 2009). In parallel, emerging studies suggest that adult neurogenesis may be preferentially regulated in the dHi or the vHi, depending upon the stimulus (Tanti and Belzung 2013, O'Leary and Cryan 2014). Indeed, in our recent study (Levone et al., unpublished; Chapter 4) we found that corticosterone reduced neuronal differentiation and maturation preferentially in NPCs derived from the iHi and vHi. Moreover, some studies have reported that antidepressant-induced increases in cytogenesis and neurogenesis occur preferentially in the vHi under both non-stress conditions in mice (Paizanis *et al.* 2010) and in rats (Banasr *et al.* 2006, Soumier *et al.* 2009) and under stress conditions in mice (Tanti *et al.* 2012) and in rats (Jayatissa *et al.* 2006); chronic corticosterone also reduced neurogenesis preferentially in the vHi of female rats (Brummelte and Galea 2010). In addition, *in vivo* studies in C57BL/6 mice have shown that the effects of

fluoxetine in the proliferation of NPCs occur preferentially in the transition between intermediate and ventral hippocampus (Zhou *et al.* 2016). Moreover a recent study also showed that the behavioural effects of fluoxetine both in a model of chronic stress and chronic corticosterone are dependent on the neurogenesis in the vHi (Wu and Hen 2014). Correspondingly, post-mortem human brain studies have reported that antidepressants increase the proliferation of neural progenitor cells preferentially in the anterior hippocampus (equivalent to vHi in rodents) in humans (Boldrini *et al.* 2009, Boldrini *et al.* 2012).

Together, these data suggest that adult hippocampal neurogenesis, specifically in the iHi and vHi, may be an important for the neurogenic and behavioural effects of antidepressant drugs. However, whether the preferential effects of antidepressants on neurogenesis in the vHi are due to increased intrinsic sensitivity of the neural progenitor cells residing in this hippocampal sub-region to antidepressants is unclear. Thus, the aim of the present study is to assess whether neural progenitor cells derived from the dorsal, intermediate and ventral hippocampal sub-regions have differential intrinsic sensitivity to the effects of fluoxetine alone and to fluoxetine modulation of corticosterone-induced reductions in neurogenesis. To this end, we isolated neural progenitor cells from dHi, iHi and vHi, and treated them with fluoxetine with or without corticosterone and measured cell proliferation, neuronal differentiation and maturation.

5.2. Methods

5.2.1. Animals and preparation of hippocampal neural progenitor cell (NPC) neurospheres

Procedures were performed under individual authorisations issued by the Health Products Regulatory Authority (HPRA) Ireland and in accordance with the European Parliament and the Council of the EU Directive (2010/63/EU). The dHi, iHi and vHi (Fig 5.1A) were dissected from male Sprague Dawley rats (postnatal day 28), bred at Biological Services Unit, University College Cork) as previously described (Walker and Kempermann 2014) (Chapter 4; Levone *et al*, unpublished). To obtain a sufficient number of NPCs per independent experiment, NPCs from each sub-region of 6 individual rats were pooled together. NPCs were seeded at a density of 10 x 10^4 cells per well and allowed to proliferate as neurospheres Neurobasal A (A2477501, Gibco), 2% B27 minus vitamin A, 0.5mM Glutamax, 50 units/ml Penicillin/Streptomicin, 2µg/ml heparin, 20 ng/ml FGF and 20 ng/ml EGF 4 days *in vitro* (DIV) prior to experimental treatment (Fig 5.1B).

5.2.2. Dissociation of neurospheres and treatment of NPCs with fluoxetine and/or corticosterone

After 4 DIV under proliferation conditions, neurospheres were dissociated and seeded at a density of 5 x 10^4 per well in a volume of 40 µl onto Poly-D-Lysine-coated (1mg/ml, Sigma Aldrich, UK) coverslips in a 24-well plate. NPCs were allowed to adhere to the coverslips for 1 h prior to the addition of 460 µl of the treatment medium (for proliferation experiments: Neurobasal A (A2477501, Gibco), 2% B27 minus vitamin A, 0.5mM Glutamax, 50 units/ml Penicillin/Streptomicin, 20 ng/ml FGF and 20 ng/ml EGF; for differentiation experiments: Neurobasal A (10888022, Gibco), 2% B27 with vitamin A, 0.5mM

Glutamax, 50 units/ml Penicillin/Streptomicin) containing the relevant experimental treatment.

To measure the effects of fluoxetine treatment alone on cell proliferation, NPCs were dissociated and allowed to proliferate in growth medium (Neurobasal A, 2% B27 minus vitamin A, 0.5mM Glutamax, 50 units/ml Penicillin/Streptomicin, 20 ng/ml FGF and 20 ng/ml EGF) containing 0.2 µM BrdU or growth medium containing 0.2 µM BrdU and different concentrations of fluoxetine (FLX; 0, 0.1 µM and 1 µM) for 4 h *in vitro* prior to fixation (Fig 5.1C).

To measure the effects of fluoxetine treatment alone on neuronal differentiation and maturation, NPCs were dissociated and allowed to differentiate in growth medium (Neurobasal A, 2% B27, 0.5mM Glutamax and 50 units/ml Penicillin/Streptomicin,) alone or growth medium with different concentrations of fluoxetine (0, 0.1 µM, 1 µM). Cells were allowed to differentiate for 4 DIV (Fig 5.2A) or 2 DIV (Fig 5.2B) prior to fixation.

To measure the effects of fluoxetine on corticosterone-induced reductions in neuronal differentiation, neuronal maturation, cell viability, and nuclear GR expression, NPCs were allowed to differentiate in growth medium (Neurobasal A, 2% B27, 0.5mM Glutamax, 50 units/ml Penicillin/Streptomicin and DMSO 0.1%) or growth medium with corticosterone alone (CORT, 1 µM, diluted in DMSO 0.1%), or growth medium with CORT 1 µM plus different concentrations of fluoxetine (0.1 µM and 1 µM). Cells were allowed to differentiate and mature for 4 DIV (Fig 5.3A) prior to fixation.

5.2.3. Immunocytochemistry

Cells were washed with warm Hanks' balanced salt solution (HBSS), fixed with ice cold methanol at -20ºC for 10 min followed by 4% paraformaldehyde at room temperature for 10 min. Cells were then washed 3x5 min with PBS-0.02%TritonX-100 (PBS-T) and blocked overnight at 4ºC with 5% donkey serum (DS; Sigma Aldrich) diluted in PBS-T. 18 h later, cells were washed 3x5 min with PBS-T and incubated overnight at 4ºC in the appropriate primary antibody diluted in 5% DS PBS-T [(rat monoclonal anti-BrdU (1:100, ab6326, Abcam) or goat polyclonal anti-DCX (1:200, sc-8066, Santa Cruz)]. 18 h later cells were washed 3x5 min in PBS-T and incubated for 2 h at room temperature in the respective secondary antibody diluted in 5% DS PBS-T [(Alexa Fluor 594 donkey anti-rat (1:1000, A21209, Invitrogen), Alexa Fluor 488 donkey anti-goat (1:500, A-11055, Invitrogen)]. For experiments requiring dual labelling immunohistochemistry, cells were then washed 3x5min and incubated overnight at 4ºC in the appropriate primary antibody diluted in 5% DS in PBS-T [(goat polyclonal anti-Nestin (1:200, sc21249, Santa Cruz); rabbit polyclonal anti-GR (1:500 sc-1004, Santa Cruz)]. 18 h later, cells were washed 3x5 min with PBS-T and incubated for 2 h at room temperature in the respective secondary antibody diluted in 5% DS in PBS-T [(Alexa Fluor 488 donkey anti-goat, (1:1000 A-11055, Invitrogen), Alexa Fluor 594 donkey anti-rabbit (1:500, A21207, Invitrogen)]. After 3x5 min washes, cells were incubated in DAPI (1:1000, from a stock of 1ul/ml, Sigma-Aldrich, UK) diluted in 2% DS in PBS-T for 10 min and then washed with PBS. Coverslips were mounted onto slides using DAKO Fluorescence mounting medium.

5.2.4. Fluorescence microscopy and image analysis

Fluorescent images of immunopositive cells were viewed under an Olympus BX53 upright microscope and photomicrographs were captured (all with the same exposure time) at 40x magnification using an Olympus DP71 camera and cellSens Entry camera software™. For each treatment condition, five fields of view were randomly captured per coverslip, there were three coverslips per treatment per plate, and each independent experiment was repeated three times. Software Image J Version 1.44 was used to count cells, to measure the length of dendritic processes and to measure fluorescence intensity of GR-immunopositive nuclei.

In the proliferation experiments, the percentage of NPCs [(Nestin$^+$ cells/total DAPI$^+$ nuclei) x 100] and the percentage of proliferating cells [(BrdU$^+$ cells/DAPI cells) x 100] were calculated.

In the neuronal differentiation experiments, the percentage of immature neurons [(DCX$^+$ cells/ DAPI$^+$ cells) x 100] was calculated. As an index of neuronal maturation, the average length of processes per DCX$^+$ cells was measured [(sum of all processes length)/ (number of DCX$^+$ cells)].

To determine the effects of fluoxetine treatment on glucocorticoid receptor (GR) translocation to the nucleus, the fluorescence intensity of nuclear GR of all cells in each field of view was measured. For each treatment condition, five fields of view were randomly captured per coverslip, there were three coverslips per treatment per plate, and each experiment was repeated three times. The software Image J Version 1.44 was used to draw around each nucleus and assess the fluorescence intensity, as a measure of nuclear GR expression.

5.2.5. Cell viability analysis [Thiazolyl Blue Tetrazolium Bromide (MTT) assay]

An MTT assay were used to determine whether the effects of fluoxetine on corticosterone-induced reduction in neuronal differentiation and maturation were due to altered cell viability. Briefly, following 4 DIV under differentiation conditions the media was removed and growth media containing 10% MTT solution (5 mg/ml, Sigma Aldrich, UK) was added. Four hours later, the medium was discarded and 100 µl of DMSO was added to each well to dissolve the formazan crystals. 75 µl from each well were plated in a 96-well plate and the absorbance at 570 nm wavelength was measured in an absorption spectrophotometer (Flexstation 3 Multimode Plate Reader, Molecular Devices). For each treatment condition, there were three samples per plate, and the experiment was repeated three times.

5.2.6. Statistical analysis

Data were analysed using IBM SPSS Statistics 23. For the experiment measuring the effects of fluoxetine on NPC proliferation and differentiation, two-way ANOVA was used. For the experiment measuring the effects of fluoxetine on corticosterone-induced reductions in neuronal differentiation and maturation, three-way ANOVA was used. If a main effect reached statistical significance, group-wise comparisons were made using Fishers LSD post-hoc test. Data are expressed as the mean + SEM. Criteria for statistical significance was set at $p<0.05$.

5.3. Results

5.3.1. Fluoxetine did not affect cell proliferation in dHi, iHi or vHi NPC cultures

We first characterised the NPCs cultures from dHi, iHi and vHi by calculating the percentage of cells in the culture that were indeed NPCs ($Nestin^+$/total number of DAPI nuclei). We observed that 90-95% of cells were $nestin^+$, thus confirming the purity of the NPC cultures.

The effects of fluoxetine on cell proliferation in NPC cultures derived from the dHi, iHi or vHi are shown in Fig 5.1D, E. Two-way ANOVA revealed no main effect of fluoxetine ($F_{2,72}$=0.31, p=0.73), hippocampal sub-region ($F_{2,72}$=0.97, p=0.39), nor a fluoxetine x hippocampal sub-region interaction ($F_{4,72}$=0.08, p=0.99) on cell proliferation.

