Dissecting the functional and morphological contributions of the glucocorticoid receptor gene in neural progenitor cells of the hippocampus

By

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Abstract

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Stress hormones are known as one of the strongest and most ecologically relevant mediators of adult neurogenesis. A lingering question in adult neurogenesis is whether these hormones, known as corticosteroids (cortisol in humans, corticosterone in rodents), act directly on neural progenitor cells (NPCs), or indirectly through secreted factors or changes in network activity. Additionally, the functional contributions of this impact are largely speculative. To address these unknowns, we generated a transgenic mouse model whose glucocorticoid receptors (GRs) could be inducibly inactivated specifically in NPCs. GRs are the main target that corticosteroids bind to when elevated during stress. We investigated the effect of this cell-specific GR knockout model on hippocampal survival and differentiation and found them to be similarly affected by chronic corticosterone treatment compared to controls. This implies that corticosterone-suppressed neurogenesis and its impact on morphology is indirect, and GR in other cells may be mediating the effects. Furthermore, mice with GR inactivation in newborn neurons behaved similarly to controls in all tasks observed under basal levels of corticosterone. When mice were chronically treated with corticosterone, however, controls exhibited an anxious phenotype in novelty-suppressed feeding (NSF), light/dark box, and elevated Omaze, whereas transgenic mice behaved like untreated control groups in all anxiety measures except latency to feed in NSF. Neither corticosterone nor inactivation of GR in adult-born neurons altered depression-like behaviors in the forced swim test, nor percent freezing in contextual fear discrimination. Lastly, we found that corticosterone increased the rate of learning in 1-trial contextual fear conditioning, an effect not mediated by reducing GR signaling in the neurogenic pool. These results highlight the functional contributions of adult neurogenesis as well as how their GRs mediate anxiety-relevant behaviors irrespective of suppressed neurogenesis.
Acknowledgements

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I am also so fortunate for the opportunity I had to form a collaboration with Francois Tronche. Francois not only helped make Paris my home, but he also made his team a welcoming and comfortable environment for me. I owe him great thanks for pulling me out of lab at night to get dinner or a coffee so I didn’t slip away into the ether of endless hours at the computer. He taught me how to live well, and how to incorporate that into a busy scientist’s schedule. I greatly benefited from not only Francois’s expertise and deep concern for the quality of my work, but also the time and consideration that he took to thoroughly read my proposals and dissertation, as well as to just talk science with me for an afternoon. During my time in his lab, I improved my skill set in designing, conducting, and interpreting experiments. Francois’s attentiveness to my development as a scientist is a privilege of which I am very appreciative. Throughout my time in both labs, Daniela and Francois introduced me to many opportunities and other scientists that expanded my knowledge base and aided my experiments and analyses. This work would not have been possible without the support, guidance, and enthusiasm of these two people that I hope know the magnitude of which I value not just their advice, but also their friendship.

Aside from my mentors, my progression through my doctorate would not have been possible without the support of the UC Berkeley community. I would like to thank my thesis committee members, John Ngai, Darlene Francis, and Wally Wang, for their enthusiasm, thoughtful consideration and advise over the years. Also to be acknowledged are my lab members, colleagues, and students who helped in this research. The Kaufer lab members, both past and present, have not only inspired and motivated me with their discussions and insights, but have been a surrogate family of support and generosity. I am thankful for all their lighthearted humor and always so generously helping me troubleshoot with my own projects and concerns at a moments notice. They made my life easier and brighter, and I am so grateful for all of their advice, concern, and smiles. In particular, Id like to thank Kereshmeh Taravosh-Lahn for help troubleshooting with tissue staining; Shawn Shirazi for his dependable technical support; and a very special thank you to Liz Kirby, for not only her time and advice on my data, but her critical help with statistics and lightning speed email responses; you are ridiculously kind.
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it was the best of times,
it was the worst of times;
I wouldn’t trade it for the world.
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LIST OF ABBREVIATIONS

abGC  adult-born granule cell
ACTH  adrenocorticotropin
ADX   adrenalectomy
ANOVA analysis of variance
AVP   arginine vasopressin
BDNF  brain derived neurotrophic factor
BrdU  bromo-deoxyuridane
CamKII Ca\(^{2+}\)/calmodulin dependent protein kinase
Cort  corticosteroids (cortisol in humans, corticosterone for rodents)
CreERT2 Cre recombinase estrogen receptor T2
CRH/F corticotrophin releasing hormone/factor
DBD   DNA binding domain
DCX   doublecortin
Dex   dexamethasone
DG    dentate gyrus
EE    enriched environment
ERT2  estrogen receptor T2
FGF2  fibroblast growth factor 2
FST   forced swim test
GC    glucocorticoid
GCL   granule cell layer
GFAP  glial fibroblast-associated protein
GFP   green fluorescent protein
GR    glucocorticoid receptor
GRE   glucocorticoid response element
GR\(_{NPCKO}\) glucocorticoid receptor gene inactivation in neural progenitor cells
HPA   hypothalamo-pituitary-adrenal
IGF   insulin growth factor
KD    knockdown
KO    knockout
LBD   ligand binding domain
LTD   long term depression
LTP   long term potentiation
MR    mineralocorticoid receptor
NE-SAM norepinephrine-sympathetic adrenomedullary
NeuN  Neuron-specific nuclear marker
NG    neurogenesis
NMDA  N-methyl-D-aspartate
NPC   neural progenitor cell
NSF   novelty-suppressed feeding
OE    overexpression
PBS   phosphate buffered solution
PCNA  proliferating cell nuclear antigen
PFA   paraformaldehyde
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<td>post traumatic stress disorder</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>SGZ</td>
<td>subgranular zone (of the dentate gyrus)</td>
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<td>SVZ</td>
<td>subventricular zone</td>
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<td>TLX</td>
<td>Tailless gene</td>
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CHAPTER 1

INTRODUCTION
OUTLINE

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1.1 Scope of thesis

The research in this thesis is an investigation of the role the glucocorticoid receptor (GR) plays in hippocampal neurogenesis and functioning. To achieve this, we have generated a novel mouse model that inactivated the GR gene in nestin-expressing cells of the adult mouse brain.

In this dissertation, I report abolishing GR expression specifically in a population of neuronal progenitor cells (type 1 and 2a; NPCs) of the hippocampus by using an inducible Cre/loxP recombinase strategy. We found that GR gene inactivation in these cells did not change the survival or differentiation rate of neurogenesis for mice under basal levels of corticosterone. Similar to controls, differentiation of NPCs was also sensitive to chronic corticosterone treatment in mice with GR gene inactivation. Functional studies further revealed that GR gene inactivation in NPCs reduced anxiety-like behaviors only when mice were under chronic treatment of stress hormones. There was no effect of GR gene inactivation on depression-like behaviors, contextual fear conditioning, or pattern separation, a hippocampal-dependent associative memory ability. This was true regardless of whether mice were treated with corticosterone or not. These results provide a novel mouse model to examine GR-mediated processes in NPCs and suggest a functional role in anxiety-like behaviors mediated perhaps independently from changes in neurogenesis.
1.2 Stress and neurogenesis in the mammalian central nervous system

1.2.1 The stress response in the brain

The body strives to maintain homeostasis, or optimal biological functioning\textsuperscript{1-3}. In order to achieve this, it needs to have systems in place that respond to the ever-changing external and internal events or stimuli that it faces. External events can range from being physical (e.g. injury, heat stroke, starvation), to psychological, such as fear of threat. These psychological events, whether real or imagined, stimulate the stress response. Two of these responses are called the norepinephrine-sympathetic adrenomedullary system (NE-SAM) and the hypothalamic-pituitary-adrenocortical system (HPA). These two systems work together to increase (or redirect) energy resources, particularly critical when an organism is in survival mode (for review\textsuperscript{4,5}).

When the brain perceives a stressor, this triggers the stress response (Figure 1; for review\textsuperscript{6}). The hypothalamus activates the NE-SAM system- releasing epinephrine (adrenaline) and norepinephrine into the bloodstream for fast reactions\textsuperscript{7}. These are the immediate physiological changes that occur, such as an increase in heart rate, breathing, and metabolism, and a decrease in digestion and growth. The hypothalamus, also activated by the HPA system, releases, via a portal system, corticotrophin-releasing hormone or factor (CRH/F) and vasopressin onto the anterior pituitary to evoke the release of adrenocorticotropin (ACTH). ACTH then reacts with the adrenal glands to secrete glucocorticoids (i.e. cortisol in humans, corticosterone in rodents; hereafter referred to as cort) into the bloodstream\textsuperscript{5}. These lipophilic hormones can then pass through the blood brain barrier and circulate throughout the brain, indirectly affecting DNA transcription. This cascade of events in the HPA axis can take approximately 20 minutes\textsuperscript{5}, however, the effect on protein synthesis can be persisting\textsuperscript{8}. 
Under basal conditions, the HPA axis functions in a circadian rhythm on a 24-hour cycle\textsuperscript{3,4}. This typically consists of lower cort secretion as the animals go to sleep and greater secretion as the animals begin to wake up (Figure 2). Although this secretion is pulsatile, averages lie predictably along this rhythm\textsuperscript{10-14}. 

Figure 1. The HPA axis. When the brain perceives a threat, a coordinated cascade of events occurs in response. Neurons in the hypothalamus release CRH and arginine vasopressin (AVP), which induce the secretion of ACTH from the pituitary. ACTH then triggers the adrenal cortex to produce glucocorticoids known as corticosteroids (cort). Cort can pass through the blood brain barrier and interact with two receptors, MR and GR. Activation of these receptors can trigger feedback loops that can inhibit further activity of the HPA axis and return the system to a homeostatic point. Photo credit\textsuperscript{9}.

Figure 2. Cort secretion throughout day in freely moving rat. Cort plasma is released in a circadian pattern that consists of ultradian (pulsatile) oscillations. During rodents’ night cycle (awake), cort secretion is higher and as they enter their light cycle (sleep), levels begin to decline. Photo credit\textsuperscript{15}. 

![Image of the HPA axis](image_url)
Under stress conditions, circadian rhythms are overridden, and cort secretion is at its peak\(^1^6,^1^7\). The effects this asserts can depend on things such as whether the stressor is acute, chronic, predictable, or controllable\(^1^8\). The HPA axis has a negative feedback loop, such that cort can inhibit both the further production of ACTH and the secretion of CRF, all with the goal of stabilizing the response\(^1^9\). However, as many people have experienced, when this system is not balanced, it can lead to more serious conditions, anxiety-related disorders, depression, or even memory impairment\(^2^0–^2^2\).

Whether under basal or stress conditions, cort can indirectly affect transcription through two steroid receptors: the mineralocorticoid receptor (MR), and the glucocorticoid receptor (GR)\(^4,^2^3,^2^4\). The MR has a 10-fold higher affinity for binding cort, and thus, is usually fully occupied under basal conditions\(^2^5\). The expression pattern of MRs is more dense in the hippocampus\(^4,^2^3\), an area of the brain highly relevant for stress and memory\(^2^6\). When conditions are more stressful and thus cort is at greater levels, it begins to occupy GRs\(^1^6,^5\). These receptors are ubiquitously expressed throughout the brain, with higher expression density in the limbic regions, particularly the hippocampus\(^4,^2^3\). Both MR and GR can be found in the cytosol and upon binding cort, translocate into the nucleus where they can directly promote or inhibit transcription (Figure 3)\(^4,^2^7\).

![Figure 3](image)

Figure 3. The glucocorticoid receptor can regulate gene transcription. Due to their size and lipophilic nature, glucocorticoids (i.e. cort) can pass through the cell membrane. When they bind to GR, this induces a conformational change that releases GR from a complex with heat-shock proteins (hsp) allowing GR to translocate into the nucleus. Here, GR can affect transcription as a dimer bound to GREs, as well as a monomer, interfering with other factors (e.g. AP-1 and NF-kB). Photo credit\(^2^5\).

GR is comprised of an N-terminus region, followed by a DNA-binding domain (DBD), a hinge region, a ligand-binding domain (LBD), and a C-terminus (Figure 4)\(^2^8–^3^0\). In the cytosol, GR is already bound to other chaperones inhibiting its DBD from being exposed\(^3^1\). When cort binds to the LBD, GR changes its conformational structure, releasing previously bound chaperones, and allowing it to dimerize and pass into the nucleus\(^3^2,^3^3\). Here it can directly affect transcription by binding onto DNA fragments known as glucocorticoid-response-elements (GRE), located upstream of a gene promoter (Figure 3)\(^3^4\). By binding to GREs, GR can promote
or inhibit (negative GREs) transcription of RNA that codes for proteins. Alternatively, GR can bind to other nuclear transcription factors, sequestering their activity, and thus, indirectly promoting or inhibiting transcription. Without the actions of this steroid receptor, the animal could not survive, as we know from genetic mutations that GR gene inactivation is lethal at birth\textsuperscript{27}.

Although the focus of this research is on GR-mediated stress, there are other stress-related molecules that may play a role in the stress response, including dopamine, serotonin, BDNF, VEGF, glutamate, and NMDA\textsuperscript{35,36}. Despite these other mediators of the stress response, we focused on cort because of its robust effect on NPCs. While mature neurons are found to express both MR and GR, newborn neuronal cells only express GR (see Figure 5)\textsuperscript{37}. Thus, it may not be surprising that high levels of circulating cort, such as that caused by environmental stressors, affect the development of these cells.

![Figure 4. Structural organization of glucocorticoid receptor. Schematic 1 dimensional (1D) amino acid sequence of nuclear receptors, such as GR. Sequence C represents the DBD (where receptor interacts with DNA) and sequence E represents the LBD (where hormone binds), both shown below as 3 dimensional (3D) structures.](image)

### 1.2.2 Neurogenesis in the hippocampus

During brain development, neural stem cells, responding to both internal and external cues, multiply (proliferate), migrate, and mature (differentiate) into neurons (a process referred to as neurogenesis) or glia (referred to as gliogenesis) (for review\textsuperscript{38}). Gliogenesis, more specifically, refers to astrocytes (astrogenesis) or oligodendrocytes (oligodendrogenesis). These mature cells are some of the main players that constitute the brain and contribute to its function. This developmental process goes through stages that become increasingly more restrictive in cell-type and self-renewal\textsuperscript{39}. It culminates with the mature cell, neuron or glia, that is fully differentiated and no longer mitotic (Figure 5)\textsuperscript{40}. Referred to broadly as neurogenesis (since most cells become neurons), this process occurs in the pre- or early post-natal stages of all...
vertebrate mammals. Grandfather of neuroscience, Ramón y Cajal, established the long-held dogma that after this initial period, the brain no longer retained this regenerative capacity: “In the adult centres, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated”.

It was not until the 1960s, with more sophisticated tools and scientific methods, that it was discovered that neurogenesis takes place in the adult brain as well. Although the results were not widely accepted, Altman and colleagues (1965) first presented evidence of mammalian adult neurogenesis using autoradiography and light microscopy of general cytological stains. This result was further supported by combining autoradiography and high-resolution electron microscopy to show evidence that adult-born cells with tritiated thymidine uptake, a marker of cell division, exhibited definite neuronal morphology. Still, mammalian adult neurogenesis was not firmly established until the 1990s, when the use of more advanced techniques in cell culturing and immunohistochemistry provided evidence of adult neural progenitor cells that were multipotent in vitro, and in vivo. Now widely accepted, adult neurogenesis has been demonstrated across species, including bird, tree shrew, mouse, rats, monkeys, and humans. Not only did the discovery of this phenomenon overturn a long-held central dogma of neuroscience, but it also enlightened us to a new form of adult plasticity that could contribute to learning processes and be a potential source for treating damage to, disorders, or diseases of the brain.

There are two locations where adult neurogenesis is found to occur: the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). Within the hippocampus, as cells proliferate, some daughter cells become NPCs which further mature into neurons (approximately 70-90%), or other glial cells (~10%). Typically, these maturing cells migrate into the granular cell layer, differentiate into granule neurons, and integrate into the circuitry by extending the appropriate projections to their CA3 target area and acquiring electrophysiological properties that make them indistinguishable from the adjacent, older neurons (Figure 5). Across species, the rate and degree of neurogenesis is varied. For example, rats have ~9000 newborn cells surviving after 1 week and ~70% of these cells are already neurons by 2 weeks; whereas mice have ~3000 newborn cells surviving after 1 week (although they have similar levels of newborn cells per area to rats at this time) and still, less than 50% have developed into mature neurons by the 4 week time point. Nonetheless, in a matter of weeks depending on the species, adult NPCs can become functionally responsive neurons. Although NPCs are restricted to two discrete brain regions and comprise less than 10% of the total DG neuronal population, their long-term, regular self-renewal has remained a conserved mechanism across species. Understandably, this begs the question of what significant role they play in brain function.
1.2.3 Measuring neurogenesis

There are different methods of measuring neurogenesis. Four of these strategies involve labeling newborn cells through the use of exogenous markers, endogenous markers, retro-viral markers, or genetic techniques. Exogenous labeling of proliferative cells involves injecting a solution of either tritiated thymidine, or a thymidine analog called 5’-bromo-2’-deoxyuridane (BrdU), which incorporates itself during DNA replication by replacing the thymidine nucleoside. Thus, when BrdU is present, any replicating cell in S-phase will take up this analog into its DNA structure and by using immunofluorescence, anti-BrdU antibody can identify them throughout their lifecycle. By thoughtfully planning the paradigm and temporal order of events, one can examine proliferation, survival, and differentiation of the cells. For
example, differentiation can be qualitatively and quantitatively measured with anti-BrdU co-labeled with other cell markers if the timing between BrdU administration and tissue fixation provided enough time for the newborn cell to mature. These other cell markers can identify distinct or overlapping stages of development, such as nestin for progenitor cells, doublecortin (DCX) for immature neurons, or NeuN for mature neurons (Figure 5). By co-labeling with antibodies for distinct cell types, one can for track cell fate.

Another method of measuring neurogenesis involves immunofluorescence of endogenous markers of cell division such as Ki67 or proliferating cell nuclear antigen (PCNA). Ki67 is a protein that is naturally expressed during the S-phase of the cell division cycle. PCNA is a protein that also naturally exists during cell division. By immuno-labeling with antibodies against themselves, one can quantitatively and qualitatively measure proliferation. A drawback of this method, however, is that the cell is only identified while replicating, making survival or differentiation measures impossible. Still, it is a less-invasive and simpler way of examining proliferation at the time of death.

Labeling of adult neurogenesis is also possible using particular retroviruses. These retroviruses are a type of virus that only enters the cell when it is dividing. Thus, this method only targets proliferating cells. If the retrovirus is bound to a fluorescent protein, similar to the BrdU method, when the virus is present, any replicating cell will be infected and using immunofluorescence, it can be identified throughout its lifecycle.

The last method mentioned includes genetic techniques such as Cre/lox recombination. Cre is a recombinase protein that can be inserted into the promoter region of any gene such that when the gene is being expressed, Cre will be active concomitantly. When active, Cre locates and excises any DNA contained between (floxed by) head-to-tail loxP fragments. Then Cre recombines the genetic coding (minus the floxed DNA segment) and from that point onward, the cell’s genetic code is permanently altered, including its progeny. Both Cre and loxP are genetic manipulations not occurring naturally in mammals. One way this technique can be used to label progenitor cells could be by using a mouse that has a gene with Cre in the promoter region of a proliferative cell marker (i.e. nestin) and a gene with loxP floxing a STOP codon preceding a yellow fluorescent protein (YFP). The Cre will remove the STOP codon, causing YFP to be visible only in nestin-expressing cells. This method, although more intricate, would label all nestin-expressing cells from birth. A more clever alternative technique is through the use of CreERT2, a form of Cre that is inducible at any selected time point. This type of Cre is controlled by a mutated estrogen receptor (ERT2) that is only active when induced exogenously by an estrogen analog such as tamoxifen. Unlike Cre, which becomes active as soon as nestin (for example) is expressed, CreERT2 becomes active when both tamoxifen is bound to its ERT2 and nestin is expressed. Thus, using this inducible CreERT2 genetic technique, proliferative cells can be labeled and tracked for proliferative, survival, and differentiation analyses.

1.2.4 Stress-induced regulation of neurogenesis

Both the stress response and neurogenesis appear to be well-conserved mechanisms that provide an organism with the ability to cope and perhaps more importantly, adapt to future occurrences. It is not so surprising then that the hippocampus is the brain structure where the stress-sensitive limbic system and one of two neurogenic niches converge. The hippocampus is critically involved in both learning and memory, two plastic processes that necessitate the
continuous need to receive, reorganize, relay, and readjust to new information\textsuperscript{26,92–94}. The regenerating NPCs of the hippocampus are well situated to react to the environment as they are found predominantly in the direct vicinity of the vasculature\textsuperscript{95}. This would allow for stress hormones circulating in the blood to quickly communicate with these regenerating cells. Thus, one of the first discovered and widely studied mediators of neurogenesis was stress\textsuperscript{96}.

Neurogenesis is not only regulated by stress, but also other stimuli that are associated with elevated cort, such as voluntary exercise, learning tasks, and enriched environments\textsuperscript{97–101}. The effects of stress on neurogenesis can be during the proliferation, survival, or differentiation of the cell. The responses can vary depending on the type of stressor (i.e. restraint, predator smell, tail suspension) and the duration of its application (i.e. acute, chronic)\textsuperscript{102}. Each stage of neurogenesis appears to be a plastic progression with the potential to be influenced by stress and cort.

### 1.2.4.1 Stress and glucocorticoids affect cell proliferation

The first stage in neurogenesis is when the cell undergoes mitosis and multiplies itself. This proliferative stage can be sensitive to environmental stressors depending on the duration of the stress. The effect of acute and chronic stress has been studied in a variety of animals, including mouse, rat, tree shrew, and marmoset monkey, and is generally found to be inhibitory.

**Acute**

Many studies have found that acute stress causes decreases in proliferation\textsuperscript{7,103}. Acute stress usually involves one instance or one day of a stressful paradigm. In one study, rats were exposed to odors from a fox, a natural predator, or nonthreatening stimuli (mint or orange). Only acute exposure to fox odor was found to decrease the number of newborn cells\textsuperscript{104}. Additionally, rats underwent adrenalectomies to remove glucocorticoids from their system, and then were given low levels of cort for maintaining normal functioning. When these rats were exposed to the odors now, there was no effect on proliferation, implying that the stress effect suppressing proliferation was driven by raised cort levels\textsuperscript{104}. Other researchers confirmed these results, finding predator odor caused inhibition of proliferation\textsuperscript{105,106}. Decreased proliferation was also seen in acute exposure to a social defeat paradigm\textsuperscript{107}, but not always significantly\textsuperscript{108}. A psychosocial stress, also referred to as resident-intruder, was found to inhibit proliferation of NPCs\textsuperscript{60,109}, as well as an acute duration of unpredictable stressors\textsuperscript{110}, such as forced cold swim and cold immobilization. Similarly, after a day of inescapable shock, proliferation again was shown to be suppressed\textsuperscript{111}. During early life, rat pups exposed to an adult male odor showed suppressed proliferation\textsuperscript{112}. While all these studies refer to suppressed neurogenesis in the SGZ of the hippocampus, neurogenesis in the SVZ has also been examined but found to show no effect of acute stress on cell production\textsuperscript{111,113}. This suggests that the effect of stress on proliferation is specific to the hippocampus and not due to the uptake or labeling of BrdU. The duration of the decreased proliferation may vary if experienced during the early postnatal stage or adulthood. In one study that looked at rat pups, they found that early life stress from maternal separation caused reduced proliferation rates that lasted into adulthood\textsuperscript{111} However, other groups found that acute stress effects on proliferation normalized within 24 hours, but these were performed on adult mice\textsuperscript{107,114}.

Although many studies show acute stress induces inhibition of newborn neurons, there are several studies that do not confirm those results. There was no change found in proliferation
in two studies of acute restraint on rats\textsuperscript{115,116}. Likewise, acute stress from both psychosocial stress or a predator odor were not always found to suppress proliferation\textsuperscript{117,118}, although these studies were criticized for administering BrdU shortly before the stressor, and thus were not a direct measure of proliferation change caused by the stressor\textsuperscript{36}. Recently, Kaufer and colleagues (2013) found that acute immobilization stress increased proliferation\textsuperscript{119}. In this study, researchers were sensitive to pre-handling procedures that prepared both experimental and control rodents for handling the day of the stress, which may have made a significant difference as well. Increased proliferation from acute stress is also found in female rodents\textsuperscript{110,120} and attributed to estradiol effects that are neurogenesis enhancing. In one of these studies, when female rats were exposed to predator odor, there was no change seen in proliferation, but after an ovariectomy and exogenous estradiol was added, there was an increase in cell birth\textsuperscript{120}. Overall, it seems as though acute stress can regulate proliferation, although these effects are sensitive to the type of stressor experienced, the protocol of the paradigm, the age of the animal, the species being studied, and the time course in which proliferation was examined.

**Chronic**

While acute stress effects on proliferation appear to be variable, chronic stress appears to be a strong inhibitor of proliferation. Chronic stress refers to any stressor experienced repeatedly over a course of a few days to several weeks. In a chronic unpredictable stress paradigm, that can include various stressors such as forced cold swim, cold immobilization, isolation, vibration, shaking, overcrowding, wet bedding, restraint, odors, altered light schedules, or strobe lighting, rodents were found to have significant reductions in the birth of new neurons\textsuperscript{110,121–124}. Chronic restraint for 6 hours per day for 2-3 weeks also induced inhibition of cell proliferation\textsuperscript{115,116}. Likewise, reduced proliferation was found after chronic psychosocial stress\textsuperscript{125–127} as well as chronic shock exposure\textsuperscript{128}, and repeated social defeat\textsuperscript{108}. During early life, rat pups that had prolonged maternal deprivation were found to have significantly reduced basal proliferation rates as adults\textsuperscript{113}.

These effects appear to last longer than those from acute stress. Chronic stress experienced during early life exhibits inhibited proliferation into adulthood\textsuperscript{113} while chronic stress experienced as an adult appears to recover from inhibited cell birth after 3 weeks\textsuperscript{114,123}.

Whether acute or chronic, the question remains whether decreases in proliferation are due to a slowing or a pausing of the cell cycle, or if the cells are exiting the cell cycle or dying. This question was investigated and researchers found that when dexamethasone, a GR-specific synthetic glucocorticoid, was applied \textit{in vitro}, it concomitantly reduced proliferation and increased p21 protein\textsuperscript{129}. P21 is an enzyme involved in cell-cycle arrest by inhibiting progression from the G1 to the S phase in the cell cycle\textsuperscript{130}. Similarly, increased p21 expression was found in NPCs\textsuperscript{131} as well as HT-22 cells exposed to dexamethasone\textsuperscript{132}. In one study \textit{in vivo}, while stress was found to downregulate newborn cell births, this correlated with an upregulation in p27Kip1, another enzyme of cell cycle arrest\textsuperscript{130}. Although the pathway in which stress or glucocorticoids enacts its effects on cell proliferation is still unknown, particularly whether its effect is directly or indirectly through NPCs, these results suggest that the stress and GC-induced inhibition of proliferation is mediated by a slowing of the G1-S phase in a cell cycle, and not just due to cell cycle exit.
1.2.4.2 Stress and glucocorticoids affect cell survival

Although thousands of newborn neurons are born each day, approximately 50% of these cells die within 3 weeks\(^{68,78}\). This stage is referred to as survival, and this pruning process is also sensitive to environmental input\(^{133,134}\).

**Acute**

Results from measurements of cell apoptosis after an acute stressor are conflicting. Cell survival was measured at different time points post-BrdU uptake from rats exposed to an acute psychosocial stressor\(^ {118}\). These time points assessed the immediate, short-term, and long-term survival of newborn neurons. The study found no change in survival rates immediately after BrdU administration, but did find a decrease in both the short-term and long-term survival rates\(^ {118}\). Similarly, rats exposed to acute unpredictable stress had increased apoptosis in the hilus, SGZ, and granule cell layer (GCL) of the hippocampus\(^ {110}\). The opposite effect, however, was seen in rats exposed to acute predator odor\(^ {120}\). In this study, the acute stressor suppressed cell death in the DG, and thus survival rates were higher than controls\(^ {120}\). In addition to decreased and increased survival rates from acute stressors, some studies find no change at all\(^ {107,121}\), although these were performed with mice, suggesting that stress effects are more deleterious on particular species. The variability in the intensity, duration, and protocol of the stressor, along with differences in BrdU time course and measurement methods can perhaps account for the conflicting results found from stress on cell death.

Although most experiments are done with male rodents to control for the neurogenic enhancing effects of estrogen in females\(^ {135}\), one study also tested cell survival rates in females exposed to the same predator odor as males were and found no change\(^ {120}\). This emphasizes sex differences in stress-induced effects on neurogenesis, particularly since ovariectomized female rodents had vast cell death in the hippocampus that was ameliorated by exogenous estradiol hormone\(^ {120}\). This could imply that any suppressive effect on survival induced by stress may be counteracted by the enhancing effect of estrogen.

**Chronic**

The effects of chronic stress on cell survival appear to be clearer. Cell death was increased in the DG of adult rats after experiencing chronic stress paradigms such as psychosocial stress\(^ {125}\), restraint stress\(^ {115}\), and unpredictable variable stress\(^ {110,136,137}\). One study that measured cell death determined that the effects were restricted to the GCL, and not seen in the hilus\(^ {136}\). Interestingly, when a GR antagonist, mifeprisone, was administered for 4 days after the chronic unpredictable variable stress paradigm, cell survival increased\(^ {137}\). This suggests that the suppressive effects on cell survival induced by chronic stress are mediated through the GR. These effects may be similar as well if stress is experienced in early life. Gould and colleagues examined rats that experienced prolonged maternal deprivation as pups and found that these pups also had lower survival rates for newborn neurons in adulthood\(^ {113}\). This effect persisted for 1 week, however, dissipating after 3 weeks\(^ {113}\). Studies on cell survival after chronic stress all seem to concur that stress, perhaps mediated through the GR, increase apoptosis of newborn NPCs.
1.2.4.3 Stress and glucocorticoids affect cell differentiation

In the rodent hippocampus, after NPCs proliferate and survive the pruning process, they begin to take on morphological, molecular, and functional characteristics of more mature cell types\textsuperscript{44}. This process is referred to as differentiation and typically, 70-90\% of these surviving cells become granule neurons\textsuperscript{68,69}. Although most of the research on stress effects on neurogenesis point to the proliferation stage as the mediator of neurogenic changes, differentiation appears to also be sensitive to environmental input.

**Acute**

Unlike the proliferation stage, there are not many studies showing an effect of acute stress on cell fate. Adult rats subjected to acute restraint were found to have no significant differences in cell differentiation\textsuperscript{115}. Similarly, rats exposed to an acute psychosocial stressor had no changes in percentage of BrdU-positive cells that co-labeled with mature neuronal markers\textsuperscript{118}. Since there was an overall significant reduction in the number of neurons, but not in the percentage of newborn cells that differentiated into neurons, this would indicate that changes in neurogenesis from acute stressors probably derive from the initial changes seen on proliferation.

**Chronic**

The effects of chronic stress on cell differentiation appear to vary from decreased neuronal fate or no change in cell fate. Both adult rats and 3-week-old mouse pups subjected to chronic restraint showed reduced neuronal differentiation\textsuperscript{115,138}. Mice that experienced chronic social isolation displayed suppressed neuronal differentiation as well in both the GCL and SGZ, but not the hilus\textsuperscript{139}. Likewise, exposure to a chronic shock paradigm reduced neural cell fate in rats\textsuperscript{128}, although not always\textsuperscript{140}. Chronic exposure to a psychosocial stressor showed suppressed neuronal differentiation in tree shrews\textsuperscript{127}, but no effect in rats\textsuperscript{125}. The effects on neuronal differentiation from chronic unpredictable stressors or chronic mild stressors, which can include cage tilting, wet bedding, predator sounds, empty cages with water on the bottom, reversal of the light/dark cycle, sporadic light changes, restraint, forced cold swim, water deprivation, pairing with a stressed littermate, or cage switching, were found to be nonsignificant in rats\textsuperscript{114,136}, but significantly reduced in mice\textsuperscript{141,142}. One study that looked at 7 weeks of chronic mild stress on mice, however, did not see this reduction as both stressed and control groups had 73\% of BrdU positive cells co-labeling with NeuN\textsuperscript{143}. One study examined a similar paradigm on three strains of mice and found a significant suppression of neuronal fate in both the males and females of all strains\textsuperscript{142}. None of these studies found any shift in astrocytic cell fate; however, a recent study found that chronic restraint stress not only decreased neuronal differentiation in rats, but increased oligodendrogenesis\textsuperscript{144}. In this study, not only stress induced this shift in cell fate from neurons to oligodendrocytes, but also lineage tracing of NPCs in vivo showed that after administering cort, oligodendrocytic fate was increased, implying that this stress effect is a cort-mediated mechanism\textsuperscript{144}. Overall, it appears that while acute stress does not change the fate of newborn cells, chronic stress can often reduce or alter the differentiation of newborn cells through elevated cort.
1.2.4.4 Other environmental factors regulating neurogenesis

As evident from stress effects, all stages of neurogenesis appear sensitive to environmental factors, although the degree and direction of the effect is sensitive to the duration and the context of the stressor. Interestingly, while many of the studies suggest the underlying mechanism involved is cort-mediated, it is clearly more complicated than an indirect relationship between cort levels and neurogenesis production. Like stress, cort levels are also increased after other environmental stimuli such as learning, environmental enrichment, and voluntary exercise\(^{97-101}\). However, unlike stress, these cort-associated stimuli have a more enhancing effect on neurogenesis\(^{97-99,101,145-148}\). Thus, it is suggested that the psychological outlook or context, such as predictability or controllability, of an environmental stimuli impacts cell development.

**Learning**

Training on hippocampal-dependent learning tasks can alter the number of newborn neurons in the DG, however, the effect can be enhancing or suppressing of cell survival depending on the protocol\(^{149}\). In studies on rats, the learning task known as eyelink trace conditioning, a classical conditioning of the eyelink response using a trace protocol, was found to increase proliferation and survival specifically in the DG and not in the SVZ\(^{99,150}\). There were no changes in differentiation, but interestingly, although cort levels were greater in rats being trained versus untrained, this was not the case among the rats learning the paired associations versus unpaired associations\(^{99}\). This may imply that cort is not mediating the learning effect on neurogenesis. During spatial navigation learning in the Morris water maze, rats showed enhanced survival as well\(^{134,150,151}\), although this increase was only evident in the rostral part of the external blade of the GCL\(^{134}\). This enhancement, however, was only apparent for the short-term survival of newborn neurons, since survival rates actually decreased after a week\(^{151,152}\). It is thought that the age of the newborn neurons at the time of training plays a role in whether they survive or die\(^{152}\). The duration of learning appears to impact cell survival since more trials in the spatial navigation task, as well as more days of training in a social transmission of food preference task both decrease survival of NPCs\(^{149,152,153}\). Whereas one day of training in social transmission of food preference showed increased cell survival, two days of training resulted in reduced survival of newborn neurons\(^{153}\). In this study, cort levels were also found to be unchanged between the learners and the controls\(^{153}\). Whether it is the age of the newborn cells that matters, or learning induces an initial increase in survival and then greater cell death ensues with time, more research is needed to elucidate in what ways learning regulates the survival of newborn NPCs\(^{149}\).

**Exercise**

Voluntary exercise seems to be a strong inducer of neurogenesis. Although running can be seen as stressful and both stress and exercise increase cort levels\(^{154}\), it is important to distinguish involuntary exercise (i.e. forced swim), which has suppressive effects on neurogenesis, from voluntary exercise (i.e. wheel running), which has enhancing effects on neurogenesis\(^{147}\). Rodents given access to a running wheel, running approximately 48km per day\(^{146}\) were compared to rodents with an immobilized running wheel in their cage to control for environment. In each study of exercise-induced changes on neurogenesis, an increase in the number of newborn neurons was significantly greater in the rodents allowed to exercise\(^{61,97,98,145,147}\). This number was often greater than double compared to controls\(^{145}\). This
increase in proliferation went down over time, however, was still greater than age-matched controls, suggesting exercise contributes to successful aging\textsuperscript{98}. Additionally, the survival of these NPCs was promoted when examined 4 weeks after BrdU injections\textsuperscript{61,146}. Voluntary exercise also increased cell fate toward a neuronal type in most studies\textsuperscript{61,97,145}, but not all\textsuperscript{146}. This increased neurogenesis was specific to the hippocampus and not found in the SVZ or olfactory bulb\textsuperscript{97} and appears to affect both sexes\textsuperscript{61,97,98,145,146}. Thus, it appears that exercise is an environmental stimulus that can promote each developmental stage of neurogenesis.

**Environmental enrichment**

Neurogenesis can also be modulated by an enriched environment (EE). An EE entails more nesting material, tunnels, toys, a running wheel, and extra food treats, such as cheese, crackers, apples, and popcorn. When exposed to this environment, while most studies find no changes in proliferation of hippocampal NPCs\textsuperscript{97,101,147,148,155}, there was an enhanced survival effect for these newborn neurons for both mice\textsuperscript{101,147} and rats\textsuperscript{148,156}. Interestingly, there does not appear to be sex differences, as many of these studies showed similar results for both male and female rodents. Although some studies found increased neuronal cell fate due to this stimulating environment\textsuperscript{97,147,155}, it was not seen in others\textsuperscript{101,148}. This effect was specific to the SGZ of the DG as it was not found in the SVZ or migratory NPCs in the olfactory bulb\textsuperscript{97}. Cort levels were found to be increased in both mice and rats exposed to EEs\textsuperscript{157,158}, suggesting the effect could be cort-mediated. It is suggested that this enhancement of neurogenesis is either due to the novelty of the environment, since mice continuously living in an EE compared to mice removed from an EE have reduced survival rates\textsuperscript{159}, or it is simply from increased motor activity (and not learning), since all cages have running wheels and exercise alone showed similar results\textsuperscript{147}.

### 1.3 The role of glucocorticoids in hippocampal neuroplasticity

Environmental stimuli, such as stress, learning, exercise, and enriched environments, are all associated with elevations in cort levels, yet can have opposing effects on neurogenesis. Therefore the mechanisms driving these changes appear to be more complex. It is important thus to distinguish potential mediators, such as cort, and assess how these factors affect neurogenesis in isolation.

#### 1.3.1. Glucocorticoids modulate neurogenesis

**Glucocorticoids directly modulate neurogenesis**

Although stress is found to reduce neurogenesis in the DG, the underlying mechanisms are far from understood. Substantial evidence suggests that cort plays an important role\textsuperscript{103}. Whereas the hippocampus is both richly endowed with adrenal steroid receptors and shows structural and functional changes due to cort manipulations\textsuperscript{160}, it is now clear that cort alone mediates neurogenesis. Experiments that removed all endogenous cort by adrenalectomy (ADX) found that proliferation of newborn neurons was increased in both young\textsuperscript{53,161,162} and aged rats\textsuperscript{163}. Although ADX generally induced more apoptosis throughout the hippocampus, NPCs had enhanced survival without circulating cort after 4 weeks\textsuperscript{53,164}. Additionally, a lack of cort also suppressed neuronal differentiation\textsuperscript{53,161,163,165}. Another study blocked HPA-axis activity by blocking receptors for corticotrophin-releasing factor (CRF) and vasopressin (V1b), two upstream regulators of cort secretion\textsuperscript{143}. Researchers found that the decrease in the birth and
differentiation of newborn cells induced by chronic stress was reversed by this method of inhibiting cort secretion\textsuperscript{143}. Adding a low dose of cort post-ADX helps maintain diurnal rhythms, while controlling for any stress-induced surge in cort levels. This would just allow for MR occupation, but suppressed GR activity since there is no rise in the cort levels. In this circumstance, the increase in proliferation from a stressor was prevented\textsuperscript{53,104} although one study reported no change with differentiation\textsuperscript{161}. Whereas removing or reducing cort availability promoted neurogenesis, increasing cort availability inhibited neurogenesis\textsuperscript{131,161,162,164,165}. Both acute and chronic treatment with cort reduced proliferation in vivo\textsuperscript{119,131,161,162,164,165} and in vitro\textsuperscript{119,131}, and was found reduced specifically in the dorsal region and not ventral region of the hippocampus\textsuperscript{119}. This reduction of newborn NPCs was \textasciitilde 30\% compared to NPC production in controls\textsuperscript{162}, but only seemed to occur from cort exposure within the first 18 days of cell birth\textsuperscript{164}. Survival rates of NPCs were also decreased by exogenous increases in cort\textsuperscript{164}, as well as differentiation to a neural cell fate\textsuperscript{131,165}. Thus, it appears clear that cort alone can regulate all stages of neurogenesis.