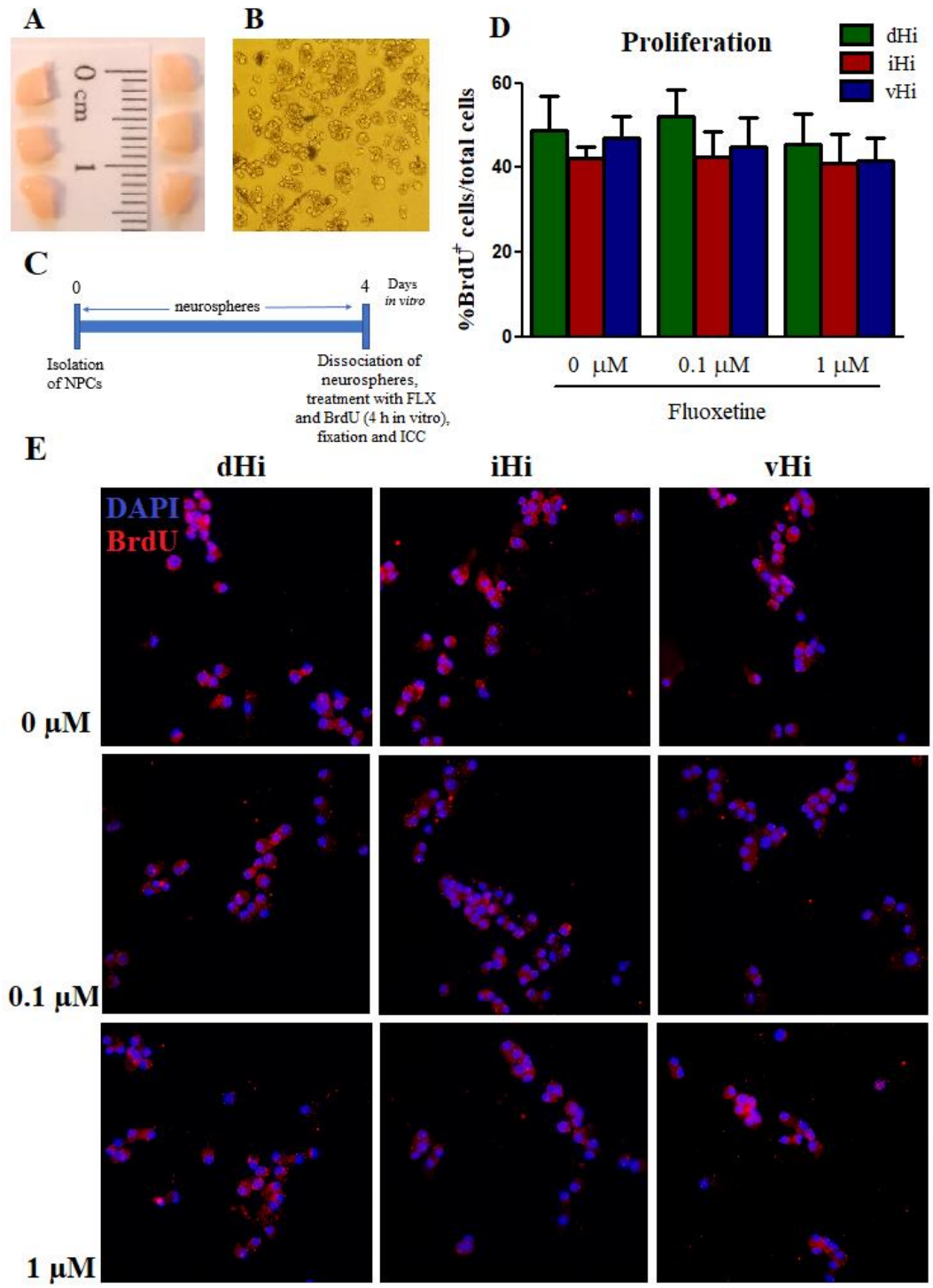

Figure 5.1*: Effects of acute fluoxetine exposure (4 h in vitro) on cell proliferation from NPCs derived from dHi, iHi and vHi. A, dHi, iHi and vHi dissected from P28 Sprague-Dawley rats. B, NPCs growing as neurospheres. C, Experimental timeline: NPCs were isolated and cultured as neurospheres for 4 DIV, then dissociated and treated with fluoxetine (0.1 μM or 1 μM) for 4h in vitro prior to fixation and immunocytochemistry. D, Fluoxetine did not affect cell proliferation (BrdU$^+$ cells) in NPCs derived from the dHi, iHi or vHi. E, Representative images of proliferating cells derived from the dHi, iHi and vHi; stained with DAPI (blue) and BrdU (red).*

5.3.2. Fluoxetine treatment alone did not affect neuronal differentiation

The effects of 4 days treatment with fluoxetine on neuronal differentiation (percentage of DCX^+ cells) of NPCs from the dHi, iHi and vHi are shown in Fig 5.2C, E. Two-way ANOVA revealed no main effect of either fluoxetine ($F_{2,72}$=0.44, p=0.65), hippocampal sub-region ($F_{2,72}$=0.04, p=0.96), nor a fluoxetine x hippocampal sub-region interaction ($F_{4,72}$=0.43, p=0.79) on neuronal differentiation.

We noticed that in the control groups, the majority of cells had differentiated into neurons (i.e. 90% - 95% were DCX^+). Such a high starting point may have precluded any potential fluoxetine-induced increases in neuronal differentiation from being observed. Therefore, in the next experiment we reduced the time allowed for differentiation to 2 DIV and then measured the effects of fluoxetine on neuronal differentiation (percentage of DCX^+ cells) in dHi, iHi and vHi NPC cultures (Fig 5.2D). Again, two-way ANOVA revealed no main effect of fluoxetine ($F_{2,72}$=1.94, p=0.15), hippocampal sub-region ($F_{2,72}$=0.51, p=0.6) nor a fluoxetine x hippocampal sub-region interaction ($F_{4,72}$=1.26, p=0.29) on neuronal differentiation.

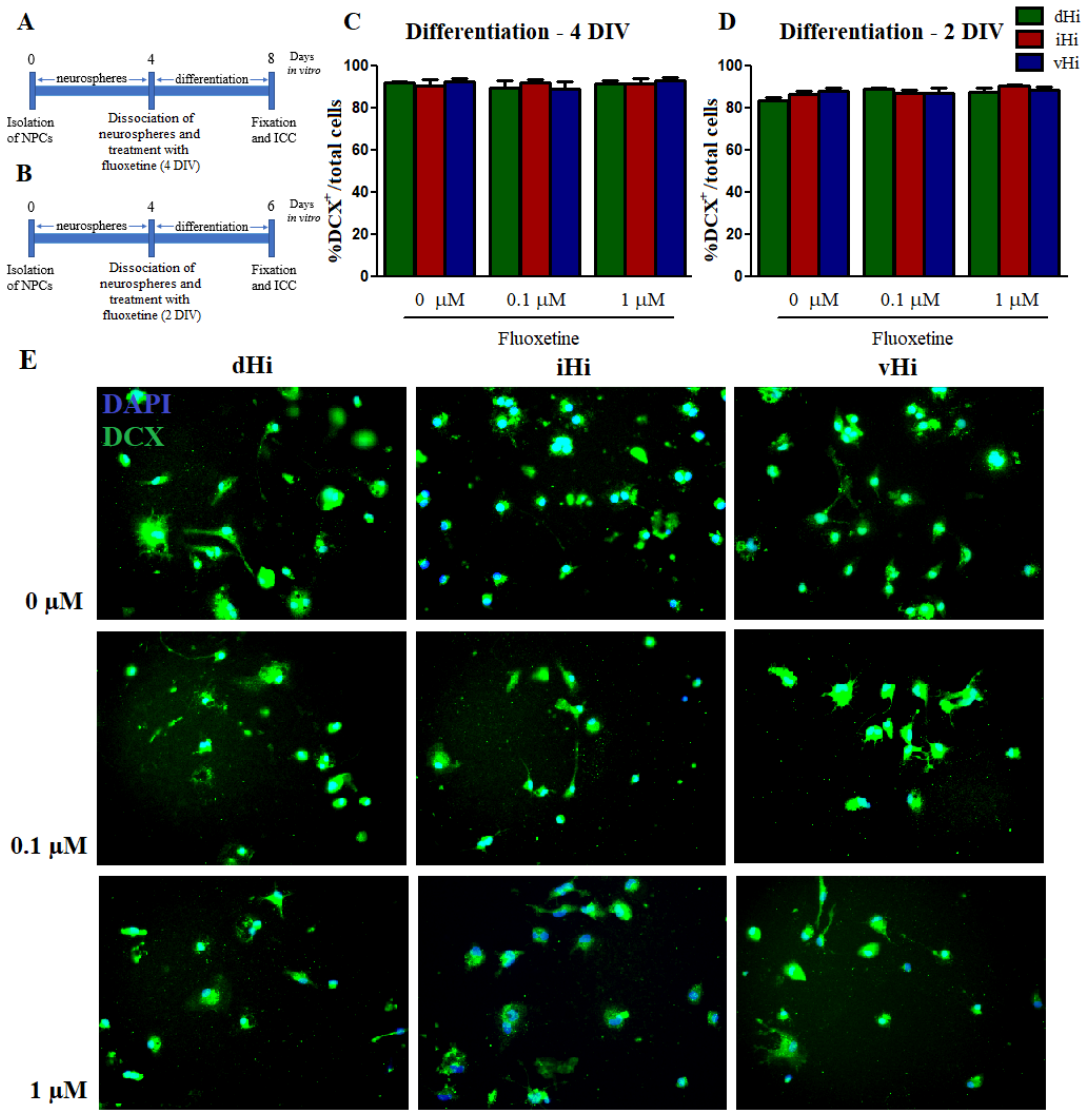

Figure 5.2*: Effects of fluoxetine on neuronal differentiation of NPCs derived from the dHi, iHi and vHi. A, Timeline of the 4 DIV exposure experiment: neurospheres were dissociated after proliferating for 4 days and exposed to fluoxetine for further 4 DIV under differentiation conditions prior to fixation and immunocytochemistry. B, Timeline of the 2 DIV exposure experiment: neurospheres were dissociated and exposed to fluoxetine for 2 DIV prior to fixation and immunocytochemistry. C, 4 DIV exposure to fluoxetine had no effects on neuronal differentiation of NPCs derived from either the dHi, iHi or vHi. D, 2 DIV exposure to fluoxetine had no effects on neuronal differentiation of NPCs derived from either the dHi, iHi or vHi. E, representative images of immature neurons differentiated from dHi, iHi and vHi NPCs; stained with DAPI (blue) and DCX (green).*

5.3.3. vHi and iHi NPC cultures were more sensitive to fluoxetine prevention of corticosterone-induced reductions in neuronal differentiation

There are some reports from *in vivo* studies that the effects of antidepressants on hippocampal neurogenesis are sometimes more apparent under stress conditions

(Tanti *et al.* 2012) or in the presence of corticosterone (Rainer *et al.* 2012). Indeed, it has been reported that antidepressant-induced neurogenesis from a human hippocampal progenitor cell line (HPC03A/07) are mediated by the glucocorticoid receptor (Anacker *et al.* 2011) and we have previously found that 4 DIV treatment with corticosterone decreased neuronal differentiation in hippocampal NPCs from P28 rats (Levone et al., unpublished; Chapter 4). Thus, we next investigated whether fluoxetine could counteract corticosterone-induced reductions in neuronal differentiation and whether such effects might be differentially affected in NPCs isolated from the dHi, iHi or vHi.

The effects of fluoxetine on corticosterone-induced reductions in neuronal differentiation are shown in the Fig 5.3B, C. Three-way ANOVA revealed a main effect of corticosterone ($F_{1,96}$=14.66, p<0.001) and of fluoxetine ($F_{2,96}$=5.98, p=0.004) but not of hippocampal sub-region ($F_{2,96}$=0.32, p=0.73), nor corticosterone x hippocampal sub-region ($F_{2,96}$=0.17, p=0.85) nor fluoxetine x hippocampal sub-region ($F_{4,96}$=0.4, p=0.81). Post-hoc analysis revealed that corticosterone significantly reduced neuronal differentiation from dHi NPCs (p=0.043) and vHi NPCs (p=0.009) and a similar trend was observed in the iHi cultures (p=0.06). These corticosterone-induced reductions in neuronal differentiation were prevented by 1 μM (but not 0.1 μM) fluoxetine in the iHi (p=0.038 vs. 1 μM CORT) and vHi (p=0.008 vs. 1 μM CORT) cultures. Interestingly, the fluoxetine-treated dHi cultures were not significantly different to the dHi cultures treated with corticosterone alone (0.1 μM, p=0.5; 1 μM, p=0.3 vs. 1 μM CORT), thus suggesting that the vHi and iHi cultures were more sensitive to the effects of fluoxetine.

5.3.*4. Neither fluoxetine alone nor fluoxetine plus corticosterone significantly affect neurite length of neurons differentiated from dHi, iHi or vHi NPCs*

The average neurite length per DCX^+ cells was measured as an index of neuronal maturation. The effects of 4 days treatment with fluoxetine alone on neuronal average neurite length in DCX^+ cells differentiated from dHi, iHi and vHi NPC cultures are shown in Fig 5.4A. Two-way ANOVA revealed no effects of fluoxetine ($F_{2,72}$=0.005, p=0.99), hippocampal sub-region ($F_{2,72}$=0.036, p=0.97), nor a fluoxetine x hippocampal sub-region interaction ($F_{4,72}$=0.65, p=0.63).

The effects of fluoxetine in combination with corticosterone on average neurite length per DCX^+ cells differentiated from dHi, iHi and vHi NPC cultures are shown in Fig 5.4B. Three-way ANOVA revealed a main effect of corticosterone ($F_{1,96}$=4.15, p=0.044), but no effect of fluoxetine ($F_{2,96}$=0.32, p=0.73) or hippocampal sub-region ($F_{2,96}$=2.19, p=0.12), nor corticosterone x hippocampal sub-region ($F_{2,96}$=0.12, p=0.89) nor fluoxetine x hippocampal sub-region ($F_{4,96}$=0.46, p=0.77). However, post hoc analysis revealed no significant differences between individual groups.

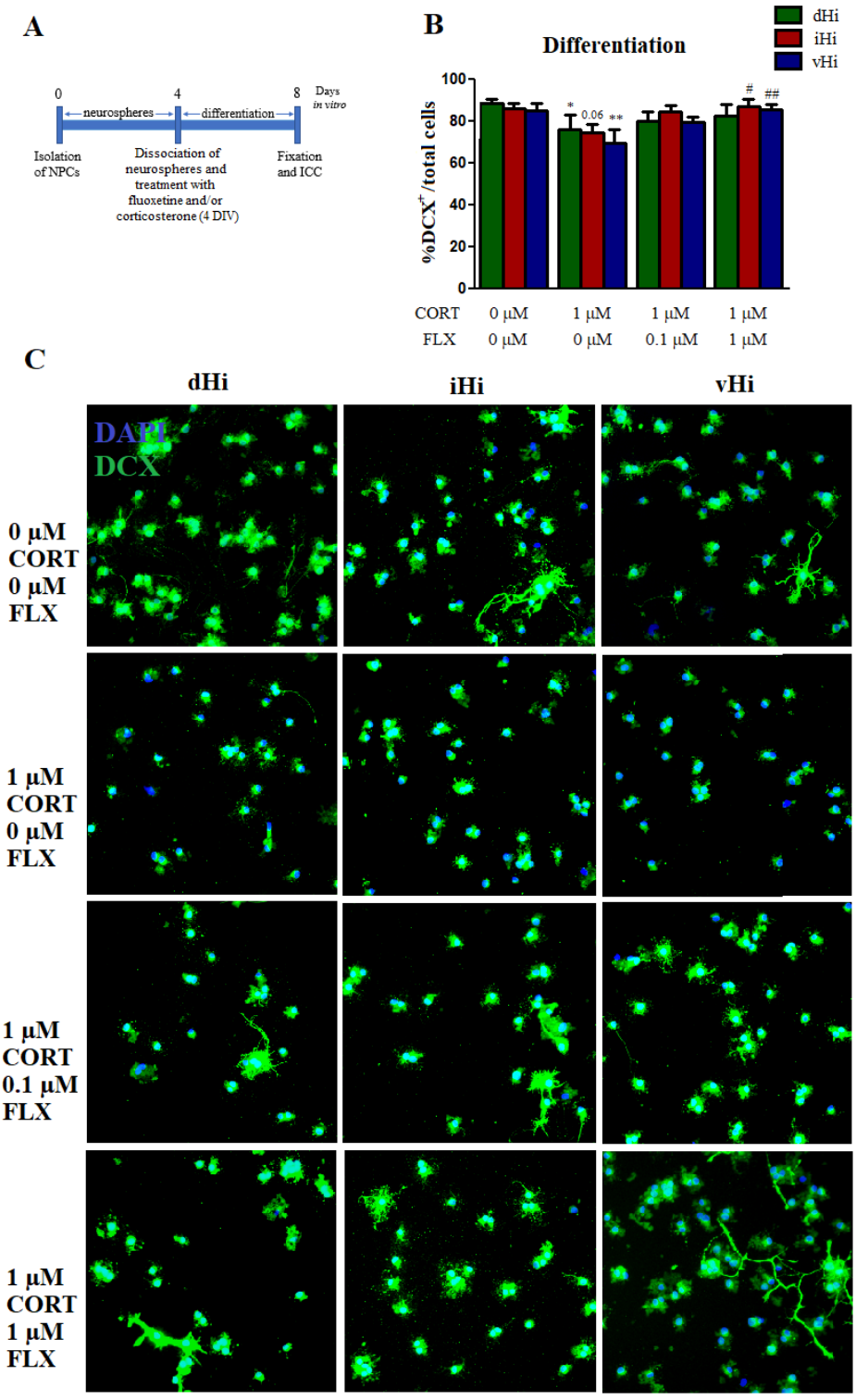

Figure 5.3*: Neural progenitor cells of the iHi and vHi are more sensitive to fluoxetine prevention of corticosterone-induced reductions in neurogenesis than those from the dHi. A, Timeline of the experiment: neurospheres were dissociated after 4 days growth and exposed to either corticosterone alone or corticosterone and fluoxetine (0.1 µM or 1 µM) for 4 DIV. B, Fluoxetine prevented corticosterone-induced reductions in neuronal differentiation, preferentially in cells derived from the iHi and vHi. C, Representative images of immature neurons differentiated from the dHi, iHi and vHi; stained with DAPI (blue) and DCX (green). *p<0.05, **p<0.01, compared with respective 0 µM group, #p<0.05, ##p<0.01, compared to corticosterone alone group, according to Fishers LSD post hoc test.*

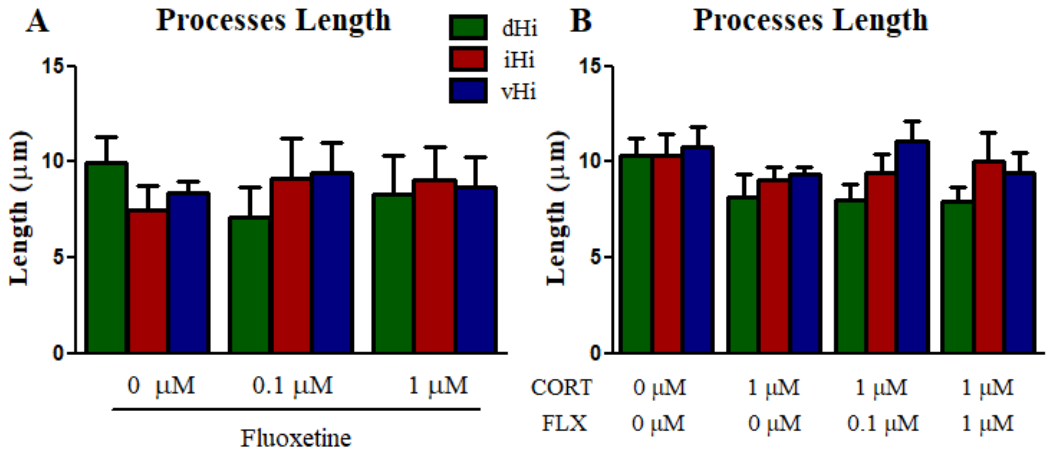

***Figure 5.4**: No differences were found on the average total length of neurites per DCX⁺ cell treated with fluoxetine alone for 4 DIV (A) or corticosterone and fluoxetine for 4 DIV (B).*

5.3.5. The sub-regional effects of fluoxetine prevention of corticosterone-induced reductions in neuronal differentiation are not due to alterations in cell viability or nuclear GR expression

The effects of corticosterone and fluoxetine on neuronal differentiation are not due to alterations in cell viability, as shown in Fig 5.5A. Three-way ANOVA demonstrated that there was no effect of corticosterone ($F_{1,96}$=2.06, p=0.16), fluoxetine ($F_{2,96}$=1.7, p=0.19), or hippocampal sub-region ($F_{2,96}$=1.01, p=0.37). Similarly, there were no significant interactions [corticosterone x hippocampal sub-region ($F_{2,96}$=0.2, p=0.82); fluoxetine x hippocampal sub-region ($F_{4,96}$=0.09, p=0.99)].

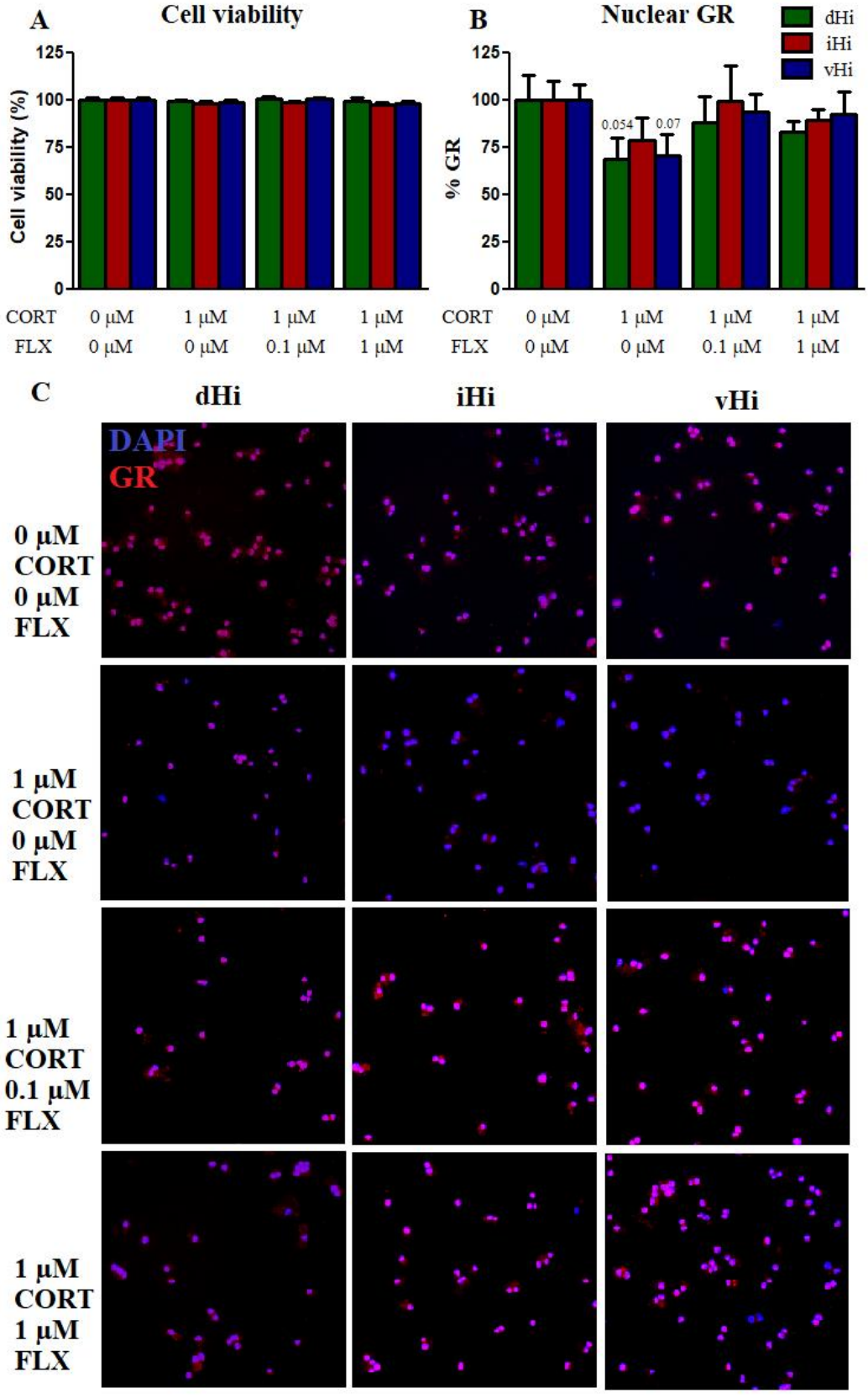

Figure 5.5*: Effects of fluoxetine and corticosterone on cell viability and nuclear GR expression. A, Neither corticosterone alone nor corticosterone plus fluoxetine co-treatment affected cell viability as assessed with an MTT assay. B, Fluoxetine seems to alleviate corticosterone-induced reductions on nuclear GR expression irrespective of the hippocampal sub-region cells were derived from. C, representative images of cells derived from the dHi, iHi and vHi stained with DAPI (blue) and GR (red).*

The effects of corticosterone and fluoxetine on nuclear glucocorticoid receptor (GR) expression are shown in Fig 5.5B, C. Three-way ANOVA revealed a significant effect of corticosterone ($F_{1,96}$=8.71, p=0.004), a trend for an effect of fluoxetine ($F_{2,96}$=2.75, p=0.069) and no effect of hippocampal sub-region ($F_{2,96}$=0.24, p=0.79). There was no interaction between hippocampal sub-region and corticosterone ($F2_{,96}$=0.11, p=0.9) or sub-region and fluoxetine ($F_{4,96}$=0.07, p=0.99). Post-hoc analysis revealed that while there were trends for corticosterone to decrease nuclear GR in cells derived from the dHi (p=0.054) and vHi (p=0.07), but not iHi (p=0.19) when compared their respective vehicle group, this pattern of effects was not apparent in fluoxetine-treated cells from any hippocampal sub-region (dHi, 0.1 μM p=0.23, 1 μM p=0.38; vHi, 0.1 μM p=0.16, 1 μM p=0.18 vs. respective vehicle group).

5.4. Discussion

Understanding the cellular actions of antidepressants is a key goal in developing novel more efficacious strategies for treating depression. The present study investigated whether NPCs isolated from the dHi, iHi or vHi sub-regions of the hippocampus exhibit differential intrinsic sensitivity to the neurogenic effects of the antidepressant, fluoxetine, in the absence or presence of the stress hormone, corticosterone. We found that fluoxetine alone did not affect neurogenesis in any of the NPC cultures. However, NPCs of the intermediate and ventral hippocampus were more sensitive to fluoxetine prevention of corticosterone-induced reductions in neurogenesis than those from the dorsal hippocampus.