**GR modulates neurogenesis**

Studies suggest that the mechanism by which cort mediates neurogenesis is through the GC receptor. As described earlier, cort binds to two receptors, MR and GR, at different affinities. Whereas MR is fully occupied at basal levels of cort secretion during the diurnal rhythm, GR activity is induced when cort levels are raised, such as that seen during stressful encounters\textsuperscript{5,25,166}. Additionally, GR is expressed in NPCs and throughout their maturation, whereas MR is only expressed in the mature stage\textsuperscript{37}. Thus, GR appears to be a direct mediator of cort-induced suppression of neurogenesis. When adrenalectomized rats given low doses of cort to maintain basal levels were treated with an MR antagonist, proliferation was enhanced (such as that seen in adrenalectomized-only rats)\textsuperscript{167}. When the same rats were treated with a GR antagonist, there were no effects, most likely because GR is not occupied at this level of cort, but when rats were given additional cort, blocking GR reversed the cort-induced suppression of neurogenesis\textsuperscript{167}. Similarly, other studies found that pharmacological blockade of GR with antagonist, mifeprisone, blocked the effects on proliferation, survival, and differentiation from both cort-induced\textsuperscript{168} and chronic stress-induced suppression\textsuperscript{137,169}. Not only does blocking GR stop suppression of neurogenesis from cort or stress, but also stimulating GR further drives suppression of neurogenesis\textsuperscript{129,131,167,170,171}. Several reports have confirmed that treating NPCs in vitro with a GR-specific agonist, namely dexamethasone (Dex), causes reduced proliferation\textsuperscript{129,131,167,170}. Suppression of neuronal differentiation is also seen from Dex-treated NPCs\textsuperscript{171}, but not always\textsuperscript{129}. Overall, it appears that the GR is a potential mediator for the effects of cort on NPCs.

**Other cort-driven mediators of neurogenesis**

Cort-induced suppression of neurogenesis may directly act through GR, but there are also several other mediators through which cort may indirectly cause this effect. These could be growth and hormone factors, cell cycle inhibitors, or excitatory amino acids like those involved in glutamate signaling.

Although the mechanism is unclear, cort can inhibit expression of growth factors as well as their receptors\textsuperscript{172–174}. One of the growth factors suppressed by chronic cort treatment is BDNF\textsuperscript{175–177}. Interestingly, while cort levels are increased during both stress and exercise, it has opposing effects for these stimuli; BDNF, however, while suppressed during stress, is actually
increased during exercise\textsuperscript{177}. Furthermore, changes in BDNF are also correlated with changes in neurogenesis\textsuperscript{178–180}. Similarly, insulin growth factor (IGF) was found to promote neurogenesis\textsuperscript{181}. This growth factor can also be regulated by glucocorticoids\textsuperscript{172,182,183}. Other growth and hormone factors that have been shown to be responsive to cort include fibroblast growth factor 2 (FGF2)\textsuperscript{119,184,185}, epidermal growth factor\textsuperscript{186,187}, and CRF\textsuperscript{143}. Additionally, all of these growth and hormone factors have also been shown to alter the ability of NPCs to proliferate and differentiate (see review\textsuperscript{69}). Thus, stress and cort-specific effects on hippocampal plasticity may indeed be indirectly mediated by these factors.

The effect of cort on neurogenesis can be derived from its slowing or pausing of the cell cycle during NPC proliferation\textsuperscript{102}. Although cort treatment is associated with changes in cell cycle inhibitors, such as cyclin D1, cyclin D2, CdK4, CdK6, p21Cip1, and p27Kip1\textsuperscript{69,188,189}, it is not clear the pathway in which it is able to mediate them. Despite this, cell cycle inhibitors, while controlling proliferation and development, can thus control neurogenesis as well.

Levels of cort availability also regulate glutamate signaling\textsuperscript{35,190,791}. Whereas cort-free adrenalectomized rodents have decreased glutamate in the hippocampus\textsuperscript{192}, high levels of cort can induce excess glutamate secretion\textsuperscript{193}. This excitatory amino acid has been a long-recognized modulator of neurogenesis as well since both stress- and cort-induced suppression of neurogenesis can be saved by blocking glutamate receptors, namely NMDA receptors\textsuperscript{162,194–196}. It appears thus that cort may exert its influence on neurogenesis by indirectly acting through glutamate and the NMDA receptor.

### 1.3.2 Glucocorticoids modulate anxiety and cognitive performance

Although there is a wealth of literature describing how various stress-induced hormonal, morphological, and synaptic changes can affect behavior and cognition, for purposes of this review, I will focus on corticosteroids (for review\textsuperscript{5,197}). Research from both animal and human studies have demonstrated acute and chronic cort influences on anxiety as well as cognitive performance, particularly on memory tasks\textsuperscript{8,198–203}. Furthermore, whereas my research focused on the hippocampal structure, I will describe these cort-associated changes in hippocampal-dependent functions.

**Memory**

One hippocampal-dependent process that is most impacted by cort is memory. Not only are there many different forms of memory, but also each form can be further broken down into stages\textsuperscript{204}. Two forms of memory most affected by adrenal hormones are declarative memory, which describes the storage of facts and events, and associative memory, which describes the forming of relationships between facts and events\textsuperscript{204}. All of these measures of memory performance, it should be noted, are sensitive to many factors such as species and strain, time of day tested, task protocol, what is measured in the task, concentration of cort, administration route of cort, and duration of cort treatment. Declarative memory can be subdivided into different phases of acquisition, consolidation, and retrieval\textsuperscript{205}. The cort-induced impact on declarative memory is most often measured using spatial maze tasks, and the cort-induced impact on associate memory can be measured by contextual conditioning, trace conditioning, and pattern separation.

Although the literature on declarative memory can appear confusing, it has become apparent that the strongest effect of cort is on the consolidation and retrieval phases of
memory. More specifically, increased cort availability, particularly during or after emotionally arousing experiences, leads to enhanced consolidation of memories. In contrast, it also suppresses retrieval of this information if elevated shortly before retention testing. When endogenous cort is removed by ADX, studies have found that the consolidation of memory for emotionally arousing events was impaired. This effect was attenuated if GR was activated with agonist Dex after training. Furthermore, acute cort treatment given post-training improved the consolidation of emotionally arousing memories. Chronically high doses of cort, however, can have less of an effect or even impair consolidation in a dose-dependent inverted-U fashion, although due to the chronic nature of the dosing, it is difficult to tease apart which stage of memory it is acting upon. Overall, these effects are believed to be GR-dependent since blocking GR with intra-cerebroventricular infusions of GR antagonists before or immediately after training impaired spatial memory in a water maze and mice with partial GR gene inactivation also show poor memory consolidation. These results clearly show cort, through GR activation, is able to manipulate the consolidation stage of memory.

While consolidation of spatial memories is mostly improved with cort, retrieval of the memory is impaired. Memory retention was tested in rats 24 hours after being trained in a spatial navigation task called Morris Water Maze. Rats that received cort treatment shortly before testing displayed impaired retrieval. Humans also show this effect when given stress doses of cortisone one hour before a retrieval test of recently learned verbal material. Similarly, high levels of circulating cort as well as infusions of GR agonist also worsen memory recall of previously learned information. These studies demonstrate that cort impairs memory retrieval, which depends, at least in part, on GR activation.

Another aspect of hippocampal-dependent memory that is affected by cort is associative memory. When examined through trace conditioning, a hippocampal-dependent eyeblink association task, it was shown that acute administration of cort enhanced trace conditioning 30 minutes post-injection, but not 24 hours later. Furthermore, adrenalectomized rats did not demonstrate a stress-induced enhancement, even when low dose cort was added to maintain basal levels. This implies that not only do high levels of cort help improve associative memory, but that it may act through the GR. Indeed, GR-knockdown mice showed impaired memory association in a contextual fear-conditioning task. Compared with controls, mice with reduced GR levels in newborn DG cells had reduced context-induced freezing, a measurement of associative memory ability. This can also be compared to rats under chronic cort treatment, which had enhanced freezing to the context. Overall, these studies show that both acutely and chronically increased cort levels help consolidate memories in an associative memory task and that GR plays a role in this enhancement.

Just as important it is to be able to associate events and facts together, it is also important to be able to distinguish between similar events occurring close in time or that contain similar features. This control of associative memory is termed pattern separation. Aging, which is associated with increased cort levels, adversely affects pattern separation in both rodents and humans. Although still unknown, given evidence of cort enhancing the duration and association of memories, but aging-induced increased cort levels impairing pattern separation, it will be interesting to discover whether cort plays a role in this ability to separate distinct memory patterns.
Anxiety

The association of a fear (real or imagined) with a neutral stimulus can lead to a maladaptive emotional state referred to as anxiety. High levels of cort, like those induced by repeated chronic stress, can play a role in this fearful behavior. These behaviors, induced by maladaptive associations, are often measured and quantified using the following tasks: open field, light/dark box, elevated Omaze or plus maze, and novelty-suppressed feeding. Each of these tasks compares rodents’ natural inclination to avoid open spaces, bright lights, and elevated platforms with their curiosity to explore novel spaces. So while a secure, calm rodent will spend more time exploring in the center of a novel arena, an anxious rodent will hug the borders of the arena and avoid lit or exposed spaces. In studies of acute cort treatment, researchers found increased exploration of open arms in the elevated Omaze, but only increased risk assessment behavior, and not conventional anxiety measures, in the elevated plus maze and open field tests. Even acute inhibition of cort by metyrapone, a cort synthesis blocker, only affected the risk assessment behaviors associated with a passive coping response to anxiety. When rodents were under chronic cort treatment, they had increased latency to feed in novelty-suppressed feeding, and they spent less time in the open arms of the elevated plus maze, the center of the open field, and the lit compartment of the light/dark box (for review). Depending on the dose, duration, and task, it appears cort can regulate various aspects of anxiety-relevant behaviors.

These effects on anxiety may also be attributed to GR activity. Tronche and colleagues developed mice lacking GR in brain cells and found inhibited anxiety in the light dark box and elevated Omaze. In the light dark box task, mice showed decreased latency to exit into the lit compartment, and spent more time exploring in this compartment. In the elevated Omaze, the number of entries and time spent in the open arms was increased in the GR mutants. Similarly, a separate study using mice with genetically reduced GR function also found the number of entries and time spent in the open arms of the elevated Omaze were increased, demonstrating reduced anxiety-like behavior once again. These studies indicate a role for GR in regulating anxiety behaviors.

There is a vast amount of literature suggesting that cort may play a role in depression (for review). This complex pathology is often associated with increased immobility (and decreased mobility) in the Porsolt forced swim test, a behavioral assay used for screening antidepressant compounds. Rodents are presented to an inescapable swim task and assessed for depression-like behaviors such as increased time spent immobile, decreased time spent actively climbing, and decreased latency to become immobile. Whereas depression and antidepressants are both associated with opposing levels of cort secretion, results from studies of cort-induced depression-like states are unclear. Rats with no endogenous cort due to ADX spend less time immobile, and acute administration of GR-agonist Dex increases immobility time to that of control levels. This GR-induced increase was not seen in rats given acute cort injections, suggesting acute cort treatment and acute GR activity does not cause greater immobility compared to controls. Yet another study found that cort acutely administered to mice through their drinking water 24 hours prior to forced swim actually reduced immobility, implying cort may even have an antidepressant-like effect. When cort levels are kept chronically elevated, mice showed no differences in the forced swim task, however rats show reduced mobility and increased immobility in a dose-dependent manner. Both brain-specific GR mutant mice and GR antisense mice showed reduced depression-like behavior. In contrast, partial GR mutant mice exhibited increased helplessness, a feature of depression, after
stress exposure, and overexpression of GR evoked reduced helplessness after stress exposure\textsuperscript{249}. While cort, through GR activation, may be involved in depression-like behaviors, assessment of this psychopathology is clearly sensitive to species, cort concentration, treatment duration, method of administration, and protocol parameters.

1.4 The role of neurogenesis in hippocampal function

Evidence has been accumulating that describes a role for adult neurogenesis in hippocampal functioning, yet the exact function is unclear. The contribution of neurogenesis to hippocampal behavior is complicated whereby neurogenic niches along the dorsal-ventral hippocampal axis have differential connectivity, and new neurons at different stages in their maturation will display distinct properties\textsuperscript{45,250,251}. Regardless, collective research suggests adult neurogenesis is involved in cognitive processes such as learning, memory, and emotional regulation\textsuperscript{44,45,252}.  

1.4.1. Neurogenesis modulates cognitive performance

Albeit controversial, roles for adult neurogenesis in learning and memory have been demonstrated in studies examining trace eyeblink conditioning, trace fear conditioning, contextual fear conditioning, spatial memory, place and object recognition memory, and pattern separation (for review\textsuperscript{44,252}). Several methods have been employed to ablate or decrease neurogenesis by low-dose irradiation, systemic antimitotic drug treatment, and genetically engineered mice that target neural progenitor cells\textsuperscript{44}. Rats treated with an antimitotic drug to eliminate proliferating cells showed impaired conditioning in the trace eyeblink and trace fear conditioning, but not contextual fear conditioning\textsuperscript{253}. Likewise, reduced neurogenesis in conditional tlx\textsuperscript{-}knockout mice had no effect on contextual fear conditioning\textsuperscript{254}, however other studies have found impaired learning on contextual fear conditioning when examined in irradiated rodents and conditionally-induced glial fibrillary acidic protein (GFAP) knockout mice\textsuperscript{255,256}. Effects on spatial memory are also elusive\textsuperscript{149}. Irradiation on both newly weaned mice and inducible tlx-knockout adult mice caused poor spatial memory in the Morris water maze, but not in the Barnes maze\textsuperscript{254,257}. Conversely, irradiated adult mice had impaired spatial memory in the Barnes maze and not the Morris water maze\textsuperscript{258}. Moreover, many other studies did not find differences in spatial learning at all when neurogenesis was reduced or ablated\textsuperscript{253,255,259–261}. Deficits in place and object recognition memory were found after irradiation\textsuperscript{256,259}, however these effects may be sensitive to the time interval between learning and testing as well as contextual setting since shorter intervals in a simpler context did not produce object recognition impairment in rats that were irradiated nor treated with antimitotic drugs\textsuperscript{259,262}. As for pattern separation abilities, increased neurogenesis improved contextual fear discrimination\textsuperscript{263} and correlated with better spatial pattern separation\textsuperscript{264}. Correspondingly, ablated and reduced neurogenesis impaired spatial pattern separation\textsuperscript{265–267}. Pattern separation, however, is broadly defined and the role of neurogenesis may be better discerned if this term is conceptualized in terms of memory resolution\textsuperscript{268}. Overall, more research is necessary to specifically identify cognitive processes regulated by neurogenesis since current research results vary most likely due to differences in the species, experimental designs, and the assumptions and techniques used to target the contributions of newborn neurons\textsuperscript{252}.  

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1.4.2 NG modulates anxiety and depression

Aside from changes in cognitive abilities, neurogenesis may contribute to emotional behaviors as well\textsuperscript{269}. It has been suggested that neurogenesis is involved in depression since blocking the production of newborn neurons also blocked the behavioral response to antidepressants\textsuperscript{10,11,13,270}. Additionally, some studies have supported the hypothesis that neurogenesis has potential relevance to anxiety disorders\textsuperscript{271}. The literature remains controversial though as to whether neurogenesis directly contributes to anxiety or the etiology of depression\textsuperscript{269,270}. It is important to note that anxiety and affective disorders (i.e. depression) are diagnostically different and current methods to examine these states look at symptoms and not etiology\textsuperscript{91}.

The effects of reduced or ablated neurogenesis on anxiety- and depression-like behaviors appear to depend on whether the animals are naïve or stressed prior to examination\textsuperscript{91}. Most of the current research agrees that ablating neurogenesis in naïve or nonstressed rodents does not impact depression-like behaviors\textsuperscript{91}. When examining whether a lack of neurogenesis exhibits an anxious phenotype, Abrous and colleagues found that naïve transgenic mice with pro-apoptotic protein over-expressed in newborn neuronal cells showed reduced time spent and number of entries in the open arms of the elevated plus maze, as well as increased latency to emerge and time spent in the cylinder of the light/dark emergence test\textsuperscript{272}. These anxiety-related behaviors appear to be due to an increase in the fear of potential threats and not just a decrease in novelty exploration since depleted neurogenesis increased predator avoidance but not novelty exploration\textsuperscript{272}. Interestingly, these neurogenesis-depleted mice did not exhibit depressive-like behaviors in the forced swim test. Another study found stressed GFAP knockout mice, which had inhibited neurogenesis, showed increased latency to feed in the novelty-suppressed feeding task; however, these mice did exhibit depressive-like behaviors in the forced swim task and sucrose preference, a test of anhedonia\textsuperscript{273}. In contrast, many studies altogether did not find that ablated neurogenesis created an anxious or depressive phenotype\textsuperscript{10–14}. Overall, a meta-analysis of all the current research found that most studies do not show that reduced neurogenesis yields depression-like behavior when the animal is naïve\textsuperscript{91,274,275}, although it may yield affective behavior if the animal is stressed\textsuperscript{91}. Interestingly, upregulated neurogenesis using a genetic approach had no effect on novelty-suppressed feeding, forced swim, nor open field exploration in both low and high stress conditions\textsuperscript{12}. It is still also unclear whether intact neurogenesis contributes to antidepressant efficacy. Whereas the underlying mechanisms that cause depression most likely rely on various aspects of brain functioning, like changes in neurotrophic factors or synaptic plasticity, as well as other brain regions, such as the frontal lobes or amygdala, it would be surprising if adult hippocampal neurogenesis influenced them all\textsuperscript{91}. Thus, although there may be a link between adult hippocampal neurogenesis and mood, it is not clear how this association is mechanistically connected\textsuperscript{276}.

1.5 Functional implications of stress-induced changes to neurogenesis

Whether stress hormones impact adult neurogenesis in a functionally significant way is largely unanswered. As discussed, cort is elevated during experiences such as stress, learning, and exercise, all of which can affect memory and emotional states. These hormones can directly alter transcription through the MR and GR, only the latter of which is expressed in newborn cells of the hippocampus. The significance of this expression is unknown, but the activity of GR in
these cells can potentially regulate the proliferation, survival, and differentiation stages of neuronal development. Like stress, these changes in neurogenesis are associated with changes in hippocampal-dependent memory processes as well as anxiety and depression, although more research is needed to determine the nature of its effect. Interestingly, these functional changes seem to arise when the animal is under duress and not in a naïve state. Both stress and neurogenesis are well-conserved mechanisms designed to help the organism learn and adjust to future environmental stimuli. Thus, in expanding our understanding of the functional role of neurogenesis, it would be important to know if the changes incurred during neurogenesis due to stress reactivity lead to changes in behavior or cognition. Equally important, whether cort-activation of GRs in newborn neurons modifies anxiety, depression, or hippocampal-dependent memory processes remains a lingering question and a compelling new avenue of investigation.
2.1 Abstract

Although it is well known that glucocorticoids (GCs), corticosterone in rodents or cortisol in humans (hereafter referred to as cort), can potently regulate neural stem/progenitor cells (NPCs), the underlying molecular mechanisms that mediate this effect remain unknown. When elevated, as occurring during stress, cort binds to the GC receptor (GR), which can directly bind to and affect cellular DNA. We sought to determine whether chronic treatment of cort suppresses adult neurogenesis by directly acting upon GRs in NPCs or indirectly through other mediators. To investigate this, we generated a novel mouse model lacking GR gene function only in NPCs. These mice then received chronic treatment of cort through their drinking water and were examined for changes in NPC survival and neuronal differentiation. Although all groups examined had comparable numbers of NPCs surviving after 4 weeks, chronic cort treatment ultimately suppressed neurogenesis by reducing neuronal differentiation. This effect was not blocked by GR gene inactivation in NPCs (GR\textsuperscript{NPC\textsubscript{KO}}). These results demonstrate that chronic cort treatment can regulate the differentiation of NPCs into mature neurons, and that this effect may be mediated indirectly through other signaling pathways of cort.
2.2 Introduction

In the adult brain, a small population of NPCs continually multiplies and matures into functioning neurons that integrate into the cytoarchitecture and contribute to the functioning of the hippocampus. These nestin-expressing stem cells mostly become neurons by progressing through stages of proliferation, survival, and differentiation, collectively known as neurogenesis. The hippocampus is a structure critical for memory and learning; correspondingly, neurogenesis appears to play an important role in the consolidation, retrieval, and association of memories.

One of the most studied and pervasive regulators of this phenomenon is stress, or more specifically, stress hormones known as glucocorticoids. During the stress response, glucocorticoids known as corticosteroids (cort) are elevated and can inhibit neurogenesis. Interestingly, during learning and exercise, elevated cort is also associated with promoting neurogenesis. Cort is both an ecologically and medicinally important hormone that can be both damaging and beneficial to brain function. Despite this, the underlying mechanisms by which cort can manipulate neurogenesis in the hippocampus remain unclear.