In vivo studies have reported that chronic fluoxetine increase cell proliferation in rats (Malberg *et al.* 2000) and in mice (Zhou *et al.* 2016), preferentially in the iHi and vHi (Zhou *et al.* 2016). However, in the present *in vitro* study we did not see an effect of fluoxetine alone on cell proliferation thus suggesting that fluoxetine does not exert its effects via direct actions on NPCs. In contrast to our findings, other studies have demonstrated that fluoxetine increases cell proliferation *in vitro* (Hui *et al.* 2014, Wang *et al.* 2014). The primary pharmacological target of fluoxetine is the serotonin transporter, but studies have shown that it is not expressed on hippocampal NPCs (Schmitt *et al.* 2007) which may perhaps explain the absence of a direct effect of fluoxetine on the cell proliferation *in vitro*.

In addition to not affecting cell proliferation, we also found that fluoxetine had no effect on neuronal differentiation of NPCs after 4 DIV exposure to fluoxetine. We observed in the control group that the majority of cells had differentiated into neurons, and this may have precluded any potential fluoxetine-induced increases in

neuronal differentiation from being observed. Therefore, in the next experiment we reduced the time allowed for differentiation to 2 DIV and then measured the effects of fluoxetine on neuronal differentiation. However, most cells had differentiated into neurons, and again no effects of fluoxetine were observed. This suggests that the fluoxetine-induced increases in neuronal differentiation reported in i*n vivo* studies (Wang *et al.* 2008, Wang *et al.* 2011) are via indirect mechanisms. However, it is worth noting that fluoxetine has been shown to increase neuronal differentiation from embryonic hippocampal NPCs of rats (Wang *et al.* 2014). In addition, an *in vitro* study demonstrated that sertraline, which like fluoxetine is a selective serotonin reuptake inhibitor, increased neuronal differentiation from isolated human NPCs and that this effect was mediated through activation of the glucocorticoid receptor (Anacker *et al.* 2011). As described above, several *in vivo* studies report fluoxetine increase AHN, but negative findings have also been reported *in vivo*, for example, a study showed that chronic fluoxetine has no effect on neuronal differentiation in mice (Wang *et al.* 2008). In fact, some studies have shown that antidepressant-like drugs, including fluoxetine, increase neurogenesis in the presence of stress only (Banasr *et al.* 2006, Jayatissa *et al.* 2006, O'Leary *et al.* 2012, Tanti *et al.* 2012, O'Leary and Cryan 2014).

Given the reports that some antidepressants exert neurogenic effects via GR and that some studies only observe neurogenic effects of fluoxetine under stress conditions, we then assessed the effects of the fluoxetine on corticosterone-induced reductions of neuronal differentiation. We have previously observed that 4 DIV exposure to corticosterone reduced neuronal differentiation at the same extent in NPCs isolated from the dHi, iHi and vHi, while 7 DIV exposure to corticosterone reduced neuronal differentiation preferentially in NPCs derived from the vHi

(Levone et al., in preparation). Thus, we decided to use 4 DIV exposure rather than 7DIV to fluoxetine to avoid any confounding effects of preferential effects of corticosterone on vHi NPCs. Here, we replicated the findings of our previous study (Levone et al., in preparation), in which we observed that 4 DIV exposure to 1 µM corticosterone reduced neuronal differentiation. However, fluoxetine (1 µM) significantly prevented this effect in iHi and vHi-derived while only attenuating it in dHi cultures. Although these are modest effects, this is the first time to our knowledge that fluoxetine shows a preferential neurogenic effect on isolated iHi and vHi-derived NPCs.

Although we found a promising effect of fluoxetine on preventing corticosterone-induced reductions on neuronal differentiation, here we found no effects of fluoxetine alone, corticosterone alone, or co-treatment of fluoxetine plus corticosterone on neurite length of dHi, iHi or vHi NPCs. In contrast, an *in vivo* study showed that chronic fluoxetine treatment increases neuronal maturation in mice, increasing the number of DCX^+ cells with tertiary dendrites (Wang *et al.* 2008). Chronic corticosterone has been shown to reduce maturation of immature neurons (Fenton *et al.* 2015), an effect that was prevented by chronic co-treatment with imipramine, a tricyclic antidepressant. However, the present study found that only a very low percentage of cells had secondary processes, thus we hypothesise that if neurons were allowed to mature for a longer time, more robust effects of corticosterone and fluoxetine on neurite length and branching might be observed. Indeed, in our previous study (Levone et al., in preparation), we found that the effects of 7 days treatment with corticosterone on neuronal maturation in these cultures were much more robust than the findings shown here. Further studies should assess the roles of longer term fluoxetine treatment on corticosterone-

induced reductions in cell proliferation and neuronal maturation in these hippocampal sub-region-specific NPC cultures.

To determine if differential sensitivity of the NPC cultures to fluoxetine prevention of corticosterone-induced reductions on neuronal maturation were due to differential GR sensitivity, we measured nuclear expression of GR. Although 1 μM corticosterone did not cause a statistically significant reduction in nuclear GR expression, cells derived from the dHi and vHi showed a trend for reduced nuclear GR expression. This trend was no longer observed when any of the cell cultures were co-treated with either concentration of fluoxetine. Thus, differential effects of fluoxetine on preventing corticosterone-induced decreases in neuronal differentiation in different NPC cultures were not due to differential sensitivity of GR to fluoxetine plus corticosterone.

In summary, here we showed that iHi and vHi NPCs were more sensitive to fluoxetine in preventing corticosterone-induced reductions in neuronal differentiation than those from the dorsal hippocampus. Interestingly, fluoxetine alone had no direct effect on cell proliferation, neuronal differentiation and maturation on NPCs derived from the dHi, iHi or vHi. Taken together, we suggest that fluoxetine neurogenic effects reported *in vivo* are likely through indirect mechanisms. However, fluoxetine showed a direct neurogenic effect *in vitro* under stress-like conditions, and these effects were predominantly observed in the iHi and vHi thus suggesting that NPCs along the longitudinal axis of the hippocampus likely exhibit differential intrinsic sensitivities to antidepressants under stress conditions.

CHAPTER 6

General Discussion

6.1. General Summary of Results

The overall aim of this thesis was to determine whether specific sub-regions of the longitudinal axis of the hippocampus play differential roles in the behavioural and neurogenesis responses to stress, the stress hormone corticosterone and an antidepressant drug. To achieve this goal, this thesis investigated whether neural progenitor cells (NPCs) derived from either dorsal (dHi), intermediate (iHi) or ventral hippocampus (vHi) have differential intrinsic sensitivities to the stress hormone, corticosterone, and to the glucocorticoid receptor (GR) agonist, dexamethasone. Using hippocampal sub-region-specific ibotenic acid-induced lesions, the roles of the dHi, iHi and vHi on emotional behaviours and neuroendocrine responses under chronic psychosocial stress conditions were then investigated. Then using the same lesion approach, the roles of the dHi, iHi and vHi on emotional behaviours and the neuroendocrine response under baseline and under antidepressant (fluoxetine) treatment were assessed. Finally, whether NPCs derived from the dHi, iHi or vHi have differential intrinsic sensitivities to the reported neurogenesis-promoting effects of fluoxetine was then investigated.

As summarised in Table 1, the most important findings in this thesis were that the vHi plays the most predominant role in regulation of emotional behaviours followed by the iHi, while the contribution of the dHi, particularly in the absence of stress was comparably smaller. In parallel, we found that NPCs derived from the iHi and especially the vHi were more sensitive to long-term exposure to corticosterone and fluoxetine. These results indicate that both the iHi and especially the vHi are important areas in the regulation of emotional behaviours and thus are promising target areas for the study of stress and antidepressant drug development.

In Chapter 4, we determined that NPCs derived from the dHi, iHi and vHi did not exhibit differential sensitivity to acute (4 h *in vitro*) corticosterone-induced reductions in cell proliferation or to short-term (4 DIV) corticosterone-induced reductions in neuronal differentiation and maturation. However, longer-term (7 DIV) exposure to corticosterone or the GR agonist dexamethasone decreased neuronal differentiation in cultures derived from the vHi but not the dHi, while iHi cultures showed an intermediate response to those of the dHi and vHi. A similar gradient-like response was observed on effects on nuclear GR expression after corticosterone or dexamethasone. These results suggest that cells derived from the vHi and to some extent iHi, are intrinsically more sensitive to the detrimental effects of corticosterone and dexamethasone on hippocampal neurogenesis *in vitro*.

Given the increased sensitivity of iHi and vHi-derived NPCs to the stress hormone corticosterone, we next explored in Chapter 3 whether there may be differences in the expression of genes related to the stress response and neurogenesis across the different hippocampal sub-regions. Indeed, we found differences in the expression of some such genes along the longitudinal axis of the hippocampus thus suggesting potentially different contributions of the dHi, iHi and vHi in response to stress. To test this hypothesis, we lesioned the dHi, iHi or vHi and examined its impact on the behavioural and neuroendocrine responses to chronic psychosocial stress. We observed that iHi lesions increased stress-induced social avoidance. Stress-induced increases in anxiety in the novelty-induced hypophagia and marble burying tests were prevented by all lesions irrespective of hippocampal sub-region. However, only vHi lesions attenuated stress-induced anxiety in the open field test. Stress-induced anhedonia was reduced by dHi and vHi, but not iHi lesions. Interestingly, only vHi lesions induced an antidepressant effect in the forced swim test and

increased the latency to adopt a defeat posture during social defeat thus indicating increased active coping behaviour in vHi lesioned mice. Taken together, these results suggest specific sub-regions along the longitudinal axis of the hippocampus modulate specific behavioural responses to chronic psychosocial stress.

Since we found that the vHi was involved in antidepressant-like behaviour in the FST, we then decided to investigate the roles of the dHi, iHi or vHi in the regulation of social, anxiety, anhedonia and depressive-like behaviour in the absence or presence of the antidepressant fluoxetine (Chapter 2). In the absence of fluoxetine, vHi lesions reduced anxiety, while none of the lesions affected sociability, anhedonia or depressive-like behaviour under these conditions. On the other hand, only vHi lesions prevented the antidepressant effects of acute fluoxetine treatment in the tail suspension test and the anxiolytic effects of chronic fluoxetine treatment in the novelty-induced hypophagia test. Interestingly, only iHi lesions prevented the antidepressant effect of chronic fluoxetine treatment in the forced swim test, while dHi lesions did not impact antidepressant- or anxiety-related behaviour either in the absence or presence of fluoxetine. These data showed for the first time that both the iHi and vHi play distinct roles in fluoxetine-induced anxiolytic and antidepressant-like behaviour.

Since we observed a role for the vHi in modulating the effects of fluoxetine in the novelty-induced hypophagia test, a behaviour previously reported to be dependent on adult hippocampal neurogenesis (AHN) (Santarelli *et al.* 2003), we then decided to investigate whether dHi, iHi and vHi NPCs may exhibit differential sensitivity to fluoxetine. While we found no effects of fluoxetine alone on neurogenesis in any of the cultures, fluoxetine prevented corticosterone-induced reductions in neuronal

differentiation of NPCs derived from the iHi and vHi, while only attenuating those effects in NPCs derived from the dHi (Chapter 5).

Taken together, these data suggest that the vHi regulates anxiety and the anxiolytic and acute antidepressant behavioural effects of fluoxetine, and antidepressant-like behaviour under chronic stress conditions. On the other hand, like the dHi, the iHi does not seem to regulate emotional behaviour under baseline conditions. However, the iHi has a role in mediating social avoidance under chronic stress conditions and also plays a role in the antidepressant effects of chronic fluoxetine treatment. In parallel, we observed that vHi NPCs were more sensitive to the effects of long-term exposure to the stress hormone corticosterone when compared to those from the iHi and the dHi. However, we also observed that iHi NPCs were more sensitive to corticosterone than those from the dHi. Similarly, under stress-like conditions only (i.e. in the presence of corticosterone) we found that NPCs derived from the vHi and iHi and were more sensitive to the effects of fluoxetine than those derived from the dHi. For an overview of all findings in the present thesis, see Table 6.1. Taken together, we suggest that future studies aimed at identifying novel hippocampal-based targets for antidepressant drug development focus particularly on the iHi and vHi.

Table 6.1. *General summary of results*

	dHi	iHi	vHi
***In vitro* findings**			
Corticosterone			
4 h	↓ Proliferation	↓ Proliferation	↓ Proliferation
4 DIV	↓ Differentiation ↓ Maturation	↓ Differentiation ↓ Maturation	↓ Differentiation ↓ Maturation
7 DIV	↔ Differentiation ↓ Maturation ↓ Nuclear GR	↓↓ Differentiation ↓↓ Maturation ↓↓ Nuclear GR	↓↓↓ Differentiation ↓↓↓ Maturation ↓↓ Nuclear GR
Dexamethasone			
7 DIV	↔ Differentiation ↓ Maturation ↓ Nuclear GR	↓↓ Differentiation ↓↓ Maturation ↓ Nuclear GR	↓↓↓ Differentiation ↓↓↓ Maturation ↓↓ Nuclear GR
Fluoxetine			
4 h	↔ Proliferation	↔ Proliferation	↔ Proliferation
4 DIV	↔ Differentiation ↔ Maturation	↔ Differentiation ↔ Maturation	↔ Differentiation ↔ Maturation
Fluoxetine on corticosterone-treated cells			
4 DIV	↔ Differentiation ↔ Maturation	↑ Differentiation ↔ Maturation	↑ Differentiation ↔ Maturation
***In vivo* findings**			
Anxiety Behaviours			
Baseline conditions	↔	↔	↓ in EPM, MBT, NIH, OF
Chronic Stress	↓ in MBT, NIH	↓ in MBT, NIH	↓ in MBT, NIH ↓ in OF
Fluoxetine	↔	↔	↑ NIH
Anhedonia			
Baseline conditions	↔	↔	↔
Chronic Stress	↓ in SP, FUST	↔	↓ in SP, FUST
Fluoxetine	↔	↔	↔
Depression			
Baseline conditions	↔	↔	↔
Chronic Stress	↔	↔	↓ in FST
Fluoxetine	↔	↑ in FST	↑ in TST

Abbreviations*: FST: forced swim test, FUST: female urine sniffing test, GR: glucocorticoid receptor, MBT: marble burying test, NIH: novelty-induced hypophagia, OF: open field, SP: saccharin preference test, TST: tail suspension test.*

6.2. Stuck in the Middle: Rationale for Subdividing the Hippocampus into Three Sub-Regions

Accumulating evidence suggests that the hippocampus is segregated along its longitudinal axis into dorsal and ventral sub-regions in rodents and corresponding

posterior and anterior sub-regions in primates (Moser and Moser 1998, Fanselow and Dong 2010). A growing body of evidence has been supporting the idea that the hippocampus is actually a gradient and differential gene expression is found along its longitudinal axis (Moser and Moser 1998, Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016, Cembrowski *et al.* 2016). Using an RNAseq database (Cembrowski *et al.* 2016), we confirmed these differences in the expression of several genes in the dentate gyrus of the dHi (dDG) versus vHi (vDG) of the mouse hippocampus. In agreement, several studies have reported differential gene expression in the dDG versus vDG in rats (Christensen *et al.* 2010) and in mice (Cembrowski *et al.* 2016). Besides finding changes in several systems of neurotransmission, here we described differential expression in the mineralocorticoid receptor, growth factor genes and genes related to neurogenesis in the vDG compared to dDG, supporting that the vHi expresses more genes that favour cell proliferation, while dHi expresses more genes that favour neuronal differentiation (Chapter 3). In agreement, a recent study suggests that the dHi has more active neurogenesis and is enriched with maturing neurons markers, such as NeuroD1 and DCX, while the mouse vHi is enriched of radial glia markers, such as Sox2 and Hes5 (Zhang *et al.* 2018). Accordingly, dHi neural progenitor cells have been shown to mature faster, associated with higher basal network activity (Piatti *et al.* 2011). Interestingly, we did not observe this in our cell cultures, in which dHi and vHi NPCs had a similar rate of proliferation, differentiation and maturation. Perhaps this is not surprising, given that these cells were isolated from the surrounding brain environment.

Although these findings demonstrate a clear differential expression of some genes between dHi and vHi, recent gene expression studies support the concept that genes

are expressed as a gradient along the hippocampal longitudinal axis in mice (Thompson *et al.* 2008, Strange *et al.* 2014, Cembrowski *et al.* 2016), and as such, the presence of an intermediate hippocampus is suggested. In this thesis, we also used a database to assess whether some genes are differentially expressed along the longitudinal axis of the hippocampus, more specifically in the dorsal CA1 (dCA1), intermediate CA1 (iCA1) and ventral CA1 (vCA1) (Chapter 3). Again, we found differentially expressed genes between dorsal and ventral sub-regions, but also between intermediate and dorsal and between intermediate and ventral sub-regions. These changes include genes related to several neurotransmission systems and plasticity. In agreement, previous studies have shown that genes are differentially expressed along the longitudinal axis of the CA1 (Leonardo *et al.* 2006, Dong *et al.* 2009, Cembrowski *et al.* 2016, Pacheco *et al.* 2017) and CA3 (Thompson *et al.* 2008). Interestingly, we also found that the mineralocorticoid receptor was expressed at lower level in the vCA1 versus dCA1, while the iCA1 had an intermediate expression of this receptor, supporting a gradient expression of the MR along the longitudinal axis of the hippocampus. This may suggest that cells derived from the dHi, iHi and vHi respond differentially to stress. Indeed, a study supports the idea that the dHi and vHi have distinct epigenetic responses to acute stressors (Floriou-Servou *et al.* 2018), with the vHi being particularly more sensitive to the stressor. Moreover, the receptor NMDA3a was also expressed in a gradient along the longitudinal axis of the hippocampus, and the vHi showed higher levels of expression. Interestingly, a previous study showed that glutamatergic receptors are differently expressed between dorsal and ventral hippocampus (Pandis *et al.* 2006). Most of the previous studies, however, compared only the dorsal half versus the ventral half of the hippocampus. A study dissected the rat hippocampus into the most dorsal third and the most ventral third, ignoring the intermediate third,

and also found that some genes are differentially expressed in the vHi versus the dHi (Lee *et al.* 2017).

In addition to these gene expression studies, other studies used different boundaries to define dorsal versus ventral hippocampus (Zuena *et al.* 2008, Morley-Fletcher *et al.* 2011, Tanti and Belzung 2013). Most studies use coordinates of coronal slices of the hippocampus for the segregation into its sub-regions. Perhaps, it is important to note that a coronal brain section might not contain one specific hippocampal sub-region only but may also contain more than one sub-region. The precise number of hippocampal domains is currently unknown (O'Reilly *et al.* 2015). Although the roles of the iHi have yet to be precisely determined, the few studies on this hippocampal sub-region show that it seems to be not only a transitional area between the dHi and vHi, but also an integrative region (Bast 2007, Bast *et al.* 2009), mediating the integration of diverse sensory modalities (Bast *et al.* 2009). The intermediate hippocampus is also involved in cognitive flexibility, engaging in rapid place encoding (Bast *et al.* 2009), and, like the dHi, plays a role in processing spatial information (Kenney and Manahan-Vaughan 2013). In this thesis, we included this third hippocampal sub-region and analysed its roles in emotional behaviours under chronic stress and chronic antidepressant treatment and, *in vitro*, the isolated NPCs response to corticosterone and fluoxetine. Here, we describe for the first time that the intermediate hippocampus plays no role in emotional behaviours in the absence of stress or antidepressants, however, this hippocampal sub-region mediates the antidepressant effects of chronic fluoxetine in the FST, and the social avoidance under chronic psychosocial stress.

It is important to notice that, besides the important circuits we cited in this thesis, there are other circuits that may play a role in the behavioural effects of chronic stress or fluoxetine. For example, the bed nucleus of the stria terminalis (BNST) has been shown to play critical roles in anxiety (Lebow and Chen 2016). Besides that, the intrahippocampal circuitry may play an important role on the results found in this thesis and should be further studied in the future. In fact, based on the information that the iHi has already been shown to be as a region of association between dHi and vHi (Bast *et al.* 2009), here we suggest that the lesions of the iHi may cause a disruption on the coherence between the dHi and vHi and the results observed after iHi lesion could be due to a lack of communication between these two hippocampal sub-regions.

For the *in vivo* part of the present study, we used the coordinates shown in Fig 6.1 to segregate the hippocampus into dHi, iHi and vHi. According to the Paxinos atlas for mouse brain (Paxinos 2012), the coordinates are as follows (in coronal slices, distance from bregma: dHi: -1.22 to -2.54 mm; iHi: -2.7 to -3.5 mm (dorsal part); vHi: -3.28 to -3.88 mm (ventral part). We decided to segregate the hippocampus into three sub-regions once there is already evidence in the literature showing that the intermediate part seems to be an independent sub-region on its own, and thus we suggest that future studies should segregate the hippocampus into these three independent sub-regions. However, we acknowledge that the hippocampus might be segregated into more independent sub-regions (Zhou *et al.* 2016), which need to be further studied. Even by segregating the hippocampus into only three sub-regions, there is a heterogeneity in the cell population between these regions. Specifically, it has been described that field CA1 cytoarchitecture is heterogeneous between dHi and vHi (pyramidal neuron layers) (Dong *et al.* 2009). We suggest that

this might influence the segregation of the hippocampus, but in the present study we cannot precise the impact that this might have on the information flow. We suggest, however, that the introduction of more sub-regions would increase this heterogeneity between the regions.

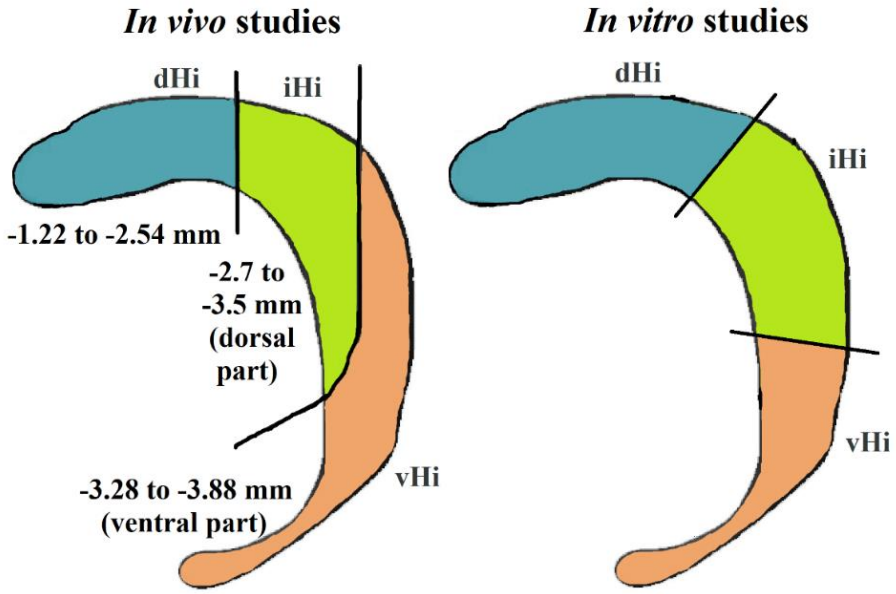

Figure 6.1*: In the left, the coordinates used to segregate the hippocampus into dHi, iHi and vHi, according to the atlas of Paxinos for mice brain (Paxinos 2012) in our in vivo studies (coordinates in relation to bregma). In the right the segregation of the hippocampus in dHi, iHi and vHi used for our in vitro studies. dHi in blue, iHi in green and vHi in pink.*

For the *in vitro* part of this study, however, the hippocampus was segregated into three equal thirds, to ease the process of gross dissection and segregation of the hippocampus. Here we acknowledge that this difference in the segregation coordinates used in the *in vitro* and *in vivo* parts of this thesis may at some extent hinder the comparison between both studies. Moreover, another confounding factor that may interfere in the comparison between the *in vitro* and *in vivo* parts of this thesis is that we used mice for our *in vivo* part and rats for our *in vitro* part. We suggest that future studies should be more consistent and use the same species for all study. We also acknowledge a potential limitation our studies, which is that we

cannot discount the possibility that behavioural effects observed could be due to the other two remaining intact sub-regions. A control lesioning the whole hippocampus could be a form of counteracting this limitation.

6.3. From the Dish to Disease: *In Vitro* Models to Predict *In Vivo* Efficacy in Stress and Antidepressant Research

It is common agreement that cells in culture do not perfectly mimic cells in a complex living system. However, isolating them to single cells in a dish might help us understand the intrinsic characteristics of the individual cells, without the interference of the environment where these are located in an organism. They are also useful tools to allow us to study the dynamics of their intracellular molecular signalling pathways. Organoids, for example, derive from cells which self-organise in three-dimensional cultures (Foley 2017) and are one of most physiologically relevant ways to have them in culture. 2D monolayer cell cultures can also be useful, as it is easier to control their homogeneous exposure to the culture medium with a treatment of choice, and they are easier to be analysed using relatively standard microscopy tools.