One of the ways in which cort can influence neurogenesis is by altering genetic expression through the mineralocorticoid- (MR) and glucocorticoid receptors (GR). When MR or GR is bound to cort, it can pass into the cell nucleus and directly affect DNA transcription. The expression of these nuclear receptors is highly enriched in the hippocampus, and play a role in emotional behavior, learning, memory, and mood disorders. Interestingly, only GR is expressed in the proliferative newborn cells, and both activation and blockage of their GR in vitro has been demonstrated to either suppress or enhance neurogenesis, respectively. Thus, evidence suggests that the GR plays an important role in regulating adult neurogenesis and is a potential mechanism for cort-induced suppression.

Other studies have attempted to elucidate the role of GR in hippocampal neuroplasticity, but have not yet isolated their role in the proliferative cells. Although GR-specific agonists and antagonists have demonstrated an effect on neurogenesis in vivo, this could be indirectly mediated by the GRs in mature neurons or astroglia. Additionally, a recent study knocked down GR with lentiviral-delivered short hairpin RNA into the hippocampal dentate gyrus (DG) to examine the role of GR in neurogenesis. They found GR knockdown increased neuronal differentiation, however this effect was examined 1 week after cell division, whereas mature neuronal differentiation usually takes at least 4 weeks in the mouse brain. If this measure is accurate, it is not clear if GR knockdown increased the speed of differentiation or the percentage of cells differentiating. Also, this effect was examined under basal cort levels, which are usually too low to even occupy GR. Furthermore, although the lentiviral-mediated knockdown targeted the neurogenic niche, it can infect any neighboring cells, including differentiated neurons and astroglia. While it appears GR can regulate neurogenesis, it remains unclear whether cort-induced suppression of neurogenesis, as experienced during stress, is directly mediated through the GR in NPCs.

To investigate this hypothesis, we generated a mouse with adult NPCs that lacked GRs. By pairing mice that express tamoxifen-inducible Cre recombinase under the control of the nestin promoter and mice that contained Cre sensitive GR alleles (GRloxP/loxP), we created a mouse model that can be induced to lose GR gene function specifically in nestin-expressing cells and their progeny. Nestin is an intermediate filament protein used to identify immature type 1 and type 2a NPCs, which are the only NPC types found to express GR. The synthetic
CreERT2 recombinase was generated by fusing the Cre open reading frame with a mutated estrogen-receptor ligand-binding domain that binds tamoxifen, but not estrogens. Adult mice were induced with tamoxifen, activating Cre activity leading to GR gene ablation. Using this novel mouse model, we examined how chronic treatment of cort affects neurogenesis and whether this effect is mediated by the GR in NPCs.

2.3 Results

Generation of inducible GR gene inactivation in adult Nestin+ cells

In order to examine how the GR in adult hippocampal NPCs contributes to neurogenesis, we used a transgenic approach allowing for temporal control of genetic recombination restricted to NPCs (Figure 1). First, mice expressing a tamoxifen-inducible form of Cre recombinase (Cre\textsuperscript{ERT2}) under nestin (marker for NPCs) transcriptional control\textsuperscript{84} were bred to a R26R-YFP reporter line to generate mice with a surrogate marker for recombination (Nestin-CreERT2/R26R-YFP). Next, we mated mice carrying \textit{GR}\textsuperscript{loxP/loxP} alleles\textsuperscript{239} with the Nestin-CreERT2/R26R-YFP line for at least two generations to produce a \textit{GR}\textsuperscript{(loxP/loxP);R26YFP+/-;Tg(Nestin-CreERT2)} (thereafter called \textit{GR}\textsuperscript{(NestinCreERT2)}) mouse line with a YFP reporter (Fig 1a). DNA from mouse tails was carefully extracted and analyzed by PCR to verify genotype (Fig 1b). Both \textit{GR}\textsuperscript{loxP} and R26R-YFP alleles are sensitive to Cre recombinase, which catalyzes site-specific recombination between the loxP sites. Thus, upon tamoxifen administration, GR was selectively inactivated and YFP expression was selectively induced only in cells expressing nestin and all their progeny thereafter (Fig 1a). Mutant mice (i.e. tamoxifen-treated \textit{GR}\textsuperscript{NestinCreERT2} mice) are denominated \textit{GR}\textsuperscript{NPCKO}. Untreated \textit{GR}\textsuperscript{NestinCreERT2} mice were used as control animals.

Verification of the inducible GR gene inactivation in adult Nestin+ cells

We used two approaches to verify the efficacy of recombinase activity \textit{in vivo}. Using immunohistochemistry, we quantified and analyzed YFP expression and co-labelling of GR and BrdU in the hippocampus of \textit{GR}\textsuperscript{(NestinCreERT2)} mice after administrating vehicle (control) or tamoxifen. Tamoxifen binds to the mutated estrogen receptor (ERT2) of Cre in cells expressing Nestin, allowing the Cre recombinase to excise the STOP codon in the YFP reporter and the exon 3 segment in \textit{GR}\textsuperscript{loxP} alleles. Thus, mice receiving vehicle (-tam) maintained an inactive YFP reporter (Fig 1c), while mice receiving tamoxifen (+tam) expressed YFP in nestin-expressing cells and their progeny (Fig 1d). Similarly, adult –tam mice maintained active GR expression in the proliferating (BrdU+) hippocampal cell population (Fig 1e), while in +tam mice, the GR gene was inactivated in the majority of this population (Fig 1f). Quantitative analysis revealed a significant difference between −tam and +tam mice for the percent of BrdU+ cells expressing GR (two-way ANOVA cort x genotype, effect of genotype: \textit{p}<0.0001, \textit{F}(1,31)=72.59, cort: \textit{p}=0.25, interaction: \textit{p}=0.73) (Fig 1g).

Chronic cort-treatment does not affect NPC survival

To examine adult neurogenesis in this cell-specific \textit{GR}\textsuperscript{(NestinCreERT2)} model, the experimental design was as follows: \textit{GR}\textsuperscript{(NestinCreERT2)} mice were treated with tamoxifen or vehicle (controls) at 5-6 weeks of age and then allowed at least two weeks for recovery (Fig 2a). Afterward, half of each of the +tam and −tam cohorts were chronically treated with corticosteroids through their drinking water for the next 20-26 weeks (age and treatment time...
were counterbalanced across all groups). Four weeks prior to perfusion, 6 mice within each group were given BrdU for analysis of cell survival and fate (Fig 2a). Control mice had similar levels of cell survival regardless of cort treatment (Fig 2b). This effect was not altered by GR ablation (two-way ANOVA, effect of cort: p=0.64; tam: p=0.96). Area measured for cell counts did not affect results as BrdU+ cells per m² was not significantly different either (data not shown). GrloxP/loxP mice were used as controls to verify NPC survival was not affected by CreERT2 genotype or tamoxifen alone (3way ANOVA, p>0.05; Fig 2b).

**Cort-suppressed differentiation of NPCs may be indirectly mediated**

Finally, we sought to determine whether chronic cort treatment reduced neurogenesis by reducing neuronal differentiation, and if this effect was directly mediated through the GR in NPCs. Mice from each group were administered BrdU 4 weeks prior to perfusion. This allowed for cell lineage tracking from newborn precursor to mature neuron. We measured the number of hippocampal cells that were BrdU positive (proliferating cell marker), and of those, which were co-labeled as NeuN positive (mature neuronal marker) in the DG and hilus (Fig 3). When comparing the percent of BrdU positive cells co-labeling for NeuN, a two way ANOVA of genotype x cort revealed a significant effect of cort (p=0.003, F (1,15)=12.8), but no effect of genotype nor interaction (Fig 3a). This suggests that chronic cort-treatment reduced neurogenesis overall in both control and GRNPCKO groups. Further post-hoc analysis revealed a significant effect of cort between controls (p=0.0087) as well as between GRNPCKO's (p<0.05; Neuman-Keuls), but no significant differences between noncort-treated groups or between cort-treated groups (Fig 3a). Again, examining the number of co-labeled NeuN+ cells per area by two-way ANOVA showed a significant effect of cort (p=0.018, F (1,15)=7.072) (Fig 3b). Neither tam treatment nor CreERT2 genotype alone affected differentiation (data not shown). All groups had a comparable number of BrdU positive cells (Fig 2b) and no significant differences in areas measured (data not shown). These results show that chronic cort treatment reduces neuronal differentiation in vivo, and it is not reversed or attenuated by GR gene inactivation in NPCs.

### 2.4 Discussion

We have generated mice using an inducible Cre-lox system to conditionally lose GR function in an adult population of NPCs. We were able to show that chronic cort treatment in vivo did not affect the survival rate of NPCs in the mouse hippocampus, however it did inhibit neuronal differentiation. This effect was not blocked by loss of GR in NPCs. In the present study, we show that GR gene function in NPCs is not necessary for cort-induced suppression of neurogenesis, implying that this effect of cort may be mediated indirectly.

This is the first mouse model of GR function that specifically targets adult NPCs and did not require surgery. Previous studies have investigated the function of the GR in vivo using pharmacological agents and found they played a significant role in the survival and proliferation of NPCs. These studies, however, looked at the general role of GR in the brain and were not specifically targeting any cell type making it difficult to assess whether the effect of cort on neurogenesis is mediated by cell-autonomous GRs. Comparatively, Tronche and colleagues created a transgenic mouse with GR gene inactivation in NPCs, but since it was not inducible, all neural cells lost GR function from birth, making it impossible to study the role of GR in adult neurogenesis. A recently published study used RNA-interference to knockdown GR within the adult neurogenic niche, however these mice had intrahippocampal injections of lentivirus, which
is invasive and not specific to proliferating cells. Here we report that we were able to isolate the direct role of GR function in a discrete population of proliferative cells.

In our study, chronic treatment of cort does not appear to affect survival. Although this supports other results, our results are unclear without more information on the effects on proliferation. Many studies have previously demonstrated that chronic stress, as well as chronic cort treatment, significantly suppresses the proliferative nature of NPCs. Thus, if in fact cort treatment also inhibited proliferation in our study, since there was no difference in the number of NPCs at 4 weeks post-BrdU injection, this would mean that noncort-treated mice had greater pruning among NPCs. In other words, cort-treated controls had less cell death. If proliferation was not affected in the GR NPCKOs, this would imply that GR NPCKOs have greater cell death, regardless of cort treatment. It is worth noting that proliferation was affected in the transgenic mice with GR gene inactivation in all brain cells (F. Tronche, unpublished data), however as mentioned, whether this is also the case for NPC-specific GR gene inactivation is currently unknown. Moreover, if cort had a similar effect on proliferation of GR NPCKOs compared to controls, this would imply that GR gene inactivation in NPCs has no effect on NPC survival. Given these points, in addition to a larger sample size, it would be important to know the effect of GR NPCKOs on proliferation in order to conclude that GR in NPCs does not affect survival rates.

Although many studies report chronic cort treatment reduces neurogenesis, this effect appears to be due to reduced proliferation. It is important to examine each stage of NPC development to understand how glucocorticoids regulate the rate of neurogenesis. This information will allow for greater elucidation of cort-mediated pathways that affect hippocampal cytoarchitecture. In line with other studies, we find that chronic cort treatment can also inhibit the differentiation of NPCs into mature neurons. This cort-induced suppression appears to be mediated independently of GR activation in NPCs, however, a larger sample size would make these results more convincing.

If in fact GR ablation in NPCs does not block cort-induced suppression of neuronal differentiation, there could be several explanations. First, it could be due to experimental design. It is possible that in our mouse model, GR was not sufficiently knocked out of enough NPCs. Whereas tamoxifen-induced Cre recombinase is not 100% efficient and some NPCs (i.e. type 2b) do not express nestin and so their GR gene remains functional, thus, it remains possible that not enough of the population was affected to block cort influence. Furthermore, it could be that our chronic cort-treatment was too extended that other cort-induced pathways compensated for the lack of GR. This latter explanation also suggests another interpretation of the results, however— that neurogenesis can be regulated indirectly.

Although NPCs can express GR, GR is not expressed ubiquitously in NPCs. Whereas GR was not found to be expressed at all in one earlier study, a more recent finding showed that only 13% of newborn BrdU+ cells expressed GR. According to this study, GR was only expressed in approximately half of NPCs at each developmental stage, with the exception of type 2b cells (0% express GR). It was not until cells reached a post-mitotic stage that they all started expressing the GR. Although this study was done on female mice, there is no known reason to assume that male mice would have a greater percentage of NPCs expressing GR. Thus, if cort does indeed suppress neurogenesis by directly acting through GRs in NPCs, it can only be affecting at most 50% of the NPC pool. This lack of GR in earlier stages of neurogenesis may imply two contrasting hypotheses: either GR expression during early stages is not as functionally active as it is during the post-mitotic stage and thus, cort indirectly influences NPC behavior; or
GR expression is always functionally active and it is such that cort directly influences the fraction of GR-expressing NPCs, which is sufficient enough to cause overall suppressed differentiation. Since our results show that when mice have a significant inactivation of GR genes in NPCs, even further reducing the sub-population of GR-expressing NPCs, they still show similarly reduced differentiation, this implies that the former hypothesis is more accurate. That being, cort might be indirectly influencing NPC behavior.

One of the cort-mediated pathways indirectly influencing NPC behavior could still be through GR activation, but through GR in mature neurons or astroglia. Since much of the literature has shown that GR plays a role in cort-induced suppression of neurogenesis, it may be that the GR in NPCs alone is insufficient to drive this effect. Many studies have demonstrated GR expression in mature neurons, astrocytes, and oligodendrocytes as well. Interestingly, it has been shown that GR activation in astrocytes can induce secretion of different factors that can mediate neurogenesis, such as basic fibroblast growth factor (FGF2) and cell cycle inhibitors. Additionally, stress effects on neurogenesis were found concomitantly with increases in FGF2 mRNA in the dorsal hippocampus. They examined this in vitro by treating NPCs with conditioned media from cort-treated astrocytes. Not only did this affect neurogenesis, but also treating NPCs with similar levels of FGF2 alone caused a change in proliferation. Furthermore, neutralizing the FGF2 blocked the effect. These results suggest that cort may regulate NPC behavior indirectly through activating GR in astrocytes.

Similarly, neurogenesis can be regulated when GR activation in either astrocytes or neurons dysregulates cell signaling by inhibiting both glutamate uptake and N-methyl-D-aspartate (NMDA) receptors. It had been shown that GR activation reduced glucose transport into both neurons and astrocytes. This effect resulted in a cascade of increased levels of damaging glutamate in the synapse, which overactivated NMDA receptors, and thus, increased free cytosolic calcium. Not only does increased cytosolic calcium (Ca2+) signaling damage the postsynaptic neuron, but it has also been demonstrated to regulate all stages of neurogenesis (for review). Furthermore, blocking NMDA receptor activity prevented GR-induced suppression of neurogenesis. Although more research is needed to elucidate these pathways in vivo, it remains another possible explanation for how cort can indirectly influence neurogenesis.

Another way that cort can influence neurogenesis indirectly is through serotonin (5HT1A) receptors. GR antagonists were shown to block the effect of cort treatment on the upregulation of serotonin transporter protein levels in the hippocampus, demonstrating that cort-induced GR activity promotes serotonin transporter production. Similarly, other studies have demonstrated cort can regulate 5HT1A receptors in the hippocampus. Both activation and inhibition of 5HT1A receptors and transporters can cause changes in proliferation and differentiation of NPCs. These studies suggest that GR-mediated increases in 5HT1A transporter and receptor protein may regulate neurogenesis. Regardless of whether this effect is mediated directly through altered levels of 5HT1A receptors on NPCs, GR activity induced in mature neurons or astroglia may be the initial mechanism that indirectly affects NPC development.

Overall, we found that chronic cort treatment did not disrupt survival rates of NPCs in the adult murine hippocampus, however it did reduce neuronal differentiation. Knocking out GR in the NPCs did not attenuate this effect of cort-suppressed differentiation. Since GR is implicated in regulating neurogenesis, our results suggest that cort-suppressed neurogenesis is mediated indirectly through GR in other cells of the stem cell niche. Furthermore, it would be interesting.
to know if cort-suppressed proliferation is also unaffected by GR\textsuperscript{NPCKO}, further suggesting that the effects of cort are indirectly mediated. Both corticosteroids and neurogenesis are implicated in psychopathologies and mood disorders\textsuperscript{5,8,197,276,304}. Additionally, they are shown to play a role in regulating memory processes\textsuperscript{44,45,204,252}. Our novel mouse model of GR gene inactivation specific to NPCs in the adult brain allows for a better understanding of the molecular mechanisms that are mediated by cort to regulate adult neurogenesis. This is relevant both to endogenously elevated cort as induced by stress, learning, and exercise, as well as exogenously administered for medicinal purposes. Furthermore, our transgenic mice will allow for a better understanding of the role that GR plays in newborn neurons and potentially contribute to the development of new neuropharmalogical therapies.

2.5 Materials and Methods

**Nestin-CreERT2 and GRloxP/loxP transgenic mice.** To selectively inactivate the GR gene in neural progenitor cells, we generated GR\textsuperscript{loxP/loxP}; R26R-YFP; Tg(NesCreERT2) mice (thereafter denominated GR\textsuperscript{NesCreERT2}), by mating ad-hoc animals, all on a C57BL/6 genetic background. The GR\textsuperscript{loxP} allele contains loxP sites flanking exon 3 of the GR gene, the first zinc finger of the GR DNA-binding domain. The strategic placement of loxP around this specific part of the GR gene not only removes exon 3, but also causes a shift in the open reading frame of the gene. Potential mRNAs generated from the mutated allele would fail in translating any functioning GR protein\textsuperscript{239}. The NesCreERT2 transgene expresses the CreERT2 recombinase gene under control of the Nestin promoter\textsuperscript{84}. The R26R-YFP Cre-reporter allele (Jackson Laboratories) harbors a transcriptional STOP cassette preventing, in the absence of active Cre recombinase, the expression of the YFP open reading frame on the Rosa26 locus. Upon tamoxifen induction in GR\textsuperscript{(NesCreERT2)} mice, Cre recombinase efficiently promotes the recombination between two head-to-tail oriented loxP sites, which leads to excision of intervening DNA, selectively in nestin-expressing cells, thus creating a nestin-specific loss of GR function and active YFP expression. Vehicle-induced GR\textsuperscript{(NesCreERT2)} mice were used as controls. Vehicle- and tamoxifen-induced GR\textsuperscript{loxP/loxP} mice were also tested alongside controls to verify no effects of Cre or tamoxifen. 60 mice (n=10 per group) were used in experiment. For analysis of gene inactivation efficiency, 10 mice per group (-tam/-cort, -tam/+cort, +tam/-cort, +tam/+cort) were used, however, 5 could not be analyzed as a result of 1 death and 4 exclusions due to inefficient recombination in NPCs.

We restricted our analysis to male mice. Animals were bred and raised under a 12h light/dark cycle; temperature was 22±2°C and humidity 60±5%. Food and water were supplied ad libitum. Experiments were performed in accordance with French (Ministere de l’Agriculture et de la Forêt, 87-848) and European Economic Community (EEC, 86-6091) guidelines for the care of laboratory animals.

Genotypes were determined by PCR analysis of tail DNA samples as described previously\textsuperscript{84,239}. Adult mice were genotyped using the following primers:

- **CreERT2:**
  - CreERT2 (TK139): 5’ (ATT-TGC-CTG-CAT-TAC-CGG-TC) 3’
  - CreERT2 (TK141): 3’ (ATC-AAC-GTT-TTC-TTT-TCG-G) 3’

- **YFP:**
  - YFP (common): 5’ (AAA-GTC-GCT-CTG-AGT-TGT-TAT) 3’
  - YFP: 5’ (GCG-AAG-AGT-TTG-TCC-TCA-ACC) 3’
  - YFP (wt): 5’ (GGA-GCG-GGA-GAA-ATG-GAT-ATG) 3’

- **GrloxP:**
  - GR12F (wt): 5’ (CAT-GCT-GCT-AGG-CAA-ATG-ATC-TTA-AC) 3’
GR15R (GR\textsuperscript{loxP}): 5’ (CTT-CCA-CTG-CTT-TAA-AGA-AGA-C) 3’
GR30R (null): 5’ (GAA-TGA-GAA-TGG-CCA-TGT-ACT-AC) 3’

PCR reactions were carried out at 58ºC for both CreERT2 and YFP and 60ºC for GR\textsuperscript{loxP}, in a buffer containing 3 mM MgCl\textsubscript{2}. An aliquot of the PCR reaction was analyzed on an agarose gel. The CreERT2 allele gave a 350bp band; the YFP allele gave a 310bp band (560bp for wt); and the GR\textsuperscript{loxP} allele gave a 450bp band (400bp for wt).

**Tamoxifen induction.** Tamoxifen (Sigma T5648) was light-protected and dissolved in 10% ethanol and suspended in 90% sunflower seed oil\textsuperscript{84}. Mice received daily injections at 180mg/kg, i.p. for 5 days when they were 5-6 weeks old. Control mice received vehicle (sunflower oil). Mice receiving tamoxifen were only group-housed with other tamoxifen-receiving mice; likewise, only vehicle (oil)-receiving mice had vehicle-receiving littermates. This guarded against possible cross-contamination of tamoxifen.

**Corticosterone treatment.** Corticosterone (Sigma C2505) was prepared as described previously\textsuperscript{11}. It was dissolved at 35 µg/mL in a water solution of 0.45% (wt/vol) hydroxypropyl-beta-cyclodextrine (Sigma 332593) by sonication for two hours. Cort treatment was given to mice in opaque water bottles to protect it from light, changed twice a week, and available ad libitum.

**BrdU labeling.** To trace cell lineage, 31-32 days prior to perfusion, mice were administered with bromo-deoxyuridine (150mg/kg, i.p. dissolved in saline; Sigma B5002) twice daily for 3 days.

**Immunohistochemistry.** Mice anesthetized with pentobarbital euthanasia solution were perfused transcardially with ice cold 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were removed, fixed overnight in PFA at 4ºC, and transferred to 0.1M PBS. Serial sections were cut coronally at 35 µm using a vibrotome (Vibratome 3000 Plus; Ted Pella, Inc). Staining consisted of 3 x 5min washes in 1xPBS, blocking and permeabilizing in a 1xPBS solution of 5% normal donkey serum (Jackson 017-000-121) and 0.3% tritonX for 30-60 minutes, followed by overnight incubation at 4ºC with primaries. Tissue was then washed 3x5min in PBS, treated with secondary antibody for two hours at room temperature, washed again, fixed in 4% PFA for 15minutes, then washed again. Tissue preparation for BrdU co-labeling then proceeded with treatment of 0.9% saline for 5 minutes, followed by an acid wash in 2N HCL at 37ºC for 30 minutes. Tissue was then washed 3x5minutes, blocked, and incubated with BrdU antibody overnight. The following primary antibodies were used on free-floating sections: rat monoclonal anti-BrdU (Abcam 6326; 1:500), rabbit polyclonal anti-GR (Santa Cruz 1004; 1:500), goat anti-gfp, FITC-conjugated (Rockland 600-102-215; 1:500), and rabbit polyclonal anti-NeuN (Millipore ABN78; 1:500). Secondary antibodies used were as follows: biotinylated donkey anti-rat immunoglobulin G (Jackson 712-065-153, 1:500), donkey anti-rabbit Alexa647 (Jackson 711-605-152, 1:500), and donkey anti-goat AF488 IgG (Jackson 705-545-147, 1:200). Visualization of BrdU was performed with StrepAlexa488 for 1 hour at room temperature. Tissue was treated with Dapi (Invitrogen D1306, 1:20000) when appropriate. Sections were mounted onto slides and coverslipped with DABCO.
**Microscopy and quantification.**
Confocal laser scanning microscopy (Zeiss) was used for counting fluorescent-labeled cells. It was performed using a 40X-oil objective on a 1 in 10 series of sections through the entire DG. In all cases, 8 hippocampi per animal of each experimental group were randomly selected within each series and analyzed. For survival and differentiation analysis, 32 mice (n=4-6 per group) were given BrdU 4 weeks prior to perfusion; of these, only 27 were analyzed due to 1 death, 1 damaged brain tissue, and 3 exclusions due to inefficient recombination in NPCs: -tam/-cort, n=6; -tam/+cort, n=5; +tam/-cort, n=4; +tam/+cort, n=4; Gr^{loxP/loxP} –tam/+cort, n=4; Gr^{loxP/loxP} +tam/+cort, n=4. BrdU+ cells were counted in 8 hippocampi of 2 series and multiplied by 10 to represent total numbers throughout DG and hilus. Area of DG and hilus were measured using Stereo Investigator software.

**Statistical analysis.**
Results are expressed as means±s.e.m. Statistical analysis was performed using Student t-tests when comparing effect of cort between controls, and two-way ANOVA for comparing all groups. Analyses were followed by Dunnett’s or Newman-Keuls post-hoc tests for pairwise comparisons, as appropriate. * indicates p<0.05.
2.6 Figures and Legends

Fig 1. Generation and verification of adult mice deficient for GR in newborn neurons. (a) Strategy for inducible gene inactivation of the GR gene in adult neural progenitor cells. Transgenic mouse lines Nestin-Cre$^{ERT2}$/R26R-YFP, and Gr1$^{loxP/loxP}$, were crossed for at least two generations to create a GR$^{(NestinCreERT2)}$ mouse line. These GR$^{(NestinCreERT2)}$ mice were heterozygous for the NestinCre$^{ERT2}$ transgene, a R26R-YFP reporter with a stop codon flanked by loxP sites, and homozygous for the GR$^{loxP}$ allele in which exon 3 was flanked by loxP sites. Upon tamoxifen administration, Nestin+ cells would have an active YFP reporter and lack a functional GR. (c-d) Representative coronal sections immunostained for anti-gfp (marker of YFP reporter) and DAPI (nuclei marker) in the GR$^{(NestinCreERT2)}$ transgenic line show recombined cells (YFP+, green) in the hippocampus of induced (+tam) mice (d), but not in the non-induced (-tam; vehicle) mice (c). (e-f) Double labeling for anti-GR (green) and anti-BrdU (red) in the hippocampal DG shows co-labeling in the –tam (vehicle) mice (e), but not in the mice receiving tamoxifen (+tam) (f). Brain sections were imaged at 40x by confocal microscopy. (f) GR$^{(NestinCreERT2)}$ mice receiving tamoxifen led to significant gene inactivation of GR in newborn neurons (*, p<0.0001, n=8-10 per group). Results are expressed as mean±s.e.m. Scale bars represent 10µm in e-f.
Figure 1

a
Nestin-CreER<sup>T2</sup>
Grhl<sup>loxP/loxP</sup>
R26R-YFP

E3   E4

Tamoxifen-induced recombination

CreER<sup>T2</sup>Nes

b
Gr<sup>++</sup>  Gr<sup>+-</sup>  Gr<sup>++</sup>
Yfp<sup>++</sup> Yfp<sup>+-</sup> Yfp<sup>++</sup>

Cre<sup>+</sup>  Cre<sup>-</sup>

-camt  +tamt

Yfp+ Dapi+

c  d

- tam  + tam

GR+  BrdU+

Merge

- tam  + tam

GR+  BrdU+

Merge

g

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*
Fig 2. Effect GR\textsuperscript{NPCKO} on NPC survival. (a) Timeline of experimental protocol. BrdU was administered 4 weeks prior to perfusion. (b) Quantification of BrdU+ cells was analyzed throughout the DG and hilus of the hippocampus (n=4-6 per group). There was no difference measured in number of BrdU+ cells in the hippocampus among groups at 4 weeks. Brain sections were examined at 40x by confocal microscopy.

**Figure 2**

\[\text{Diagram showing timeline and quantification data.}\]
Fig 3. Effect of GR<sup>NPCKO</sup> on NPC differentiation. BrdU was administered 4 weeks prior to perfusion. Quantification of NeuN-/BrdU+ and NeuN+/BrdU+ cells was analyzed throughout the DG and hilus of the hippocampus (n=4-6 per group). (a) Cort-treatment had a significant effect on neurogenesis for both groups. (b) The same effect is seen in the number of newborn neurons per area. (c) Double staining for anti-NeuN (mature neuronal marker) and anti-BrdU (proliferating cell marker) shows co-labeling in the hippocampal DG of untreated mice, (d) while reduced co-labeling is seen in cort-treated mice. Brain sections were examined at 40x by confocal microscopy. Scale bars represent 10µm.
CHAPTER 3

FUNCTIONAL CONTRIBUTIONS OF THE GLUCOCORTICOID RECEPTOR IN ADULT NEUROGENESIS
3.1 Abstract

Corticosteroids (cort) are one of the strongest and most relevant environmental signals mediating adult neurogenesis. While the impact of these stress hormones on newborn hippocampal neurons has been demonstrated in terms of cellular changes, the functional relevance of these changes is unclear. To characterize these functional changes, we impaired cort-signaling in newborn neurons by irreversibly knocking out the glucocorticoid receptor (GR) selectively in adult neural progenitor cells (NPCs). Then, by using tests that measure anxiety- and depression-like behavior, as well as cognitive skills, such as contextual fear conditioning and pattern discrimination, we sought to determine whether changes in adult neurogenesis under chronic stress-like conditions affected hippocampal functioning. We found that chronic cort treatment can induce an anxious phenotype, which is prevented by reducing GR signaling in adult-born neurons. This suggests that anxiety-like behaviors may be induced thru newborn neurons via GRs. Additionally, we found that neither chronic cort treatment nor GR gene inactivation in NPCs (GR
\textsuperscript{NPCKO}) influenced depression-like behavior or performance in contextual fear discrimination. Although unaffected by GR
\textsuperscript{NPCKO}, chronic cort treatment did, however, enhance learning in contextual conditioning. Overall, these findings show that adult neurogenesis can be functionally relevant to anxious behavior under chronic cort treatment. This suggests that adult-born neurons, through GR signaling, can alter the behavioral response to stressful stimuli, an adaptive effect that can be evolutionary advantageous or dysfunctional under chronic stress.
3.2 Introduction

The conservation of adult neurogenesis across invertebrate (for review\(^{46}\)) and vertebrate (for review\(^{69}\)) species suggests a fundamental biological significance that is at the forefront of exploration. This plasticity appears to be restricted to two regions of the mammalian brain: the subventricular zone of the lateral ventricles, and the subgranular zone in the hippocampus\(^{305}\), further suggesting that the distinct functions of these structures allow them to benefit from the continual production of new neural cells. Within the mouse hippocampus, the process of a newborn progenitor cell developing into a functional granule neuron takes approximately 4 weeks\(^{306-308}\). Although this population of adult-born granule cells (abGCs) makes up only around 10% of the dentate gyrus (DG)\(^{309}\), due to their hyper-excitability, greater integration capacity, and extensive connections, it is believed to make a disproportionate functional contribution\(^{310}\). It is well known that the hippocampus is critical for memory and learning\(^{311-313}\), and significantly involved in mood-related behaviors\(^{314}\). Accordingly, abGCs are implicated in memory and learning processes such as contextual conditioning and pattern separation\(^{218,221,268,315}\), as well as anxiety-like behaviors, and depression\(^{252,272,276,316-318}\). Although adult neurogenesis contributes to these aspects of hippocampal-dependent functions, the degree to which is controversial.

Further indication of the functional relevance of hippocampal neurogenesis is their responsiveness to the external environment, particularly stress. This implies hippocampal neurogenesis may affect behaviors associated with stress and adaptation. An elevation in circulating glucocorticoids, as triggered during stress, has been demonstrated to impact the behavior of newborn neurons\(^{103,279}\). During stress, the elevated glucocorticoid known as corticosterone in rodents, and cortisol in humans (hereafter called cort), impacts hippocampal plasticity not only through neurogenesis, but also synaptic integrity, cell migration, apoptosis, long term potentiation (LTP), and CA3 dendritic remodeling\(^{279,319-321}\). To what degree any of these effects are functionally significant, however, is yet to be determined.

What is known, nonetheless, is that cort affects a variety of hippocampal functions, such as anxiety- and depression-like behaviors, as well as memory processing\(^{197,200,243,279,322}\). This can be seen in Cushing’s syndrome, a state of hypercortisolism, in which patients commonly suffer from memory dysfunction as well as mood disorders like depression and anxiety\(^{197}\). Considering that both the cort response during stress as well as adult neurogenesis are conserved mechanisms, it would seem apparent that there would be a functional advantage of abGCs being responsive to cort signals. Cort actions are mediated by two receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Whereas MR binds to cort with an affinity 10-fold higher than GR, GR does not become occupied (activated) until cort levels are high, i.e. at the circadian peak and during stress\(^{24,280,323,324}\). Furthermore, while abGCs express both MR and GR, only GR is expressed earlier on during the proliferative stages\(^{37}\). This implicates GR as an important target in newborn neurons during high levels of cort.

In the brain, GR is thought to regulate emotional behavior and cognitive functions\(^{4,283}\). While it was shown that general GR gene inactivation is lethal\(^{27}\), and brain-wide GR gene inactivation reduces anxiety-like behaviors\(^{239}\), it is unknown if GR gene inactivation specifically in NPCs is behaviorally relevant as well. Whereas adult neurogenesis appears to contribute to functions similarly affected by high levels of cort, the question remains whether abGCs mediate any of these cort-induced effects on behavior and cognition. To address this question, we irreversibly inactivated GR gene signaling specifically in adult NPCs and their progeny, thereby
reducing the capacity of the abGC population to respond to stress signals, i.e. elevated cort. In this novel GR mutant mouse model specific to adult neurogenesis (GR\textsuperscript{NPCKO}), we investigated the functional relevance of GR-signaling in abGCs. Mice received 4 weeks of chronic cort treatment through their drinking water to induce an anxious state (as described\textsuperscript{11}), and were then tested on a battery of behavioral tests under continued treatment. These behavioral tests included commonly used protocols to measure behaviors associated with anxiety and depression, as well as cognitive abilities such as contextual fear conditioning and pattern separation. This study explores not only how cort impacts behavior, but also functional contributions of adult neurogenesis, or in other words, how the response of abGCs to chronic cort treatment affects mood and memory.

3.3 Results

**Generation of mice with reduced GR signaling in adult born granule cells.**

To investigate the role played by GR-signaling in abGCs, we generated a transgenic nestin-CreERT2 mouse line with inducible GR gene inactivation (see chapter 2). CreERT2 is a chimeric Cre recombinase enzyme obtained by fusing the Cre open reading frame and a mutated ligand-binding domain (LBD) of the estrogen receptor. In the presence of tamoxifen, a synthetic ligand of the estrogen receptor, the CreERT2 protein is released from chaperone proteins in the cytoplasm and translocates to the nucleus where it excises DNA segments flanked by loxP sites. In the GR\textsuperscript{(NesCreERT2)} model, CreERT2 was only present in cells expressing nestin, an intermediate filament protein found mostly in stem cells and used to identify type1 and type 2a NPCs\textsuperscript{84,325}. These mice were homozygous for GR alleles containing exon 3 flanked by loxP. Deletion of this segment excises the DNA binding domain and abolishes the expression of the LBD in the GR gene, resulting in a nonfunctional GR\textsuperscript{239} specifically in NPCs.

Mice were induced with tamoxifen at 5-6 weeks old (young adulthood) and given at least 2 weeks for recovery (Fig 1a). This technique was successful in knocking out GR in approximately half of the proliferative cells in the hippocampus that express GR (see Fig1g from chapter 2) thereby reducing the capacity of the neurogenic pool to respond to elevated cort levels. We then chronically treated half of the induced GR\textsuperscript{NPCKO} mice and half of the non-induced control mice with low-dose cort and looked at several aspects of hippocampal-dependent behavior and cognition. First, we examined whether there was an effect on locomotion, or their physical activity in a novel environment. After 4 weeks of cort treatment (see experimental design, Fig 1a), mice were placed in a dark novel environment where their movements were measured by crossing an invisible grid. Both untreated and cort-treated control and GR\textsuperscript{NPCKO} mice behaved similarly to each other across 5 hours of their light cycle and overall (Fig 1b). Likewise, all groups exhibited comparable levels of physical activity in the open field test (Fig 1c). We measured the percent of time spent in the center (Fig 1c), corners, and sides (data not shown) of the arena, as well as the distance traveled, and velocity of movement. In all measures, GR\textsuperscript{NPCKO} mice performed similarly to controls regardless of cort-treatment.

**Cort-treatment has reduced effect on anxiety-like behaviors in GR\textsuperscript{NPCKO}.**

Next, to examine whether GR in abGCs plays an important role in mediating anxiety-like behavior in a stressful environment, we used three behavioral paradigms that test the natural avoidance behavior of mice: novelty-suppressed feeding, light/dark box, and elevated Omaze (Fig 2). In novelty-suppressed feeding (NSF), we measured how long it took before food-
deprived mice approached as well as ate food from the aversive (i.e. lit and exposed) center platform (Fig 2a). For latency to approach, two-way ANOVA revealed a significant effect of genotype (p=0.019, F(1,30)=6.18), but only a near significant interaction effect (genotype x cort, p=0.062). Further post-hoc analysis, however, showed a significant difference between the untreated and cort-treated controls (p<0.05, Dunnett), but not the GRNPCKO’s. Cort-treated controls were also significantly different from both GRNPCKO groups (p<0.05, Neuman-Keuls). Thus, under chronic cort-treatment, we found that the controls on cort took significantly longer to approach the food, while the behavior of the GRNPCKO’s were not affected by cort (Fig 2a). The latency to eat revealed only an effect of cort-treatment (p=0.002, F(1, 31)=0.596), which post-hoc analysis revealed was largely driven by a significant difference between cort-treated GRNPCKO mice and both untreated mice groups (p>0.05, Neuman-Keuls). Furthermore, there was not a significant effect between all groups in an analysis of percent of mice not feeding (p=0.09, Mantel-Cox log rank).

In light/dark box, we measured the latency to exit the dark compartment (and enter the lit compartment), and the frequency of entries (4 paws) and time spent in the lit compartment (Fig 2b). Two-way ANOVA revealed a significant effect of genotype in both the latency to enter and time spent in the lit compartment (p=0.013, F(1,3)=7.06 and p=0.020, F(1,31)=5.97, respectively), while further post-hoc analysis showed cort-treated controls took significantly longer to exit compared to untreated controls (p<0.05, Dunnett’s), as well as untreated and cort-treated GRNPCKO’s (p<0.05, Neuman-Keuls). Interestingly, cort-treated GRNPCKO’s behaved similarly to untreated GRNPCKO’s. This interaction effect was most apparent in the number of 4 paw exits, which proved significant in two-way ANOVA (p=0.037, F(1,31)=4.77) (Fig 2b).

In elevated Omaze, we measured the latency to exit the wall-sheltered segment (and enter the aversive, open, and more lit segment), and the frequency of entries and time spent in the lit, open segment (Fig 2c). Two-way ANOVA revealed a significant effect of genotype in the frequency of 4 paw entries (p=0.047, F(1,29)=4.29), as well as an effect of cort in both the frequency of 4 paw entries (p=0.007, F(1,29)=8.468) and time spent in the lit, open segment (p=0.0004, F(1,29)=16.3). Follow-up post-hoc analysis showed that these effects were driven by the cort-treated control group, which spent significantly less time in the lit, open segment (p<0.05, Dunnett and Neuman-Keuls), and had less 4 paw entries (p<0.05, Dunnett and Neuman-Keuls) compared to all other groups. Interestingly, in both measurements, untreated and cort-treated GRNPCKO’s performed similarly (Fig 2c). This again suggests that while anxiety-like behavior in control mice is affected by cort-treatment, the behavior of GRNPCKO’s is unaffected. There were no significant differences found among all groups in the latency to exit in this task.

GRNPCKO mice do not show depression-like phenotype.

Elevated levels of cort are often associated with depressive-like symptoms, and NPCs have been shown to be involved in the behavioral effects of antidepressants, thus, we sought to investigate whether the GR in abGCs plays a role in depressive-like behaviors. To examine this, we used the mouse Porsolt forced swim test, which is a well-established clinical model of stress-coping ability. In this task, the behavioral response to stress is measured in the time spent floating (immobility), active (climbing), and the latency to float (Fig 3a-c). Although there appears to be a trend of chronic cort-treatment affecting both controls and GRNPCKO’s in both the latency to float and time spent immobile, two-way ANOVA revealed no significant differences across all groups (Fig 3a-b). Similarly, mice did not differ significantly in the time spent climbing (Fig 3c). Further post-hoc analyses confirmed no
significant differences among groups, suggesting that neither chronic cort-treatment nor GR signaling in NPCs alone drives a depression-like phenotype.