While embryonic hippocampal NPCs are more numerous, easier to culture, grow better and produce more cells than hippocampal NPCs from the adult brain, they are unsuitable for our research questions involving the interrogation of NPCs from specific regions along the longitudinal axis of the hippocampus. The niche of neurogenic cells in embryonic mice has been shown to be initially located in the ventral pole of the hippocampus, and these cells migrate along the longitudinal axis towards the dorsal hippocampus during early postnatal life and is thought to be complete by postnatal day 21 (Li *et al.* 2013) and thus any time point after PND21

is a suitable time to interrogate NPCs along the longitudinal axis of the hippocampus. Here, we adapted a protocol used to isolate and culture adult mouse NPCs (Walker and Kempermann 2014) for use for the isolation of NPCs from P28 rats, thus limiting the challenges associated with working with adult hippocampus-derived NPCs. The fact that all sub-regional NPCs arise from the same neurogenic pool (initially located in the vHi) could lead to the interpretation that the NPCs from different adult hippocampal sub-regions should have the same intrinsic characteristics. However, in the present study we demonstrated for the first time that NPCs isolated from different sub-regions of the hippocampus have unique intrinsic sensitivities in response to the stress hormone, corticosterone (Chapter 4), but not to the antidepressant, fluoxetine (Chapter 5). These findings suggest that NPCs from different hippocampal sub-regions probably develop these intrinsic characteristics in response to glucocorticoids after migration, and this is probably due to the neural input influences (the connections that each hippocampal sub-region does) they receive which might cause them to differentially express some relevant genes. However, we also hypothesise that the age of the animals is a critical factor and should always be taken into consideration, as these NPCs have recently finished the migration along the longitudinal axis of the hippocampus, and whether their response to corticosterone change in an age-dependent manner is unknown. We do know, however, that it would not be possible to study the differences of dHi, iHi and vHi in embryonic NPCs or NPCs from younger animals since they are predominantly located in the ventral pole. Taken together, the differential sensitivities of dHi, iHi and vHi to longer-term corticosterone exposure may at least in part explain the in vivo reported that AHN in the vHi is particularly vulnerable to chronic stress.

Several *in vivo* studies have shown that chronic stress and chronic antidepressant treatment affect AHN preferentially in the ventral sub-region of the hippocampus, which is also a sub-region related to the control of the HPA axis in response to stress. In the *in vitro* part of this thesis, we isolated neural progenitor cells (NPCs) from the dHi, iHi and vHi, in an effort to determine whether these cells, when isolated and free from external influences, respond differentially to the stress hormone corticosterone, the glucocorticoid receptor agonist dexamethasone, or the antidepressant fluoxetine (Chapters 4 and 5). *In vivo*, corticosterone and chronic stress have been shown to reduce cell proliferation in the dentate gyrus of the hippocampus of rats (Jayatissa *et al.* 2006, Brummelte and Galea 2010). Acute stress has also been shown to transiently reduce hippocampal NPCs proliferation (Falconer and Galea 2003, Heine *et al.* 2004) in rats. *In vitro* studies show that short and long-term glucocorticoids exposure reduce cell proliferation in human (Anacker *et al.* 2013) and rat (Boku *et al.* 2009) NPCs. In agreement, this thesis reports that acute corticosterone decreases cell proliferation to a similar extent in cells derived from the three hippocampal sub-regions (Chapter 4). However, we found no effects of fluoxetine on cell proliferation (Chapter 5 of this thesis for more details).

In contrast to our findings, several *in vivo* studies have reported that corticosterone (Brummelte and Galea 2010, Anacker *et al.* 2013) and chronic stress (Malberg and Duman 2003, Heine *et al.* 2005, Kim *et al.* 2005, Mitra *et al.* 2006, Dagyte *et al.* 2009, Jayatissa *et al.* 2009) decrease proliferation of NPCs, preferentially in the vHi rather than the dHi, and that antidepressants increase neurogenesis preferentially in the iHi and vHi (Zhou *et al.* 2016). Taken together, our results may indicate that the preferential effects of corticosterone, stress and antidepressant treatment on cell

proliferation in the vHi as observed *in vivo* are not due to the intrinsic characteristics of the NPCs residing in each hippocampal sub-region, but due to other factors perhaps related to the influences that these cells receive where they reside. Another possibility is that the NPCs were not exposed to corticosterone or fluoxetine long enough.

We next exposed the cells to corticosterone or fluoxetine for 4 DIV under differentiation conditions and found that most NPCs differentiated into neurons. The preferential differentiation into neurons was intentional, as the culture media was serum-free conditions, and this is known not to favour astrocytic differentiation. Under these conditions, we found again that corticosterone reduces neuronal differentiation and maturation to a similar extent in all hippocampal cultures irrespective of the sub-regional source, and fluoxetine alone had no effect on neuronal differentiation or maturation. Interestingly however, we found that fluoxetine prevents corticosterone effects on neuronal differentiation in iHi and vHi-derived cultures, while it only attenuates these effects in dHi-derived cultures. This suggests that preferential neurogenic effects of fluoxetine observed under chronic stress conditions *in vivo* could in part be due to differential intrinsic characteristics of NPCs residing in different sub-regions along the longitudinal axis of the hippocampus. However, we observed that very few (2-4%) of the immature neurons obtained after 4 DIV had secondary processes, which suggests that they were still in the process of maturation. Thus, longer term exposure to fluoxetine may be required to obtain a more complete insight into the effects of fluoxetine and corticosterone on neuronal maturation.

Taking into consideration the *in vivo* studies that show that the vHi is more sensitive to the effects of chronic stress (Zuena *et al.* 2008, Brummelte and Galea 2010, Morley-Fletcher *et al.* 2011, Hawley *et al.* 2012, Tanti *et al.* 2012, Tanti and Belzung 2013) combined with the limited number of neurons with a more mature morphological phenotype in the 4 DIV cultures, we then decided to expose the NPCs to corticosterone for a longer period (7 DIV). 7 DIV corticosterone exposure reduced neuronal differentiation and their maturation in cultures derived from all hippocampal sub-regions. However, cells derived from the iHi and more especially those derived from the vHi were more sensitive to these effects of long-term corticosterone exposure. This is in accordance with the *in vivo* studies previously cited. Thus, here we show for the first time that cells from the iHi are also more sensitive to these CORT-induced effects on hippocampal neurogenesis and thus more attention should be given to this hippocampal sub-region in future studies that assess the effects of stress on hippocampal function. In addition, we suggest that the preferential effects of chronic stress on vHi AHN observed *in vivo* could at least in part be due to the differential intrinsic sensitivity of NPCs resident in the vHi to corticosterone.

Taking into consideration that the vHi is more sensitive to the effects of corticosterone after long-term, but not short-term exposure, we suggest that the expression of GR may play a role in differential response to corticosterone, as it is known that the expression of the GR is variable during the neuronal differentiation of transient-amplifying cells to neuroblasts and then immature neurons (Garcia *et al.* 2004, Egeland *et al.* 2015), and different expression of GR on our short-term versus longer-term experiments may have been responsible for or diverse results in cell proliferation versus longer-term cell differentiation. Indeed, we also found that

the longer-term treatment with corticosterone (7 DIV) reduced the nuclear translocation of GR, specifically in cells derived from the iHi and vHi. These results were in accordance with our results on cell differentiation and maturation. However, it has been shown *in vivo* that 5 days stress is able to decrease GR immunoreactivity, while 20 days of stress increase it, both preferentially in the dHi, but not in the vHi (Robertson *et al.* 2005), but the expression of GR specifically in NPCs or adult born neurons was not investigated thus making it more difficult to make direct comparison to our data. To follow up with these results, we then interrogated whether there were sub-regional differences in the intrinsic sensitivity of differentiating neurons to the GR agonist, dexamethasone. Indeed, exposure of NPCs to dexamethasone for 7 DIV reduced neuronal differentiation and maturation, and in a more pronounced way than corticosterone did. The sensitivity of vHi NPCs to dexamethasone was even more apparent suggesting that neurons generated from vHi NPCs display intrinsically higher GR sensitivity. Moving forward, it will be important that future studies determine whether these *in vitro* findings will translate to the *in vivo* setting. To test whether these *in vitro* findings on neurogenesis might contribute to the behavioural results from the lesion studies, the manipulation of AHN in specific areas of hippocampus (e.g. the overexpression/knockdown of TLX, etc) would be informative. Indeed, it has been reported irradiation-induced ablation of AHN in the vHi but not dHi prevents the behavioural effects of fluoxetine (Wu and Hen 2014). The roles of AHN in specific sub-regions including the iHi under chronic stress conditions has not yet been investigated.

6.4. Lesion Studies and Other Approaches on the Neuroscience Era of Circuit-Dissection

Many of the previous studies which assessed the roles of the hippocampus used specific cytotoxic lesions to unveil the differential functional roles of dHi versus vHi (Morris *et al.* 1982, Moser *et al.* 1995, Bannerman *et al.* 2002, Kjelstrup *et al.* 2002, Bannerman *et al.* 2003). More recent studies, however, have been using more modern techniques, including optogenetics and chemogenetics (Felix-Ortiz *et al.* 2013, Felix-Ortiz and Tye 2014, Carreno *et al.* 2016, Padilla-Coreano *et al.* 2016, Parfitt *et al.* 2017, Jimenez *et al.* 2018). Briefly, here we used ibotenic lesions of the dHi, iHi, or vHi to investigate whether these hippocampal sub-regions play distinct roles on emotional behaviours in baseline and in response to chronic antidepressants (Chapter 2) or chronic psychosocial stress (Chapter 3).

In Chapter 3 of this thesis, we described the roles of dHi, iHi and vHi in the behavioural and neuroendocrine responses to chronic psychosocial stress. We chose to use a protocol of social defeat and overcrowding, as previous studies in our laboratory demonstrated that this protocol of stress promotes consistent reductions in social interaction (Finger *et al.* 2011, Burokas *et al.* 2017). While stress decreased social interaction in all experimental groups, we did observe that iHi lesions exacerbated stress-induced social avoidance and that vHi lesions increased the latency to adopt a defeat posture during social defeat protocol, possibly suggesting that vHi mediates active coping behaviour. To the best of our knowledge, this is the first time that a role for the iHi in stress-induced social avoidance has been reported. However, previous studies have implicated the vHi in resilience to social defeat stress (Calfa *et al.* 2007, Kenworthy *et al.* 2014, Laine *et al.* 2017, Hultman *et al.* 2018).

Interestingly, in Chapter 2 of this thesis we showed that lesions of the vHi but not of the dHi or iHi robustly reduced anxiety in the elevated plus maze, novelty-induced hypophagia, marble burying and open field tests in mice in baseline (with no stress or antidepressant treatment). Our findings of vHi modulation of anxiety are in agreement with previous reports, which showed that vHi lesions in rats reduced hyponeophagia (Bannerman *et al.* 2002, Bannerman *et al.* 2003, McHugh *et al.* 2004, Esposito *et al.* 2005, Ge *et al.* 2006) and decreased anxiety in several behavioural tests (Bannerman *et al.* 2002, Kjelstrup *et al.* 2002, Bannerman *et al.* 2003, McHugh *et al.* 2004, Esposito *et al.* 2005, Ge *et al.* 2006, Pentkowski *et al.* 2006). Moreover, optogenetic and pharmacogenetic studies have reported that several vHi neural pathways regulate anxiety in the open field and elevated plus maze (Felix-Ortiz *et al.* 2013, Padilla-Coreano *et al.* 2016, Parfitt *et al.* 2017). However, It is also important to note that an optogenetic study demonstrated that the activation of mature granule cells of both the dHi and vHi DG reduces anxiety behaviour in the elevated plus maze in mice (Kheirbek *et al.* 2013). In Chapter 3 of this thesis we found that in agreement with previous studies, chronic stress increased anxiety in several behavioural tests (Finger *et al.* 2011, Slattery *et al.* 2012, Burokas *et al.* 2017). Interestingly, we found that lesions of either the dHi, iHi or vHi all prevented stress-induced anxiety in the marble burying and in novelty-induced hypophagia tests. To the best of our knowledge, this is the first time that this has been shown in these behavioural tests under chronic stress conditions. While the present results might suggest that all hippocampal sub-regions mediate stress-induced anxiety, this effect may be test-dependent, as a different picture emerged in the open field test, whereby lesions of the vHi had an anxiolytic effect attenuating the stress-induced decrease in time spent in the centre

of the open field, an effect not due to alterations in locomotor activity. Again, this further highlights the predominant role that the vHi seems to have in anxiety.

In agreement with previous studies, in Chapter 2 of this thesis reported that chronic fluoxetine treatment had anxiolytic effects in the novelty-induced hypophagia test (Santarelli *et al.* 2003, Dulawa *et al.* 2004, Dulawa and Hen 2005), and in the marble burying test (Nicolas *et al.* 2006, Greene-Schloesser *et al.* 2011). vHi lesions prevented the anxiolytic effects of chronic fluoxetine treatment in the novelty-induced hypophagia test but not in the marble burying test. The reasons underlying this differential effect are currently unclear but may indicate that fluoxetine-induced anxiolytic effects occur through different mechanisms or different pathways in these two tests. For example, AHN has been shown to be important for the anxiolytic effects of fluoxetine in a similar test of neohypophagia, the novelty-suppressed feeding (Santarelli *et al.* 2003, David *et al.* 2009). Moreover, several studies report that chronic treatment with antidepressants (Banasr *et al.* 2006, Soumier *et al.* 2009, Paizanis *et al.* 2010, O'Leary and Cryan 2014) including fluoxetine (Tanti *et al.* 2012), preferentially increase AHN in the vHi. Moreover, it was recently reported that new neurons in the mouse vHi are required for the anxiolytic effects of chronic fluoxetine treatment to be manifested in the novelty-supressed feeding test (Wu and Hen 2014).