**GRNPCKO's are successful at 1-trial contextual fear conditioning.**

Contextual fear conditioning is a hippocampal-dependent function that studies have demonstrated can be impaired by cort administration\(^3\)\(^{237,326}\), as well as ablated neurogenesis\(^2\)\(^{255,327,328}\). To determine whether impairment may be due to cort effects (via GR) specifically on abGCs, we next examined contextual fear conditioning in our cort-treated controls and GR\(^{NPCKO}\) mice. On day 0, mice were allowed to explore a novel environment for 3 minutes before they received a 2sec- 0.75mV foot shock (Fig 4a). During this time before foot shock, there was minimal freezing (0-5% of total time). The next day, all mice demonstrated a significant increase in freezing compared to day 0 (three-way ANOVA, p<0.0001, \(F\)\(_{(1,33)}=100.18\)) verifying that all mice were able to learn and recall an association between contextual cues and an electrical shock (Fig 4b-c). Interestingly, when only comparing treatment within control groups, conditioning was not affected by cort per se (p=0.56), but was affected by the interaction of cort over time (two-way ANOVA, \(p=0.0457, F\)\(_{(1,16)}=4.697\)), indicating a change in the rate of learning (Fig 4b). This effect is more clearly evident by comparing the differences in %freezing in untreated versus cort-treated controls (p=0.03) (Fig 4d). Our GR\(^{NPCKO}\) mice did not significantly block this effect. According to three-way ANOVA, there was no effect of genotype (p=0.86), and only a near significant interaction effect of genotype x cort x day (p=0.06). Two-way ANOVA did not find a significant interaction for the difference in %freezing for genotype x cort (p=0.11) (Fig 4d), although follow-up post-hoc analyses showed a difference in cort-treatment among controls (p<0.05, Dunnett), but no effect of cort on GR\(^{NPCKO}\)s (Fig 4d).

**Performance in contextual fear discrimination is unaffected by GR\(^{NPCKO}\)s.**

Lastly, to examine if GR signaling in abGCs plays a role in cognitive function, we tested mice in a contextual fear discrimination paradigm (Fig 5a). Whereas adult neurogenesis is both necessary and sufficient in improving contextual fear discrimination\(^2\)\(^{266,315}\), and cort is importantly involved in modulating fear\(^2\)\(^{226}\), we sought to examine whether GR mediates this effect in the abGCs. On day 0, mice were conditioned to associate a particular context with a foot shock (Fig 5a). Over the next 9 days, freezing behavior was measured in the context consistently associated with a foot shock, which reinforced this association daily, and a similar context never associated with a foot shock. All mouse groups showed significantly greater freezing over time in the shock-associated context (A), indicating successful learning of this association (p<0.0001, \(F\)\(_{(5,160)}=58.62\), repeated measures two-way ANOVA, data batched to account for missing values) with no differences between groups (Fig 5b). However, this association extended to the unconditioned context (B) since percent freezing values increased over time as well (p<0.0001, \(F\)\(_{(5,160)}=15.05\), repeated measures two-way ANOVA, data batched to account for missing values), suggesting low pattern discrimination (Fig 5c). Since mice appeared to reach a learning plateau after 9 days of training, we added a rest day (no training) as a challenge, and then resumed three more days of training. Although percent freezing in context B increased over time, each experimental group of mice was able to discriminate the contexts overall, as indicated by repeated measures two-way ANOVA (Fig 5d-g). Interestingly, only our GR\(^{NPCKO}\)s on cort treatment had a significant interaction effect of time and context when analyzed within group (p=0.039, \(F\)\(_{(11,156)}=1.93\)), although this is not significant when analyzed across groups (Fig 5g).
Further within group analysis using Bonferroni post-hoc tests showed a significant freezing difference between contexts on day 13 within untreated controls (Fig 5d), day 9, 12, and 13 within cort-treated controls (Fig 5e), day 4, 8, 12, and 13 within untreated GR\(^{NPCKO}\)s (Fig 5f), and day 12 and 13 within cort-treated GR\(^{NPCKO}\)s (Fig 5g).

Although all mice appeared able to learn the contextual fear association, and discriminate contexts within group, this ability was not significantly different between groups (Fig 6). The ability to discriminate these environments was measured per day with the following ratio: the difference in percent time spent freezing in chamber A (foot shock) versus chamber B (neutral) divided by the sum of percent time spent freezing in both chamber A and B. Repeated measures three-way ANOVA revealed a significant effect of day (p=0.0013, F\(_{(11,33)}=2.87\)), but no effect of genotype (p=0.218), cort (p=0.965), nor an interaction effect (p=0.096) when comparing discrimination ratios across groups (Fig 6a-b). Comparing just the effect of cort on control mice revealed a significant interaction between day and cort treatment (p=0.0002, F\(_{(11,17)}=3.52\)), repeated measures two-way ANOVA), which appears to be driven by the last 2 days since analysis of days 1-9 showed no effect (Fig 6a). Three-way ANOVAs comparing the effects of context, genotype, and cort across individual days revealed an overall difference in context for days 2-13 (p<0.05), however again, no effect between groups. Thus, although Bonferroni post-hoc analysis showed no individual group differences in discrimination between all groups on day 4 (Fig 6c), cort-treated controls with significant difference in discrimination by day 9 (Fig 6d), cort-treated controls and all GR\(^{NPCKO}\)s groups with significant differences in discrimination by day 12 (Fig 6e), and all groups with significant differences in discrimination by day 13 (Fig 6f), these individual group differences are not more significant than the differences in contextual freezing for other groups. It should also be noted that these scores (Fig 6c-f) are compared between groups per day, while scores in figure 5 (d-g) are compared within group across time. Overall, these results suggest all groups had comparable abilities in discriminating similar contexts.

### 3.4 Discussion

To distinguish GR signaling pathways within abGCs that mediate behavior from those throughout the nervous system, we generated an inducible GR mutant mouse line in which nestin-driven, Cre-mediated inactivation of the GR gene takes place selectively in NPCs. By waiting until mice were adults to induce gene inactivation, we were able to examine how adult neurogenesis is behaviorally relevant to hippocampal functioning. We found that the functional significance of GR gene inactivation in abGCs was not apparent in our tests unless mice are experiencing chronic cort exposure. In all tests administered, GR\(^{NPCKO}\) mice exhibited normal behavior under basal conditions and were indistinguishable from their control littermates. It was not until mice were experiencing chronic stress-like conditions that differences in behavioral phenotypes emerged. Whereas chronic cort treatment induced an anxious phenotype in control mice, it had a reduced effect on anxiety-like behaviors in GR\(^{NPCKO}\)s. All mice behaved similarly in locomotor and open field tests, which measure physical activity in a novel environment, indicating that changes in anxiety-like behaviors were not due to differences in their motivation or ability to explore. These results highlight the role of adult neurogenesis as being an environmentally responsive adaptive mechanism.

The selective disruption of GR signaling pathways in abGCs significantly reduced fearful behavior when mice were under chronic cort exposure. This suggests that abGCs can attenuate
anxiety in novel environments via suppressed GR activity or even that perhaps anxiety in novel environments can be induced thru GR signaling in abGCs. The anxiety-reduced phenotype of cort-treated GR<sup>NPCkO</sup> mice was measured in three different behavioral paradigms based on the natural avoidance behavior of mice: light/dark box, elevated Omaze, and NSF. In the light/dark box, cort-treated controls took significantly longer to exit the dark compartment, spent significantly less time in the lit compartment, and had significantly less number of 4 paw entries into the lit portion compared to untreated controls. These are all typical fearful behaviors found in an anxious phenotype (i.e. avoiding bright, open spaces). Interestingly, the behavior of cort-treated GR<sup>NPCkO</sup> mice was indistinguishable from untreated mice. This anxiolytic effect was confirmed in the elevated Omaze test. Again, mice are subjected to a novel environment in which although they have a natural tendency to explore, they prefer avoiding bright, open, and now elevated spaces. Similarly, cort-treated controls took longer to exit the enclosed arms (albeit not significantly), spent significantly less time in the open arms, and had significantly less number of 4 paw entries into the open arms compared to untreated controls. Once again, the behavior of cort-treated GR<sup>NPCkO</sup> mice was indistinguishable from untreated mice. Lastly, we saw similar avoidance behavior in NSF. Cort-treated controls took significantly longer to approach the brightly lit, center platform containing food, whereas GR<sup>NPCkO</sup> blocked this cort-induced behavior. Although GR is expressed in many hippocampal cells, these results suggest that the cort-activated GR signaling specifically in abGCs can induce anxiety-like behavior, or at least, can attenuate anxiety-like behavior when the signaling is deficient.

It is worth noting that this effect was not robust in all anxiety-relevant measures. For example, it was not seen in the latency to eat, which could be due to mice not being hungry enough (although all mice ate immediately post-test) or this measurement of the NSF test could be more related to depression-like symptoms rather than anxiety. Indeed, several studies have used this test as a measure of antidepressant efficacy. Interestingly, we also did not see this effect in the open field test. Although often used as a measure of physical activity and motivation, we did not see reduced time spent in the center of the open field, whereas we do see that effect in other anxiety-relevant tests. This may be due to experimental procedures, such as the size of the arena used, the length of the experiment, and what area we decided constitutes as being in the center. These changes may elucidate behavioral differences. Additionally, although there is an apparent trend of GR<sup>NPCkO</sup> blocking the effect of cort on anxiety-like behavior, there was only a statistically significant interaction effect (genotype x cort) for the number of 4 paw entries in the light/dark box, and near significant interaction effect for the number of 4 paw entries and time spent in the open arms for the elevated Omaze. An increased sample size may help clarify the strength of the effect; otherwise changes in the testing procedures or cort treatment might reveal stronger differences. Be that as it may, it could be that GR signaling in abGCs contributes finer aspects to these behaviors, and are not solely responsible for anxiety in its entirety. The contribution of adult neurogenesis to these behavioral nuances will need to be disentangled over time.

Future studies on GR<sup>NPCkO</sup> blocking anxiety-relevant behaviors will be necessary to demonstrate whether this effect is truly regulated by a cell-specific signaling pathway. It is possible that it is not abGC-specific inactivation of the GR gene attenuating cort-induced responses. First, it could be just overall reduced GR signaling in the DG that drives our effect, irrespective of cell type. Unfortunately, the ideal control group addressing this concern would be GR gene inactivation in ~10% of matured granule cells, a difficult manipulation. Secondly, there could be other signaling pathways, such as altered MR activity or a decrease in NMDA.
receptors, that also result in the same effect of blocking cort-induced anxiety-like behaviors. Thus, GR gene inactivation may not be necessary to induce or attenuate these fearful behaviors. Thirdly, it could be any disruption in hippocampal signaling can mediate anxiety-related behaviors. Lastly, there could indeed be a strong functional effect of GR gene inactivation in NPCs, however for the effect to be clearly evident, there needs to be 100% GR gene inactivation in all hippocampal stem/progenitor cell types. More thorough and advanced techniques will help address these issues for the future. In any case, our results demonstrate that reduced GR signaling in abGCs is sufficient for suppressing cort-induced anxiety-like behaviors.

Other studies have supported the idea that either GR or neurogenesis plays a role in anxiety-related behaviors. It is well documented that chronic elevation of cort levels results in an anxious phenotype. Exogenous administration of cort has shown to increase latency to feed in NSF, decreased time in center of open field, decreased time in open arms of elevated plus maze, and decreased time spent in lit compartment of light/dark box. Whereas elevated cort levels activate GR signaling, further evidence that this anxious phenotype is via GR was demonstrated in mice with brain-specific GR gene inactivation which displayed increased latency to exit the dark compartment and decreased time spent in the lit compartment of the light/dark box. Additionally, these neural GR gene inactivated mice had reduced number of entries and more time spent in the open arms of the elevated Omaze.

Soon after discovering elevated cort can inhibit proliferation of newborn neurons in the hippocampus, Gould and colleagues proposed that since high levels of cort are often found in patients with affective disorders, perhaps the changes in hippocampal neurogenesis were regulating mood. The research that addressed this proposal has been controversial. Many experiments manipulating neurogenesis saw no effect on anxiety-like behaviors, although this could be due to techniques used to target neurogenesis. Other studies, however, found that altered neurogenesis did result in an anxious phenotype. In one study, mice with a profound decrease in NPCs showed decreased number of entries and time spent in the lit portion of the light/dark box.

Since elevated cort can both suppress neurogenesis and promote an anxious phenotype (via GR), and some studies found that suppressed neurogenesis results in an anxious phenotype, we questioned whether the cort effect on neurogenesis was driving this anxious phenotype. By inactivating the GR gene in nestin expressing cells, and hence abGCs, we did not find a change in anxiety-like responses. It was only after mice were chronically exposed to excess cort did they begin to exhibit an anxiolytic effect of their mutation. This is the first study we know of that has examined mood regulation acting through GR in abGCs. Overall, our findings advance our understanding of not only the functional contributions of abGCs, but also the mechanisms underlying anxiety-relevant responses.

Aside from anxiety, we also investigated how GR signaling in abGCs affects depression-like symptoms. While it appears that elevated cort and adult neurogenesis are relevant to depression, their direct relationship with depression is not clear. Half of people suffering from depression were found to have hypercortisolism and normalizing their cort levels not only led to symptom relief, but also the extent of which predicted relapse. Likewise, half of Cushing patients have depression and correcting their hypercortisolism reduced their psychopathology. Co-treating with antidepressants and anti-glucocorticoids improves both the time it takes to treat and its efficacy in psychotic depression, and cort synthesis inhibitor, metyrapone, is found to be effective in treating this affective disorder as well. Oddly though, dexamethasone (dex), a
GR agonist, and RU38486, a GR antagonist, were both found to separately alleviate symptoms of depression.\textsuperscript{355,356} Dex, however, was not only found to poorly penetrate the brain, but also suppress endogenous cort, thus, it often acts as an antiglucocorticoid in the brain.\textsuperscript{337–339} The forced swim test has demonstrated both reliable and predictive validity on the efficacy of all antidepressants effective in humans for the last several decades.\textsuperscript{10,227,242,340,341} This test correlates increased immobility, and decreased activity and latency to immobility with a depressive phenotype.\textsuperscript{227,340} It was then discovered that x-ray ablation of hippocampal neurogenesis prevented antidepressant efficacy on this test\textsuperscript{10,287,329} and ever since, it has been proposed that antidepressants function through the abGCs.\textsuperscript{10,126,342} Additionally, it was thought that since the hippocampus is often found reduced in depression, it may be due to impaired neurogenesis.\textsuperscript{279} Although evidence appears to link neurogenesis and depression together, most studies do not find reduced neurogenesis causes depressive behavior,\textsuperscript{91,255,260,274,275} and state that they are not well correlated.\textsuperscript{255,343}

To our knowledge, we are the first to examine this relationship between glucocorticoids and neurogenesis and their effect on depression-like behavior. Although glucocorticoids are believed to play a causative role in the etiology of depression,\textsuperscript{20,344} the amount and duration of cort administration are key determinants for the expression of a depressive phenotype.\textsuperscript{197} Studies have found increased immobility in the FST after treating mice orally with 25\(\mu\)g/mL of cort for 2 weeks\textsuperscript{345} and through their drinking water with 20\(\mu\)g/mL for 2 months, although this latter experiment was in 3-week-old mice\textsuperscript{346}. We chose to chronically administer low dose cort (35\(\mu\)g/mL) through drinking water (to naturally and more ecologically mimic the circadian and pulsatile nature of cort release and the stress response) over 6–7 weeks prior to and throughout testing. Although there was a trend of increased immobility and decreased latency to float, neither measurement was significantly different from nontreated controls. Either cort was at too low of a dose for not enough time or exogenously administered cort is not a reliably effective method to induce depression-like behavior in the FST. It could also be due to the mouse line we used, whereas Balb/c mice are typically more prone to exhibiting depressive-like responses.\textsuperscript{347} Hen and colleagues found similar results to ours after treating similar mouse species with cort and not finding an increase in depressive-like behaviors in the FST\textsuperscript{11}, however only total mobility was reported, and not latency to immobility or immobility alone. They did find this treatment protocol of cort increased the latency to feed in NSF, another test used to demonstrate affective impairment,\textsuperscript{272} although we did not find the same effect in our study. Interestingly, even though mice treated with cort did not exhibit a depressive phenotype compared to untreated mice, mice treated with cort and fluoxetine, an antidepressant, showed significantly greater mobility compared to untreated and cort-treated mice.\textsuperscript{11} This underlies that actual antidepressant efficacy is independent from the etiology underlying depression, or in other words, fluoxetine may be masking a depressive phenotype only by inflating opposing behaviors.

Moreover, neither GR\textsuperscript{NPCKO} nor GR\textsuperscript{NPCKO} under cort-treatment had any significant effect on depression-like behaviors. These results may be different if mice were initially exhibiting a depressive-like state, or if we had examined how they respond to antidepressants. It was proposed that the functional importance of abGCs may only be revealed when the animal is in a depressive-like state.\textsuperscript{348} In any case, our results appear to support the majority of research (76% of current studies) demonstrating that manipulations of abGCs have no effect on mood-related tests in naïve rodents\textsuperscript{91}. It is worth noting that even though our GR\textsuperscript{NPCKO}s on cort demonstrate anxiolytic, but not antidepressant-like behavior, depression and anxiety-related behaviors may
indeed be differentially associated with the hippocampus\textsuperscript{349} as they are distinct diagnostic disorders\textsuperscript{91}.

Next, we examined hippocampal-dependent cognitive functions that may be affected by GR signaling in abGCs. The acquisition of contextual information associated with an unconditioned stimulus has been regarded as a hippocampal-dependent ability, involving the DG and amygdala\textsuperscript{350,351}. This ability can be measured in a task known as contextual fear conditioning, in which a rodent is placed in a novel environment to explore and receives a foot shock soon thereafter in this environment. Typically 24 hours later, animals are replaced in this environment to gauge their memory of the previous event. Evidence of this association is exhibited as freezing behavior, an adaptive species-specific defense mechanism in anticipation of an aversive event\textsuperscript{352}. Both cort and GR activation are involved in fear conditioning\textsuperscript{214} such that stress- or cort-mediated activation of GR promotes memory consolidation, particularly due to it being an emotionally arousing experience\textsuperscript{214,353,354}. Several studies have verified that chronic cort administration in both rats and mice enhance contextual fear conditioning\textsuperscript{219,237,326,355} and removal of cort through adrenalectomy impairs this behavior\textsuperscript{214,356,357}. Furthermore, it has also been demonstrated extensively that GR signaling affects freezing behavior in this context\textsuperscript{354,357–361} and blocking GR with an antagonist before conditioning impairs freezing behavior\textsuperscript{362}. At the same time, evidence has suggested that the cells mediating this function are granule cells in the dorsal DG, such that activation or inhibition of these cells impairs contextual encoding\textsuperscript{317}. Some studies have narrowed this cell type to the abGCs because arrest of NPCs through either X-irradiation or genetic ablation techniques has significantly impaired contextual fear conditioning\textsuperscript{255,256,309,328,363–366}. However, other studies have not found NPCs as mediators in contextual fear conditioning\textsuperscript{12,253,254,266,367,368}. For example, neither arrest of neurogenesis through x-irradiation\textsuperscript{12} or genetic ablation\textsuperscript{253,262,266}, nor enhanced neurogenesis\textsuperscript{12} affected freezing behaviors in rodents compared to controls. This conflicting data may be explained though by discrepancies in the experimental design and NPC targeting technique\textsuperscript{367}. For example, only high-dose irradiation, and not a low dose, impaired fear conditioning in one experiment\textsuperscript{369}; another experiment demonstrated only single-trial, and not multiple trial conditioning, is affected by neurogenesis arrest\textsuperscript{327,328}; furthermore, this reduced conditioning effect is only seen after at least 4-6 weeks have passed post-arrest\textsuperscript{327}. Thus, it appears that the effect of cort, via GR, in either the dorsal DG or amygdala can regulate the association of contextual and aversive information, and it is possible that abGCs are necessary for this function as well.

Thus, we decided to investigate whether GR signaling in abGCs that were at least 6 weeks old were involved in contextual fear conditioning. We found that none of our mice had impaired fear conditioning through our manipulations. Furthermore, in our control mice, cort alone enhanced the rate of learning as seen by significantly increased freezing behaviors over time. This effect was not seen between our untreated and cort-treated GR\textsuperscript{NPCKO} mice, but there was not a significant interaction for this effect compared with controls. Also, although both GR\textsuperscript{NPCKO} groups exhibited less freezing behavior than controls, it was not a significant effect. Our results verify other studies showing that cort enhances associative memory in the contextual fear conditioning paradigm, although our experimental design cannot separate whether it is enhanced acquisition or retrieval that drives the increased freezing behavior. Furthermore, we demonstrate that reduced GR signaling in abGCs is not sufficient enough to impair this type of learning and memory, and although it appears there is a reduced effect of cort treatment on
GR\textsuperscript{NPCKO} s compared to controls, this did not prove to be significant. Our data suggests that cort promotes contextual fear conditioning, but not through GR signaling in the abGCs.

Our results are in conflict with another study that investigated the role of GR signaling in NPCs\textsuperscript{218}. This latter study used lentiviral constructs to deliver short hair-RNA to knockdown GR in the DG and found impaired freezing behavior\textsuperscript{218}. Although infected cells appear to be mostly NPCs, lentivirus is not specific for NPCs. Thus, this design may have affected a wider population of cells than ours. Importantly, this study also used a different mouse line than ours, specifically they used Balb/c mice, which are known to be more sensitive to stress, have increased anxiety-like behaviors, and enhanced contextual fear conditioning compared to other strains\textsuperscript{142,370–372}. This appears to be an important distinction; one study found ablated neurogenesis affected novel object preference, another hippocampal-dependent memory task, only in 129/SvEv mice and not C57B1/6J mice\textsuperscript{327}. Additionally, Fitzsimons et al (2013) used 0.4mA for a foot shock\textsuperscript{218}, whereas our shock was 0.75mA. It may be that our aversive stimulus was too intense to see the subtle contributions of GR signaling in abGCs to this behavior. Overall, there seems to be many differences between our mice, experimental designs, and perhaps even scoring strategies that result in decreased freezing behaviors for their GR mutant mice but not in our mice.

Lastly, another cognitive ability that we tested was contextual fear discrimination. This test is often used as a measure of the ability for pattern separation, a skill highly associated with hippocampal neurogenesis\textsuperscript{268,373}. Pattern separation is described as a neurophysiological computation that disassociates similar patterns of neural activity such that similar memories (representations) can be distinguished from one another\textsuperscript{315}. Although this ability is difficult to measure because of current limitations in our understanding of how this information is neurally represented\textsuperscript{374}, it is thought to be exhibited in the contextual fear discrimination task\textsuperscript{315,373,375}. This task assesses a rodent’s ability to disambiguate two highly similar contexts from one another by measuring its fear responses in each context. This was initially discovered to be a hippocampal-dependent ability because electrolytic lesions of the hippocampus resulted in higher freezing behaviors in the nonshock context, and thus, less discrimination between the two contexts\textsuperscript{376}. Further investigation showed that reduced neurogenesis impaired discrimination of similar contexts\textsuperscript{265,266,377}. Ablated neurogenesis from X-irradiation resulted in reduced discrimination of similar spatial locations in radial arm maze\textsuperscript{265} and overexpression of the apoptotic Bax protein in NPCs reduced the difference in discriminative freezing responses in contextual fear discrimination\textsuperscript{266}. Correspondingly, enhanced neurogenesis by knocking out the Bax protein in NPCs improved contextual fear discrimination\textsuperscript{12}. Whereas cort enhances fear conditioning, we examined whether cort had a similar effect on fear discrimination. Additionally, we investigated whether this ability was mediated by GR signaling in abGCs. Comparing discrimination ability across groups showed that all mouse groups performed similarly in the contextual fear discrimination paradigm. This was not due to impaired association learning, as all groups had increased freezing levels in both contexts over the course of the experiment. This is also not due to impaired discrimination as individual analysis of each group revealed an overall significant difference in freezing levels per context. Although this analysis showed specific days in which groups began consistently demonstrating a significant difference in freezing between contexts, this does not suggest particular groups were discriminating significantly sooner when compared to other groups. It only demonstrates each group was successful at discriminating contexts at some point during the course of the experiment. Examining individual days across groups highlights this result. Here we found that
even though there may be a significant difference in freezing levels between contexts for one group on one day, does not mean that this difference is significantly different from the difference in freezing levels per context for another group. Thus, although all mice learned the contextual fear association and could discriminate the contexts at some point during the course of the experiment, their ability to discriminate was not significantly different from each other. This result is similar to previous research studying the effect of chronic immobilization stress on mice with Nr1 knockout in their NMDA receptors (NMDAR) in their CA3 neurons compared to controls. In this study, both transgenic mice and controls were equally capable of discriminating a novel context after chronic stress. Similarly, chronic cort did not appear to differentially impact our GRNPCKO's compared to controls. This further strengthens evidence that GR signaling in abGCs is not prominently involved in contextual fear discrimination.

Whereas neurogenesis has been shown to be necessary for normal pattern separation, the lack of effect of GRNPCKO's on contextual fear discrimination could indicate other mechanisms are more critical for this type of memory processing. In particular, NMDA receptors seem to be important for pattern separation. One study that knocked out the Nr1 subunit in NMDAR in the granule cells of the DG found that while mutant mice had normal fear conditioning responses, they had increased freezing behavior in the nonshock context, and thus reduced discrimination. Also, knock out of the Nr2B subunit of the NMDARs specifically in abGCs resulted in impaired fear discrimination. These NMDARs regulate the glutamatergic and calcium signaling in abGCs, which can lead to long-term changes in synaptic strength and connectivity. Thus, although the NR2B knockout mouse model did not affect cell survival, it did reduce dendritic complexity. This may affect the DG/CA3 circuit that facilitates pattern separation.

It is possible that our experimental design did not capture the role GR signaling plays in abGCs as well. One aspect that may make a difference is the timing between context presentations. A recent study on pattern separation found that odor discrimination was impaired by lesions in the ventral DG, but only if the two similar odors were presented 60 seconds apart, since there was no effect if they were presented within 15 seconds from one another. This implies that the DG is important for discriminating similar patterns within a particular time frame. It could be the case that if we altered the length of time between context exposures, it would reveal deficits or improvements in pattern separation for our GRNPCKO mice. Other possible design changes in our experiment that could exhibit behavioral differences include reducing the intensity of the foot shock, and increasing the dissimilarity between the contexts, since mice appeared to be freezing too often in the nonshock context. These changes could help elucidate whether GR signaling in abGCs is truly involved in contextual fear discrimination, or whether other mechanisms, such NMDAR signaling, are a more relevant pathway to continue investigating.

Adult neurogenesis represents a form of neural plasticity that has been conserved across species and may not only influence human brain functioning, but also be manipulated for purposes of repair. Over the last decade, much research has been dedicated to understanding the functional importance of adult neurogenesis. While evidence has suggested roles in mood regulation and memory processing, results have been inconsistent perhaps due to varied experimental designs that examine these complex cognitive abilities too broadly. Whereas stress, via cort signaling, impacts the behaviors of newborn neurons, and cort, via GR signaling in the brain, regulates similar emotional and cognitive processes as proposed relevant to adult neurogenesis, we investigated how chronic cort exposure and GR signaling specific
to abGCs affects anxiety-related behaviors, depression-like phenotypes, contextual fear conditioning, and pattern separation in a contextual fear discrimination paradigm. Our results showed that our chronic cort treatment increased anxiety-like behaviors, did not induce a depressive phenotype, promoted contextual fear conditioning, and did not significantly influence contextual fear discrimination. Reduced GR signaling in abGCs had no impact on any of the behavioral and cognitive functions measured. Interestingly, the functional contributions of our GR\textsuperscript{NPCKO} mice were not revealed until mice were subjected to chronic cort exposure. While no changes were seen in depression-like behaviors, fear conditioning, or contextual discrimination, reducing GR signaling in abGCs significantly attenuated cort-induced anxiety-like behaviors to novel environments.

These behaviors are symptomatic to anxiety disorders, the most common types of mental illness in the US\textsuperscript{383}. While the underlying psychopathology remains elusive, anxiety disorders remain a pervasive problem in society, affecting 25% of the population\textsuperscript{384} and costing the US government upwards of $45 billion per year\textsuperscript{385}. It is clear that reducing the outcome of this disorder would be a major benefit to society. Additionally, a substantial number of people receive repeated cort injections and glucocorticoid treatments without fully understanding the neurological impact. Our results not only reveal functional contributions of cell-specific GR signaling, but also highlight the relevance of abGCs in responding to environmental changes. Expanding our knowledge of these mechanisms and their functional relevance, both what they do and do not regulate, is critical for better understanding brain functioning, as well as developing more effective therapeutic strategies and novel neuropharmalogical targets.

3.5 Materials and Methods

**Nestin-CreERT\textsuperscript{T2} and GR\textsuperscript{loxP/loxP} transgenic mice.** A mouse model with inducible gene inactivation was used to remove GRs from NPCs and their progeny in the adult hippocampus (mouse line is described in more detail in CHAPTER 2). Briefly, mice homozygous for the Gr\textsuperscript{loxP} allele\textsuperscript{239} were mated with the bitransgenic line expressing the CreERT2 recombinase gene under control of the nestin promoter\textsuperscript{84} and a R26R-YFP reporter strain (Jackson Laboratories). This mating required two generations to obtain mutant animals (GR\textsuperscript{loxP/loxP};Tg(NesCre\textsuperscript{ERT2});Tg(R26R-YFP)), thereafter designated as GR\textsuperscript{(NesCreERT2)}, and a further one to amplify the colony. Activation of CreERT2 is induced by tamoxifen; this leads to Cre recombinase excising the floxed GR gene selectively in nestin-expressing cells, thus creating a temporally controlled nestin-specific loss of GR function and active YFP expression. Vehicle-induced GR\textsuperscript{(NesCreERT2)} mice were used as controls. Vehicle- and tamoxifen-induced GR\textsuperscript{loxP/loxP} mice were also tested alongside controls to verify no effects of Cre or tamoxifen. All lines were maintained on a C57BL/6 background and only male mice were used. Ten mice from each group were used in experiment; four mice were excluded from analyses, however, due to post-mortem genetic verification. Animals were bred and raised under a 12h light/dark cycle; temperature was 22±\textdegree C and humidity 60±5%. Food and water were supplied \textit{ad libitum}. Experiments were performed in accordance with French (Ministere de l’Agriculture et de la Forêt, 87-848) and European Economic Community (EEC, 86-6091) guidelines for the care of laboratory animals.

**Tamoxifen induction.** Tamoxifen (Sigma T5648) was light protected and dissolved in 10% ethanol and suspended in 90% sunflower seed oil\textsuperscript{84}. Mice received daily injections at 180mg/kg,
i.p. for 5 days when they were 5-6 weeks old. Control mice received vehicle (sunflower oil). Mice receiving tamoxifen were only group housed with other tamoxifen-receiving mice; likewise, only vehicle (oil)-receiving mice had vehicle-receiving littermates. This guarded against possible cross-contamination of tamoxifen.

**Corticosterone treatment.** Corticosterone (Sigma C2505) was prepared as described previously\(^\text{11}\). It was dissolved at 35 µg/mL in a water solution of 0.45% (wt/vol) hydroxypropyl-beta-cyclodextrine (Sigma 332593) by sonication for two hours. Cort treatment was given to mice in opaque water bottles to protect it from light, changed twice a week, and available *ad libitum*. Behavior testing started after 4 weeks of cort treatment and treatment continued throughout all behavioral testing.

**General behavioral apparatus and procedures**

**Locomotor.** Mice were introduced into circular chambers (4.5 cm width, 17 cm external diameter) crossed by four infrared captors (1.5 cm above the base) placed at every 90° (Imetronic, Bordeaux, France). Locomotor activity was measured by number of quarter turns every 5 minutes for 5 hours. Quarter turns were counted when mice interrupted two successive beams, thus having traveled a quarter of the circular corridor.

**Open field.** Motor activity was quantified over 9 minutes in a 100x100x30 cm\(^2\) white Plexiglas field with the center brightly illuminated (~980 lux) compared to the corners (~100 lux). The computer recorded the session and defined grid lines that divided the box into 9 equal squares, with the center square consisting of four lines 33.3 cm from the wall. The mice were acclimated to the room at least 15 minutes prior to testing and the open field was cleaned between mice.

**Light/dark test.** The light/dark test was conducted in a chamber measuring 45x20x25 cm. Less than half of the chamber is an enclosed opaque dark box (black PVC) with a small opening to allow passage (5 cm x 5 cm) into the lit portion (white PVC). The lit portion was approximately 50 lux, while the dark portion was approximately 2 lux. The mice were acclimated to the room at least 15 minutes prior to testing and chambers were cleaned between mice. Mice were placed in the dark chamber and testing began once the lid to the dark chamber was closed. Mice were given 9 minutes to freely explore both chambers in a quiet environment.

**Elevated Omaze.** The testing apparatus consisted of an elevated (80 cm) opaque plastic platform in the shape of an “O” (70 cm diameter). The ring was divided into 4 alternating open (100 lux) and closed (60 lux) arms. Closed arms contained 8 cm high walls. Mice were placed facing within the closed arm and allowed to freely explore the platform for 6 minutes while being recorded. A mouse was considered to exit or enter into arms when all 4 paws were introduced. Platform was cleaned between mice and any animals that fell off the platform during testing were excluded from analyses.

**Novelty-suppressed feeding.** Mice were food-deprived for 16 hours prior to testing and their weights were recorded pre- and post-deprivation. The testing apparatus consisted of a 45x45x30 cm box covered with approximately 2 cm of fresh bedding. Two food pellets were secured to a white paper platform in the center of the arena. A light was shone above the center onto the food as well, such that the box center was 800 lux and the corners were 100 lux.
were placed in the corner of the box and recorded for 30 minutes. Once a mouse ate, defined as sitting on its haunches and biting the pellet with its forepaws, or reached the 30 minutes, the center light was turned off and they were given 6g of food in the box corner and 5 minutes to continue feeding. After this, mice were reweighed and returned to their homecage. Boxes were cleaned in between mice.

*Porsolt forced swim.* As described previously\textsuperscript{241}, mice were placed into clear cylinders (30cm tall, 9.5cm diameter, filled with 15cm of 22°C-24°C water) and videotaped for the entire session. Immobility, defined as floating with minimal limb movement to keep head afloat, climbing behavior, and latency to float were measured for 6 minutes; only the last 4 minutes were analyzed.

*Contextual fear discrimination.* Mice were exposed to two contextually similar chambers, but only one of which was always paired with a two second shock (0.75mA). In this task, they were evaluated for their ability to discriminate between the two environments, as measured by their freezing behavior. Both chambers were essentially equal in appearance, including a grid floor, but with a few differences: chamber A was cleaned between mice (grid and waste tray) with 75% ethanol, and had closed doors with a houselight and fan, metal walls, and a lemon scent in the bedding under the grid floor, and the mice were transported there in an opaque enclosed bucket with a layer of bedding; chamber B was cleaned with non-ethanol cleaner, and had open doors (natural light), no running fan, blue plastic walls, and an orange scent in the bedding under the grid floor, and mice were transported there in a clear open bucket with no bedding. The experiment proceeded as follows: on day 0, mice were placed in chamber A. They were given 185s to explore, then received the shock, and were removed 15 seconds later and returned to their homecage. On day 1, mice were re-exposed to chamber A as in day 0. Two hours later, they were placed in chamber B. In chamber B, they were given 185s to explore and then returned to their homecage. For days 2-13, mice were exposed to both chambers in a random manner with a 2 hour delay between chambers. There was no training (rest) for either chamber on day 10. Mice were recorded in both chambers and freezing, defined as complete stillness except for necessary breathing movements, was measured every 5 seconds for 3 minutes. All behavior experiments were performed during the light phase of the light/dark cycle. Mice were handled for at least 5 minutes 3 days prior to all behavior testing.

**Statistical analysis.**

Results are expressed as means±s.e.m. Statistical analysis was performed using two-way analysis of variance (ANOVA) for all tests except contextual fear conditioning and discrimination, which used a repeated measures three-way ANOVA. Analyses were followed by Dunnett’s, Newman-Keuls, or Bonferroni post-hoc tests for pairwise comparisons, as appropriate. * indicates p<0.05.
3.6 Figures and Legends

Fig 1. Normal levels of activity and motivation in cort-treated and GR$^{NPCKO}$ mice. (a) Timeline for experimental protocol. (b) There were comparable levels of general locomotor and exploratory activity in a novel environment among GR$^{NPCKO}$s and controls, as measured by the actimeter. (c) There was no difference among groups on all examined measures of the open field protocol.
Figure 1

a) Timeline for the experiment:
- 5-6w: Postnatal Development
- 2w: Postnatal Development
- 4w: Corticosterone Treatment
- 20-26w: BrdU Injection
- pn: Postnatal
- Tamoxifen
- Recovery
- Behavior Testing
- Histology
- Forced swim test
- Open field
- Elevated plus maze
- Light/dark box
- Feeding

b) Locomotion

Total 

0 500 1000 1500

Tamoxifen
- - + + - +
Corticosterone
- + - + + +
CreERT2
+ + + + - -

Distance traveled (m)

0 2 4 6 8 10

Tamoxifen
- - + + - +
Corticosterone
- + - + + +

Velocity (cm/sec)

0 5 10 15 20

Tamoxifen
- - + + - +
Corticosterone
- + - + + +

Time spent in center (%)

0.0 0.5 1.0 1.5 2.0 2.5

Tamoxifen
- - + + - +
Corticosterone
- + - + + +
Fig 2. Anxiety-related behavior in GR$^{NPCKO}$ mice treated with cort. (a) Cort treatment preceding novelty-suppressed feeding significantly increased the latency to approach food for control mice, but not for GR$^{NPCKO}$s. However, cort-treatment similarly affected latency to eat for control mice compared to GR$^{NPCKO}$s. (b) Cort-treated control mice exhibited significantly increased latency to enter the aversive lit compartment compared to GR$^{NPCKO}$s. Similarly, cort-treated control mice exhibited a trend of decreased time spent in the lit compartment, but no effect of cort was seen on GR$^{NPCKO}$s. Likewise, the number of 4 paw entries showed a significant interaction effect between genotype and cort treatment. There were no differences between GR$^{NPCKO}$s with or without cort-treatment for all parameters measured in the light-dark box. (c) Cort-treatment also affected time in open arms and 4 paw entries in the elevated Omaze for control mice, but not GR$^{NPCKO}$ mice. There were no significant differences in latency to enter open arms. *, p<0.05 using Dunnett and Neuman-Keuls’ post-hoc analyses, as appropriate. Results are expressed as mean±s.e.m (n=7-10 per group).
Figure 2

a  Novelty-suppressed feeding

b  Light-dark box

c  Elevated maze

Latency to approach (s)  Latency to eat (min)  Mec not feeding (%)  Time

Latency to exit (s)  Time in lit (s)  # 4paw entries

tamoxifen  corticosterone  tamoxifen  corticosterone  tamoxifen  corticosterone  tamoxifen  corticosterone
Fig 3: GR\textsuperscript{NPCKO} mice do not express depression-like behaviors. Mice underwent the Porsolt forced swim test and time spent (a) immobile, (b) active until initial float, and (c) climbing was measured. Neither cort treatment nor GR\textsuperscript{NPCKO} had a significant effect on mice behaviors. Results are expressed as mean±s.e.m.