In Chapter 2, we found that none of the hippocampal sub-region lesions affected anhedonia (in the saccharin preference and female urine sniffing tests). Here we also found that fluoxetine did not have an effect in the female urine sniffing test. This is a relatively new test (Malkesman *et al.* 2010) which has yet to be fully characterised in terms of responsivity to different types of antidepressant drugs.

However, in Chapter 3, we found that the chronic psychosocial stress paradigm induced an anhedonic effect in the saccharin preference and female urine sniffing tests. This is in contrast with some reports, which showed that this specific stress paradigm increased anxiety without altering saccharin preference (Slattery *et al.* 2012) or sucrose preference (Hammels *et al.* 2015). However, it has been reported that chronic social defeat stress does induce anhedonia in the female urine sniffing test (Finger *et al.* 2011, Lehmann *et al.* 2013, Burokas *et al.* 2017), and in the saccharin preference test (Liu *et al.* 2018). In our study, we found that lesions of either the dHi or vHi prevented stress-induced anhedonia in both the saccharin preference test and the female urine sniffing test. Interestingly, iHi lesions did not affect stress-induced anhedonia in either of these tests. Taken together, we show for the first time that the dHi and vHi, but not the iHi play a role in the stress-induced anhedonia.

In Chapter 2, we describe that the lesion of any hippocampal sub-region in baseline did not alter depressive-like behaviour in the forced swim test. Interestingly vHi lesions prevented the antidepressant effects of acute fluoxetine treatment in the tail suspension test. To the best of our knowledge, this is the first time that the vHi is shown to mediate the antidepressant effects of acute treatment with an antidepressant drug. Here we found that iHi lesions prevented the antidepressant effects of chronic fluoxetine in the forced swim test. This is the first time that the iHi has been shown to play a role in the antidepressant effects of chronic fluoxetine. When we examined the impact of chronic stress and lesions on antidepressant-like behaviour, we found that chronic psychosocial stress did not alter immobility time in the forced swim test. In accordance with our results, a growing number of studies have reported that chronic psychosocial stress increases anxiety, without altering

depressive-like behaviours in the forced swim test (Keeney and Hogg 1999, Kinsey *et al.* 2007, Slattery *et al.* 2012, Hammels *et al.* 2015). Interestingly however, we found that stressed vHi-lesioned animals showed reduced immobility time when compared to both sham groups thus indicating an antidepressant-like phenotype. Intriguingly, this effect is not apparent in non-stressed vHi-lesioned mice (Chapter 3). Others have reported that chemogenetic activation of the vHi to medial prefrontal cortex pathway in non-stressed rats exerts an antidepressant-like effect in the forced swim test and that inactivation of this pathway is also required for the sustained antidepressant-like effects of ketamine in this test (Carreno *et al.* 2016). Here, we found that under chronic stress conditions vHi lesions induce antidepressant-like effects thus suggesting that, under chronic stress conditions, other vHi-mediated pathways regulate antidepressant like effects in this test. Taken together, we show for the first time that the vHi plays a role in modulating antidepressant-like behaviour in the mouse forced swim test under chronic stress conditions

Comparing the results of the same tests, used in different experiments, we found some differences in the performance of sham animals. Specifically, the largest difference we found was in the novelty-induced hypophagia test, in which we found, that control sham animals in the baseline, fluoxetine and stress experiments had an average latency difference of 150 s, 300 s and 70 s, respectively. Interestingly, sham animals in the antidepressant study were injected with saline daily and this *per se* could have been a stressor that might have increased latency in this test. However, the same pattern was not observed in the open field test in which we found that control sham animals in the baseline, fluoxetine and stress experiments had centre quarter time of 85 s, 80 s and 170 s respectively. Other

behavioural tests showed very consistent results across all experiments including the marble burying test (approximately 10 marbles buried by sham animals in all experiments), the saccharin preference test (approx. 80%, in the baseline experiment; 60-70% preference in the chronic stress experiment), the female urine sniffing test (80% for baseline and stress experiments; 70% for antidepressant experiment). Finally, for the forced swim test, we found an average of 140 s immobility for sham animals in the baseline experiment, 110 s for chronic stress experiment and 125 s for antidepressant experiment. Taken together, differences found might have been due to differences in experimental conditions as outlined below.

In the baseline experiment, animals started behavioural tests one week after the stereotaxic surgery. For chronic antidepressant and chronic stress studies, however, this happened 26 and 28 days after, respectively. Moreover, animals in the baseline and antidepressant experiments were group housed, while animals in the chronic stress experiment were singly housed after the surgery. The findings observed here are in contrast with a previous study that showed that singly housing increases anxiety and depression-like behaviour (Berry *et al.* 2012). In the present thesis, we singly housed the animals in the chronic stress experiment in accordance with previous studies performed in our laboratory (Finger *et al.* 2011, Burokas *et al.* 2017), but future studies should consider including a sham-group housed group.

Finally, it is important to emphasise that the hippocampus has connections with many other brain regions and plays diverse other roles not cited in the present thesis. These other brain connections, however, could also be involved in the results shown here. For example, it has been shown that an excitatory circuit from the ventral

hippocampus to the lateral septum is involved in feeding behaviour (Sweeney and Yang 2015). The novelty-induced hypophagia test, a test used here to assess anxiety, has also a feeding component. Whether the vHi to lateral septum circuit is involved with the results obtained in the novelty-induced hypophagia is unknown. Also, it has been shown by optogenetics that the stimulation of the nucleus accumbens to the ventral hippocampus circuit increases locomotor activity (Bagot *et al.* 2015). The marble burying test, a test used here to assess anxiety, also has an active/locomotor behaviour component. Whether the nucleus accumbens to vHi circuit is involved in the marble burying test, however, is still unknown. Also, the elevated plus maze (EPM), the most classical test used to assess anxiety, also involves other components, including risk assessment and spatial navigation. While the risk assessment is an emotional behaviour and thus preferentially regulated by the vHi, spatial navigation is preferentially regulated by the dHi. Interestingly, we only observed an anxiolytic effect of the vHi lesion in the EPM, but we did not use any test to assess spatial navigation, thus we cannot argument this other component of the EPM.

Finally, it is important to emphasise that the hippocampus also plays role in many other functions not related in this thesis. Indeed, the ventral hippocampus has also been shown to play roles in olfactory functions (Vanderwolf 2001), reward (Riaz *et al.* 2017) and contextual decision making (Ross *et al.* 2011), thus the ventral hippocampus lesion performed in the present study could have altered several other functions not assessed in the present thesis. Additionally, the intermediate hippocampus has also been shown to send projections to the lateral septum rostral, which in turn projects to hypothalamic medial zone nuclei, a brain region involved in the expression of social behaviour (Risold and Swanson 1996). In the present

study, the lesion of iHi did not alter social behaviour, but did prevent chronic fluoxetine-induced reductions in anxiety and antidepressant-like behaviour.

6.5. Limitations and Future Directions: Towards New Treatment Strategies for Depression and Stress-Related Disorders

6.5.1. Intermediate hippocampus

Throughout this thesis, we demonstrated that the iHi seems to be a separate hippocampal sub-region, with its own functional roles. Some studies, however, suggest that the hippocampus is composed of many independent areas along the longitudinal axis. While most of the studies segregate the hippocampus into dHi and vHi, we segregated it into three sub-regions, including the iHi, in an effort to tackle discrepancies found in the literature, which use diverse boundaries to segregate the hippocampus into dorsal and ventral regions. We suggest that future studies should consider the intermediate hippocampus as a functionally independent sub-region. Moreover, we suggest that future studies should have a consensus on the specific coordinates used for dorsal, intermediate and ventral hippocampus, both in rats and in mice.

6.5.2. Cell culture studies

Several *in vivo* and *in vitro* studies have shown that chronic stress and chronic glucocorticoid or antidepressant treatment affect neurogenesis. Whether this happens differentially along the dorsoventral axis of the hippocampus is not completely clear. In this thesis, we isolated NPCs from P28 SD rats, specifically from the dHi, iHi and vHi, although culture of NPCs from non-embryonic animals is a very difficult technique and many months had to be spent to establish the

protocol used in the laboratory. Future work by other researchers planning to use this protocol should be willing to spend several months on protocol validation. Here, NPCs were grown as neurospheres for 4 DIV, then the neurospheres were dissociated into single cells and then treated them with corticosterone for 4 h, 4 DIV or 7 DIV. We found a strong detrimental effect of 7 DIV corticosterone and dexamethasone on the differentiation and maturation of NPC-derived neurons, especially on those derived from the iHi and vHi. To confirm whether these results are really GR-dependent, future studies should focus on the reversal of these effects, using the GR antagonist RU486.

The isolation of NPCs from dHi, iHi and vHi could also be used as a tool to verify whether these cells, when isolated, show differential gene expression, both at baseline and after corticosterone and/or an antidepressant treatment. To the best of our knowledge, this assessment has never been done and would help unveil if and which genes are differentially expressed in these NPCs. After identifying the potential genes or proteins of interest, these could be silenced/overexpressed, in an effort to identify targets that could induce AHN more rapidly than current antidepressant agents.

6.5.3. Lesion studies

Many previous studies have used the lesion approach to find out the functional roles of the dHi and vHi. In the current era of optogenetic and chemogenetic neural circuitry dissection, this technique may be considered old-fashioned, but it is still useful to assess the functional roles of these regions. A limiting factor, however, is that the lesions with ibotenic acid do not allow the lesion of a specific area of the hippocampus (e.g. DG or CA), and neither a discrete cell type. Moreover, we have

limited idea of what specific vHi inputs or outputs actually mediate the various findings. To do this, optogenetics or DREADDs would be a useful approach.

In the present study, we used the lesions approach to assess whether dHi, iHi and vHi mediate emotional behaviours and neuroendocrine response under baseline, under chronic psychosocial stress and under chronic fluoxetine treatment. Here, we discussed our data using other studies that focused specifically on the network between the ventral hippocampus and other brain regions, such as nucleus accumbens, amygdala and medial prefrontal cortex. However, several other brain regions could be involved in the results found in this thesis.

Using lesion approach, future work should first assess whether dHi, iHi and vHi mediate the fluoxetine-induced recovery from stress-induced anxiety and depressive-like behaviour. Then, the specific mechanistic processes of how the vHi mediates anxiety under baseline conditions, antidepressant-like behaviour under chronic stress conditions, and anxiolytic and antidepressant effects of chronic fluoxetine treatment should be more deeply assessed. For that, *in vivo* optogenetics or DREADDs could be a possible approach. Excitation and inhibition of specific pathways between the dHi, iHi and vHi and other brain areas under baseline, chronic stress and chronic antidepressant treatment conditions would help to elucidate the mechanism underlying how these hippocampal sub-regions lesions exerted their effects.

6.5.4. Towards new treatment strategies for depression and stress-related disorders

Studying the mechanisms underlying antidepressant action can help on the development of new antidepressant drugs. The discovery of ketamine, an

anaesthetic that showed acute antidepressant effects in human trials, brought the hope that the discovery of a better antidepressant could be near. However, after finding that ketamine has adverse effects, and its effects are actually limited, we understand that much research still has to be done in this regard for the discovery of better antidepressants.

For future work, the identification and understanding of the mechanism of how newly-born neurons contribute to the response and recovery from stress, and to the response to antidepressants would make a big step towards new treatment strategies for depression and stress-related disorders. The knowledge on the roles of different hippocampal sub-regions should be deepened, aiming for a better understanding of the pathophysiology of anxiety, depression and stress-related disorders. We suggest that future studies should focus on understanding the pathways to and from the diverse hippocampal sub-regions, and how they modulate emotional behaviours and the response to stress and antidepressants. A useful tool would be to study this comparing stress-susceptible with stress-resilient animals. A gene expression study along the longitudinal axis of the hippocampus of stress susceptible versus stress resilient animals would be helpful to identify new antidepressant targets. Similarly, it would be also helpful to identify differential gene expressions along the longitudinal axis of the hippocampus of antidepressant responder versus non-responder animals.

In the near future, the role of genetics and epigenetics in the pathophysiology of depression should also be better understood. Mutant genetic code and molecular abnormalities, besides the classical neurotransmission malfunction, should be taken into consideration as possible etiologic factors, because subtle molecular

abnormalities can change the efficiency of cell signalling (Stahl 2013). Genetic polymorphisms of serotonergic, noradrenergic and dopaminergic transporters and receptors have also been identified. Finally, given that genetic factors can influence drug response (Schosser *et al.* 2012), the use of pharmacogenomics, tests which analyse an individual's genes and match the results to the available antidepressant drug, should be widely used, as this allows a personalised treatment, faster response and higher chances of remission (Hall-Flavin *et al.* 2013).

6.6. Concluding Remarks

In summary, the present thesis corroborates with the current literature, showing evidence of a functional segregation of the hippocampus into its longitudinal axis. Also, we reinforce the importance of segregating the hippocampus using a tripartite model, given that there is enough evidence that the iHi is an independent sub-region on its own right. Specifically, this thesis reports for the very first time that NPCs derived from the iHi and vHi but not the dHi are more sensitive to the deleterious effects of the stress hormone, corticosterone, on neurogenesis. These effects are correlated with reduced translocation of GR into the nucleus, especially in cells from the iHi and vHi. In vivo, iHi seems to mediate social avoidance and vHi mediates antidepressant-like behaviour under chronic stress conditions. Moreover, iHi and vHi seem to mediate fluoxetine behavioural effects. Therefore, we suggest that the iHi and vHi sub-regions are promising targets for the treatment of stress-related psychiatric disorders and future studies should focus in these sub-regions, rather than on the hippocampus as a whole.

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APPENDIX A

BUFFERS, SOLUTIONS AND PROTOCOLS

Cell culture protocols

Dissection (should take approximately 10 minutes per animal)

- Decapitate the animal;

- Dissect out the brain, open the meninges;

- Put it on a wet sterilized filter paper in a 100 mm Petri dish;

- Pour some cold HBSS on the brain;

- With the scalpel remove the brain steam and cerebellum;

- With the scalpel cut the interhemispheric fissure;

- Take out the hippocampus carefully;

- Put it in “collection medium”.

PS: While dissecting, put the enzyme at 37°C (approximately 20 minutes before starting the incubation), growth media should also be in the incubator

Cell Isolation

- Pour the hippocampi in a 100 mm Petri dish;

- With a scalpel, transfer from the petri dish to a horizontal 15 mL tube;

- Mince the tissue with the scalpel in the 15 mL tube until you can no longer see no big tissue pieces;

- Pour 5mL of the warmed enzyme mix into the 15 mL tube;

- Gently vortex;

- Incubate at 37°C for a total time of 30 minutes;

- Gently vortex every 3 minutes;

- At 20 minutes of incubation, pipette up and down with a glass Pasteur;

- At 30 minutes of incubation, pipette up and down with a micropipette (first with the p1000 30 times and then with the p200 30 times);

- Centrifugate at 300 g for 5 min at RT;

- Remove the supernatant and resuspend pellet in 1 mL of buffer;

- Centrifugate at 300g for 5 min at RT;

- Remove the supernatant and resuspend in 1mL of 20% Percoll. Then pour the rest of the Percoll and mix (5mL final volume);

- Centrifugate at 800 g for 10 min at RT;

- Remove the upper phase. Remove gently the liquid in the middle. Resuspend the pellet in 10 mL buffer;

- Centrifugate at 300 g for 5 min at RT;

- Remove the supernatant and resuspend the pellet in 1 mL of the growth medium;

- Pass the cell suspension through a 40 µm cell strainer;

- Count the cells using a hemocytometer and trypan blue;

- 1 x 10^5 cells are seeded per well in an ultra-low attachment 24-wells plate (final volume per well of 500 µL).

Solutions:

- Ice-cold HBSS for washing the brain;

Collection medium:

- Hibernate-A;

- 2% B-27 minus Vitamin A;

- 0.5 mM Glutamax.

Enzyme mix:

- 5.5 mL 1 U/ml Dispase;

- 2.5 U/ml Papain;

- 250 U/ml DNaseI;

- Mix until homogeneous and sterile filter.

Percoll:

- For 90% Percoll: 0.5 ml of 10X PBS and 4.5 ml of Percoll 100%;

- For 20% Percoll: 1.1 ml of 90% Percoll and 3.9 ml of Neurobasal-A.

Growth media (for proliferation):

- 24 mL Neurobasal-A;

- 2 % B-27 minus Vitamin A;

- 0.5 mM Glutamax;

- 50 U/mL Penicillin/Streptomycin;

- 2 µg/ml Heparin;

- 20 ng/mL bFGF;

- 20 ng/mL EGF.

Growth media (for differentiation):

- 24 mL Neurobasal-A (**Attention**, it is a different Neurobasal than the one used for the proliferation step);

- 2 % B-27 with Vitamin A;

- 0.5 mM Glutamax;

- 50 U/mL Penicillin/Streptomycin.

Dilutions for cell culture

Epidermal growth factor

EGF (25µg) (Sigma) was reconstituted using 0.2µm filtered sterile 10mM acetic acid (Sigma) containing 0.01% Bovine serum albumin (BSA) (Sigma, Ireland) to give a stock of 20µg/ml. Aliquots were stored at -20°C.

Heparin (50mg/ml)

Heparin (50mg) (Sigma) was dissolved in 1ml of sterile PBS (10mM). The solution was sterile filtered and stored at 4°C

Fibroblast growth factor

bFGF (25µg) (Millipore) was reconstituted using 0.2µm filtered sterile PBS (10mM) containing 0.1% BSA, 1mM DTT, and heparin (5µg/ml), to give a stock solution of 20µg/ml. Aliquots were stored at -20°C.

DNAseI

The DNAseI was diluted in sterile deionised water (to keep its inactivity) and stored at -20°C for no longer than 6 months. The stock solutions had a concentration of 25,000 U/ml. When use it, make sure to use a medium with Magnesium to activate it.

Poly-D-lysine

Poly-D-lysin was diluted in sterile deionised water (1 mg/ml) and kept at -20°C.

Corticosterone

Corticosterone was preferentially freshly diluted in 100% DMSO (or else frozen for no longer than 1 month). The stock solutions prepared had a concentration of 10-50 mM.

Fluoxetine

Fluoxetine was freshly diluted in sterile deionised water. The stock solutions prepared had a concentration of 1-10 mM.

Dexamethasone

Dexamethasone was preferentially freshly diluted in 100% DMSO (or else frozen for no longer than 2 weeks). The stock solution had a concentration of 10-50 mM

Protocols for perfusions

PBS (to make 1 litre):

- Dilute 5 tablets of PBS (Sigma) in deionised water;

- Filter.

PFA 4% (to make 1 litre):

- Dilute 5 tablets of PBS (Sigma) in 200 ml of deionised water;

- Dilute 40g of PFA in 800 ml of deionised water at around 70°C (warmed in microwave);

- Mix concentrated PBS with PFA and adjust pH to 7.4;

- Filter.

Perfusions:

- Clean tubing with dH2O and then ice-cold PBS;

- Anaesthetise animal and check for reflexes (feet, eyes, etc);

- Open chest and expose the heart;

- Circulate PBS from the left ventricle at the speed of 25 for around 5 min;

- Turn off pump and switch tube to PFA;

- Circulate PFA at the speed of 35 for around 8-10 min;

- Wash tubing with PBS at the speed of 45 for 3-5 min between animals.

Antifreezing solution (for free-floating slicing)

- 25% PBS 0.2M (pH 7.4);

- 30% ethylene glycol;

- 25% glycerol;

- 20% deionized H2O.

Gelatin coating slides

- 5g Gelatin;
- 250mg Chromic Potassium Sulphate;
- 500ml dH_2O;
- Microscope slides.

- Heat 500ml of dH_2O to 40°C;
- Add the gelatin slowly to dissolve, using a stir bar;
- Add the chromic potassium sulphate;
- Prepare the slides by cleaning with 70% ethanol and placing in the holders;
- Cool subbing media to 35°C;
- Pour the media into two black slide boxes and remove the foam;
- Place the slide holders into the subbing media for approximately 1 minute;
- Remove carefully, tap the edges and clean the slide holders with paper;
- Place slides in a box and dry for 1 week at room temperature or overnight at 40°C.

Cresyl violet staining from frozen sections

- 300ml dH_2O;
- 0.3g Cresyl violet;
- 30ml acetic acid;
- Add cresyl violet to dH_2O and stir for 24h;
- Filter and add acetic acid;
- Top up to 300ml to replace water lost from evaporation.

Staining:

- Remove sections from freezer and let them dry to RT;
- Place sections into 70% ethanol overnight at RT (de-fat step);
- Dry slides holders and wash in dH_2O for 2 minutes;
- Dry and place in cresyl violet solution for 10 minutes;
- Rinse with dH_2O;
- Differentiate in 95% ethanol for 5 minutes;
- Dehydrate in 100 % ethanol 2x5 minutes;
- Clear in histolene 2x5 minutes;
- Coverslip using DPX mounting medium (toxic).

RNA extraction (Mirvana kit)

- Remove tissue from -80°C and add 300uL of lysis
- Transfer to bigger Eppendorf and mash with pestil
- Add 30 uL of miRNA homogenate additive
- Leave for 10 min in wet ice
- Add 300 uL of Acid Phenol Chloroform (cold room) and vortex for 30s (do not forget APC at RT)
- Centrifuge at 7000rpm for 5 min (put elution to heat at 95°C - 100 uL per sample)
- Remove supernatant and add 3.75 ml of molecular ethanol (1.25 volumes)
- Add to new tube with filter and centrifuge for 15 s
- Throw liquid away
- Add 700 uL of wash solution 1 and centrifuge for 10-15 s
- Throw liquid away
- Add 500 uL of wash solution 2/3 and centrifuge for 10-15 s
- Throw liquid away
- Add 500 uL of wash solution 2/3 and centrifuge for 10-15 s
- Throw liquid away and centrifuge again for 10-15 s to dry the filter
- Transfer filter to a new tube and add 100 uL of elution to each tube
- Centrifuge for 30 s
- Throw away filter
- Keep tubes on ice

To measure RNA with NanoDrop:

- ND100 - nucleic acid - RNA
- Click blank (1 uL of RNA free water)
- Clean
- Put sample (1 uL), then Measure
- 260/280 has to be >1.8 and 260/230 has to be >1.0 - measure of cleanliness
- Show report - reports - save report - export report table only

ELISA kit for corticosterone

- Remove kit from the cold room 30 min before use;

- Prepare the assay buffer by diluting 1/10 in distilled water (approx. 20 ml per plate);

- Prepare the corticosterone standard: solution should be at RT; label 5 eppendorf from 1 to 5;

- Put 900 uL of buffer in tube 1 and 800 uL of buffer in tubes 2-5;

- Add 100 uL of standard CORT to tube 1 and vortex;

- Add 200 uL from tube 1 to tube 2 and vortex;

- Add 200 uL from tube 2 to tube 3 and vortex;

- Add 200 uL from tube 3 to tube 4 and vortex;

- Add 200 uL from tube 4 to tube 5 and vortex;

- USE DILUTED ST WITHIN 1H OF DILUTION.

- The dilution of the samples are as follows:

    - T0 - 1/21 - 10 µL of sample + 200 µL of buffer (or 12 uL + 240 uL, if possible)

    - T30 - 1/60 - 4.16 µL of sample + 245.84 µL of buffer

    - T120 - 1/40 - 6.25 µL of sample + 243.84 µL of buffer

- Pipet 100 uL of buffer into the NSB and B0 wells

- Pipet 100 uL of ST1-5 into appropriate wells

- Pipet 100 uL of the samples into appropriate wells

- Pipet 50 uL of buffer into NSB wells

- Pipet 50 uL of blue conjugate into each well, except TA and BLANK wells

- Pipet 50 uL yellow antibody into each well, except the BLANK, TA and NSB wells

- Every well should be GREEN, except the NSB that should be BLUE. The BLANK and TA are empty and should have no colour.

- Seal plate with the sealer and incubate the plate at RT on a plate shaker for 2h at 600 rpm

Example of plate (39 samples per plate):

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	St 1	St 5	S 4	S 4	S 12	S 12	S 20	S 20	S 28	S 28	S 36
B	BLANK	St 1	St 5	S 5	S 5	S 13	S 13	S 21	S 21	S 29	S 29	S 36
C	TA	St 2	S 1	S 6	S 6	S 14	S 14	S 22	S 22	S 30	S 30	S 37
D	TA	St 2	S 1	S 7	S 7	S 15	S 15	S 23	S 23	S 31	S 31	S 37
E	NSB	St 3	S 2	S 8	S 8	S 16	S 16	S 24	S 24	S 32	S 32	S 38
F	NSB	St 3	S 2	S 9	S 9	S 17	S 17	S 25	S 25	S 33	S 33	S 38
G	Bo	St 4	S 3	S 10	S 10	S 18	S 18	S 26	S 26	S 34	S 34	S 39
H	Bo	St 4	S 3	S 11	S 11	S 19	S 19	S 27	S 27	S 35	S 35	S 39

- Prepare the wash buffer by diluting 1/20 in distilled water (approx. 70ml per plate);

- Empty the content of the wells and wash by adding 200 uL of wash solution to each well; repeat the wash 2 more times;

- Empty the wells and tap the plate on a paper towel to remove remaining buffer;

- Remove the bubbles with the pink needle (be very careful in this step);

- Add 5 uL of blue conjugate to TA wells;

- Add 200 uL of pNpp Substrate solution to every well and check for bubbles again;

- Incubate at RT for 1h without shaking, with tin foil;

- Add 50 uL of stop solution to every well and read plate.

- Turn on machine (1st floor) and open software;

- Open the file “Corticosterone final final” in Loreto’s folder.

- Click in read plate (this will make the plate go in the machine); the plate will go out and then in again in the middle of the reading;

- Results will be in “Matrix” → Statistics → Data: Conc, well type: samples;

- An acceptable CV is 15%;

- Click in the Excel symbol and then “Save”.