Figure 3
Fig 4: Freezing behavior of mice during a contextual fear conditioning paradigm. (a) Experimental design of contextual fear conditioning paradigm. On day 0, mice were exposed to chamber A where they received a 2s-0.75mA foot shock (denoted by the lightening bolt) after 185s. The following day mice were re-exposed to chamber A and the percentage of time freezing was measured. (b-c) On day 0, all groups showed negligible levels of freezing in context A prior to foot shock. Contextual fear was measured 24 hours after the conditioning from day 0. All groups showed significantly elevated freezing in the conditioned context. (b) Chronic cort treatment improved learning. (c) GR\textsuperscript{NPCKO} s showed no differences in freezing over fear conditioning protocol regardless of cort treatment. Similarly, untreated GR\textsuperscript{NPCKO} s had comparable scores to untreated controls. (d) Chronic cort treatment appeared to only enhance fear conditioning between control mice.
Figure 4

(a) Day 0

(b) CONTROLS

(c) GR^{NPCKO}\_S

(d) Difference in % freezing (day1-day0)

| tamoxifen | - | + | - | + |
| corticosterone | - | - | + | + |
Fig 5: Freezing behavior of mice during a contextual fear discrimination paradigm. (a) Experimental design of contextual fear discrimination. On day 0, mice were only exposed to chamber A where they received a 2s-0.75mA foot shock (denoted by the lightening bolt) after 185s. Following, each day mice were exposed to both chamber A and B in a random order with a 2 hour homecage rest period in between from day 1-9. Mice had no training on day 10, and then continued training day 11-13. Chamber A always administered a foot shock, while chamber B was always neutral. Freezing was measured for the first 180s in both chambers each day. Measurements over time of the percent time freezing in chamber A (b) and chamber B (c) indicated that all mice were able to learn to associate fear with a contextual setting, though not differently. This was verified by repeated measures two-way ANOVA of each group, which also showed that overall percent freezing in chamber A was significantly greater than in chamber B for nontreated controls (effect of context, p<0.0001, F(1, 185)=31.23; effect of time: p<0.0001, F(11, 185)=5.91) (d), cort-treated controls (effect of context: p<0.0001, F(1, 204)=28.30; effect of time: p<0.0001, F(11, 204)=3.76) (e), nontreated GR_NPCKO’s (effect of context, p<0.0001, F(1, 183)=70.33; effect of time: p<0.0001, F(11, 183)=7.00) (f), and cort-treated GR_NPCKO’s (effect of context, p<0.0001, F(1, 156)=44.57; effect of time: p<0.0001, F(11, 156)=8.66) (g). Further analysis revealed specific days these contexts significantly differed within group. For nontreated controls, this was only on day 13 (d). For cort-treated controls, day 9, 12, and 13 showed significant differences (e). For nontreated GR_NPCKO’s, day 4, 8, 12, and 13 were significantly different (f). For cort-treated GR_NPCKO’s, freezing behavior was significantly different only on day 12 and 13 (g). Results are expressed as mean ± s.e.m. *, p<0.05; §, p<0.01; #, p<0.001.
Figure 5

a  Day 0  1  2  3  4  ....  9  11  12  13
2 hour delay

b  shock only
% freezing

Days

c  nonshock only
% freezing

Days

d  -tam/-cort
Freezing (%)

context A (training)
context B

e  -tam/+cort
Freezing (%)

context A (training)
context B

f  +tam/-cort
Freezing (%)

context A (training)
context B

g  +tam/+cort
Freezing (%)

context A (training)
context B
Fig 6: Contextual discrimination is not significantly affected by cort or GR^{NPCKO}. (a) Analysis of discrimination ratios for days 1-13 for controls (a) and GR^{NPCKO} s (b). There were no differences in the discriminating ability across 13 days between all groups (a-b). (c-f) Although scores of contextual discrimination were not significantly different between groups, average freezing score per context is depicted for all groups on individual days. There were no significant differences exhibited between levels of contextual freezing for any group on day 4 (c). However, by day 9, cort-treated controls were showing a significant contextual difference in freezing behavior (d). By day 12, this difference persisted in cort-treated controls, and was now found in nontreated and cort-treated GR^{NPCKO} s. This difference remained significant for these groups by day 13 and appeared in nontreated controls as well. Results are expressed as mean ± s.e.m. *, p<0.05; §, p<0.01; #, p<0.001.
Figure 6

(a) Discrimination ratio (a-b)/(a+b) over days for two conditions: No tam No cort and No tam + cort.

(b) Discrimination ratio (a-b)/(a+b) over days for +tam no cort and +tam + cort conditions.

(c) Day 4 freezing response for four conditions: -tam/cort, -tam/+cort, +tam/cort, +tam/+cort.

(d) Day 9 freezing response for the same four conditions as (c).

(e) Day 12 freezing response for the same four conditions as (c).

(f) Day 13 freezing response for the same four conditions as (c).
CHAPTER 4

DISCUSSION
OVERVIEW

4.1 Introduction to general discussion

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4.4 GR signaling in abGCs: implications for stress-related brain disorders

4.5 Future directions
4.1 Introduction to general discussion

Stress is one of the strongest and most ecologically relevant mediators of adult neurogenesis. It has been shown that this occurs through stress-induced elevations in corticosteroids, which act through GR to reduce proliferation and differentiation of NPCs (see CHAPTER 1). How GR signaling regulates this process and whether it has any impact on behavior or cognition has long been speculated but is still unknown. While many studies have shown that disrupted GR signaling impacts mood and memory functions, the functional impact of newborn neurons on brain function is still a hot controversial topic. This is due to different methods of ablating neurogenesis that only occasionally elicit behavioral deficits. To carefully manipulate the role of GR signaling within newborn neurons and investigate whether it can mediate behavior would necessitate newer animal models. Previous research on GR function using various pharmacological and genetic manipulations in animal models has been conducted. However, since GR influences a pleiotropy of functions that vary among hippocampal cell types, it is important to isolate the role GR signaling in NPCs to understand how these regenerative cells contribute to behavioral responses under stress.

To investigate whether the effects of stress on neurogenesis are responsible for some of the stress-induced emotional and cognitive changes, we examined GR signaling within NPCs. A goal of my dissertation was to isolate the effects of GR specifically in newborn neurons and characterize their contributions to cell development and behavior. We generated an inducible Cre-mediated inactivation of GR gene function specifically in nestin-expressing cells and their progeny. After this genotype was induced in adulthood, we measured changes in neurogenesis as discussed in CHAPTER 2. Our results suggested that the effect of cort directly on NPCs was not the driving force to suppress differentiation in vivo and this reduced neurogenesis must be indirectly mediated through GR in other cell types. We then characterized how this cell-specific GR gene inactivation model responded behaviorally under either basal levels of cort or chronic cort treatment. These characterizations were made in CHAPTER 3 using non-induced GR$^{NPCKO}$ mice as a control. We found that control mice had increased anxiety-like behaviors under chronic cort treatment, however, cort-treated GR$^{NPCKO}$s behaved similarly to untreated controls.

In this chapter, I will discuss the contributions of these findings to our understanding of GR function and adult neurogenesis in a broader context. I will compare our GR$^{NPCKO}$ model to other GR animal models, assessing its advantages and disadvantages. I will then propose how my results fit in with our current understanding of GR function and highlight the adaptive role of newborn neurons to novel encounters. Lastly, implications and future directions of this research will be discussed.
4.2 Cre-mediated knockdown of GR in NPCs

4.2.1 A comparison of our GR$^{NPCKO}$ mouse model with other models

Previous research has helped elucidate GR functioning through assorted pharmacological and genetic models. Various GR agonist and antagonists can be injected or consumed to manipulate GR function, however, these methods are transient, partial, nonspecific, and are typically incapable of passing the blood brain barrier$^{214,387}$. Whereas more controlled manipulation of GR is not possible (or unethical) in humans, advanced genetic techniques have generated mouse models that control expression and function of GR.

While some of these mouse models have demonstrated GR affects on anxiety- and depressive-like behaviors, spatial or contextual memory, HPA regulation, and basal cort levels, others have not (see Table 4.1). This may be due to the technique employed, the expression levels, the tissue affected, and any side effects of the manipulation that make it difficult to deduce GR influence. Our GR$^{NPCKO}$ model attempts to correct for these concerns by being cell-type specific (NPCs), inducible, centrally noninvasive, and having complete GR gene inactivation in affected cells.

Since GR has a pleiotropy of effects that are tissue-specific$^{20,386,388,389}$, we focused on adult newborn neurons and how GR signaling in this regenerative pool of cells influences function. Some of the first mouse models of GR function reduced expression, however, this reduction occurred in many different types of tissue$^{240,249,390–392}$. Indiscriminate targeting introduces peripheral changes in immune function and metabolism that conflict correlations between GR and behavior. Thus, even though all of these studies found impaired HPA regulation$^{240,249,390–392}$, unsurprisingly, the behavior results are conflicting. A more recent study knocked down GR expression in the neurogenic niche and purported direct effects of GR signaling regulating neurogenesis$^{218}$. Although this study had a more tightly controlled manipulation of GR expression, knock down was achieved by short hairpin RNA interference delivered by lentiviral injections. Lentivirus can enter any cell type, thus while it may have infected some newborn neurons in the neurogenic niche it was injected into, the possibility of infecting different cell types at different stages of maturity could not be controlled for with this technique. A more substantial ablation of GR function was achieved by the Cre/lox recombination method$^{239,393–395}$. In these studies, GR function was ablated throughout the brain$^{239}$, or throughout the limbic system$^{393–395}$. This GR inactivation model appeared to have reduced anxiety-like behaviors, however, due to increased general locomotor activity and hyperadrenalism in the paraventricular nucleus, attributing suppressed anxiety to solely GR is confounded by these added variables. To our knowledge, ours is the first study that specifically addressed GR function in a specific cell type, NPCs.

Our GR$^{NPCKO}$ model was also advantageous compared to past and current models by being inducible. Although prenatal deletion of GR is lethal$^{239,396}$, previous studies have examined partial GR deficiency from birth through adulthood$^{33,239,249,393,394,397–400}$. The results of these studies, however, are difficult to distinguish from developmental side effects and compensatory mechanisms that may arise over time. By temporally controlling GR gene inactivation, we could bypass these confounds as well as isolate the role of GR in adult neurogenesis.

There can always be inflammatory and other damaging side effects that result from injections into brain tissue. By using CreERT2 recombination, our mouse model can be induced by intraperitoneal injections of tamoxifen. Other methods that manipulated GR function used
invasive procedures that require anesthesia as well as surgical incisions into brain tissue. Although these methods allow for temporal control of GR function and use carefully constructed controls when examining differences, the possibility of anesthesia, inflammation, cell death, or infections alone or in combination with GR changes can confound behavioral and physiological results.

Another advantage of our mouse model is that the GR genes in cells targeted by Cre recombination are consequently inactivated, a mutation stably passed onto their progeny. Other methods to study GR function, such as antisense RNA inhibition, short hairpin RNA interference, and use of GR heterozygous mice (Gr−/+), can only reduce GR expression within cells. One study attempted to ablate GR function by deleting exon 2 in the GR alleles, however this deletion still expressed a GR gene that was at least partially functional. Our GRloxP alleles target exon 3, which when excised, causes a frame shift in the transcriptional readout, ultimately resulting in a nonfunctional GR. Additionally, other studies have examined overexpression of GR or impaired the morphology of GR to examine function. Importantly, these have allowed for comparison of functions, although not all of these studies were designed to examine GR function. Furthermore, impaired dimerization, although not a complete inactivation of GR gene function, allowed for GR-induced actions to be examined more mechanistically. In any case, none of the aforementioned GR mouse models can precisely examine the contributions of GR activity in adult newborn neurons to cytoarchitecture, or behavior and cognition. By being cell-type specific (NPCs), inducible, centrally noninvasive, and having complete loss of GR function in affected cells, our GRNPCKO model can address these questions.

4.2.2 Assessment of our GRNPCKO mouse model

Despite the advantages our GRNPCKO mouse model offers us in studying adult neurogenesis, there are several disadvantages to our method as well. First, nestin is a protein expressed in most NPC cell types (type 1, 2a, and 2b), however, not all types, that being type 3. This NPC type is slightly more restricted in its developmental potential compared to type 1, and though it is more proliferative, these abilities are limited. Thus, not every hippocampal NPC was affected. Additionally, our nestin-CreERT2 mouse targeted nestin-expressing cells in the subventricular zone as well. By examining hippocampal-dependent functions, we attempted to separate the role of GR in each area, however, it is always possible GRNPCKO in the subventricular zone adds to these effects. Moreover, Cre recombination is not 100% efficient and does require an injection of tamoxifen. Although controls injected with tamoxifen behaved similarly to noninjected controls, an ideal model would not require this slight stressor. Overall, GR gene inactivation in our mice was sufficiently reduced by 50%. Though this was not a complete GR inactivation in NPCs, due to non-nestin-expressing NPCs, and inefficient Cre recombination and tamoxifen induction, we believe this can also be a benefit to our model, as it more accurately resembles naturally occurring dysfunction with the GR.

Another disadvantage to our experimental model is not all controls groups were possible for comparison. In particular, GR gene inactivation specifically in ~10% of other (matured prior to adulthood) granule cells would be an appropriate control. Any differences between our GRNPCKOs and this one would suggest that the results were truly due to GR function specifically in NPCs and not just a reduction in overall GR signaling. An ideal GRNPCKO model may also include more temporal control over GR function, such as those achieved through optogenetics.
In these mice, behavior testing could be compared prior to or after GR loss, such that one could examine cognitive skills due to GR\textsuperscript{NPCKO} and then reexamine these skills once GR function was reinstated. Undoubtedly, proper controls for re-exposure to similar paradigms would need to be implemented. It is important also to acknowledge the results from mouse models may differ significantly from those performed on rats. It would be an interesting comparison to examine GR function, particularly in NPCs, within the rat species since much stress research is outlined in the rat, and neurogenesis is more proliferative and contributes more abGCs to the DG in the rat as well\textsuperscript{72}. Unfortunately, genetic manipulations of GR (or anything) in a rat model are typically not successful or currently available. In any case, these additional controls and techniques are advantages our model does not provide.

Lastly, generation of our GR\textsuperscript{NPCKO} mice for our experimental design resulted in some potential confounds. First, whereas litters had varying numbers of CreERT2-expressing male mice, it was difficult to control for the ultimate number of littermates per cage for our mice. Due to this, and any unexpected death, our mice varied in having zero to four littermates within their cage. Particularly in cages receiving chronic cort treatment, stressed littermates can add additional stress to the environmental situation confounding cort-specific effects on behavior. Additionally, due to varying litter sizes, it was difficult to generate enough mice per group within a close age range. Each experimental group controlled for age differences, however, within group, this could mask or reduce effects, and any subsequent mouse deaths or removals due to inefficient recombination in the brain, may upset the balance of age discrepancies between groups.

4.3 GR signaling in the neurogenic niche

Results from our research indicate that GR gene inactivation in NPCs did not alter neurogenesis nor produce an observable phenotype different from controls. It was not until the introduction of chronic cort exposure that we saw differences. Cort-suppressed differentiation of NPCs still occurred, similarly to controls, yet our GR\textsuperscript{NPCKO} model blocked cort-induced anxiety-like behaviors. This suggests that cort (and perhaps stress) can indirectly regulate cell maturation through GR signaling in the surrounding niche and directly regulate mood-related behaviors through GR signaling in abGCs. Furthermore, it suggests that cort (and stress) effects on neurogenesis (i.e. proliferation, survival, and differentiation) are not contributing to changes in anxiety-related behaviors. This brings up two additional queries: does adult neurogenesis become functionally relevant only when environmental stimuli (such as stressors) are introduced? And what is the GR pathway promoting anxiety-relevant behaviors or able to attenuate them when GR is blocked?

4.3.1 Newborn neurons mediate fearful behaviors only when exposed to chronic cort treatment, not to basal levels of cort

When manipulations were made to adult neurogenesis, that being reduced GR signaling within abGCs, there was no observable phenotype for baseline behaviors. This is not so surprising since GR is mostly occupied, and thus activated, when circulating cort levels are elevated\textsuperscript{4,24}. However, whereas in the absence of stress, cort can be elevated naturally due to its circadian and highly pulsatile fluctuations\textsuperscript{404}, and the simple act of handling mice or exposing them to a new environment can raise cort levels\textsuperscript{405,406}, it would not be curious to have a
behavioral phenotype prior to our chronic cort treatment. We did not find this in our manipulated abGCs; similarly, other studies manipulating neurogenesis do not find a phenotype for baseline behaviors (for review\textsuperscript{91}). It often appears that a behavioral phenotype is exhibited when the rodent is presented with novel environmental stimuli, communicated to the brain by elevated cort levels\textsuperscript{107,273,407}. This suggests that the functional contributions of abGCs are for adaptive purposes, adjusting emotional responses and memory associations to prepare for future scenarios. This role of abGCs would promote survival as well as reproductive success in a dynamic environment.

A recent article supports this hypothesis and elaborates that adult neurogenesis functions to regulate the stress response and assign stress salience to sensory context\textsuperscript{408}. This idea initially arose from evidence that the dentate gyrus (DG) helps mediate the stress response and novelty detection in both rodents and humans\textsuperscript{373,409–411}. Lesions to the DG caused a reduced cort response to a novel environment that did not habituate with repeated trials\textsuperscript{412}. This appeared to be specific to novelty since the cort response was normal for other stressors like laparotomy and ether exposure\textsuperscript{412}. Further investigation revealed that novelty detection might specifically involve abGCs\textsuperscript{265,266}. Similarly, these newborn neurons have a bidirectional relationship with stress. First, stress impacts neurogenesis, most likely in an indirect manner according to our results. Although the functional importance of abGCs is hotly contested, the sensitivity of adult neurogenesis to chronic elevations in cort levels is consistently reproduced. Secondly, neurogenesis impacts the HPA axis\textsuperscript{408}. Reduced neurogenesis resulted in enhanced cort secretion, thus, dysregulating negative feedback\textsuperscript{411}, as well as impaired recovery from a social stressor\textsuperscript{407}. Furthermore, due to their connections to the limbic circuitry and vasculature, abGCs are well positioned to sense stress and adjust the response accordingly\textsuperscript{408}. GR signaling within abGCs is specifically implicated in mediating stress recovery and the development of chronic stress-related conditions due to its role in regulating the gradual influx of calcium currents\textsuperscript{414}. Overall, this evidence suggests that abGCs may be involved in assigning stress salience to novelty during learning. Thus, if this construction is dependent on the stress state of the animal, these cells might only become specifically engaged under stressful conditions\textsuperscript{408}.

The highly plastic nature of the hippocampus and its high concentration of GRs suggest that one of its functions are to be sensitive to stressors and adapt affect and memory processing accordingly. It is proposed this may be mediated by GR signaling within abGCs, producing a behavioral phenotype conditional to stress. Thus, we suggest that this functional role is taken into consideration for future evaluations of adult neurogenesis.

**4.3.2 How is cort impacting abGC function if not through suppressed neurogenesis?**

When stress is chronic or overwhelming, this may impair neurogenesis and produce maladaptive responses, such as mood dysregulation. While the link between chronic stress (via elevated stress hormones) and anxiety-like behavior has long been recognized, the underlying mechanisms remain largely hypothetical. Neurogenesis was first proposed to be involved when Gould and colleagues reported that both stress and cort suppressed neurogenesis\textsuperscript{53,96}, a finding that correlated with postmortem tissue showing reduced hippocampal volume in depressed patients\textsuperscript{415–417} and patients with PTSD\textsuperscript{418,419}. It was then followed by a study showing that chronic antidepressant treatment increased neurogenesis\textsuperscript{420}. This established the idea that neurogenesis could be responsible for the development or treatment of affective disorders, however at this time, evidence was only correlative. To test this, Hen and colleagues compared
antidepressant efficacy in mice with ablated neurogenesis to mice with intact neurogenesis.\textsuperscript{10} When it was shown that antidepressants were not effective in neurogenesis-deficient mice, the “neurogenesis hypothesis of affective disorders” was established.\textsuperscript{91} Since then, many reviews have discussed the cumulative evidence that is highly conflicting and conclude that while intact neurogenesis may aid in antidepressant efficacy in a stressed animal model, reducing neurogenesis does not induce anxiety- or depressive-like behaviors.\textsuperscript{91,271,348,421–423} This latter association was only shown to be correlative, and not causal. Indeed, the research presented in this thesis addresses this controversy. We show a model in which neurogenesis was reduced by chronic cort treatment, yet remained resilient to cort-induced anxiety-like behaviors. Similarly, other studies corroborate our results, demonstrating neurogenesis ablated by x-ray irradiation led to increased resiliency in a social avoidance task with mice,\textsuperscript{107} and mice with reduced neurogenesis from selectively deleting cell cycle checkpoint kinase, ATR, also exhibited reduced anxiety-like behaviors in three different tasks.\textsuperscript{424} In any event, chronic cort treatment still induced anxiety-like behaviors in our control mice, and our results suggest that newborn neurons are involved. However, these behaviors were not mediated by reduced numbers of abGCs; instead, it appears they can be mediated by GR signaling within abGCs.

Our experiments demonstrate that two mouse groups with cort-suppressed neurogenesis behaved differently under chronic cort treatment. Adult neurogenesis refers to newborn cells that range in developmental stage (i.e. NPCs, immature neurons, and abGCs). Thus, at each stage, these cells have varying degrees of impact on the hippocampus. Cort-suppressed neurogenesis occurs during the NPC or immature neuronal stage, a phase in which cells are not quite functionally integrated. Therefore, a reasonable hypothesis would be that these two mouse groups with cort-suppressed neurogenesis behaved differently under chronic cort treatment due to GR signaling within the abGCs.

While this hypothesis requires further support, GR signaling within abGCs would be largely capable of regulating protein production, such as NMDA,\textsuperscript{162,295,296} AMPA,\textsuperscript{191,425} and 5-HT\textsubscript{1A} receptor levels,\textsuperscript{426,427} morphology, such as dendritic arborization,\textsuperscript{218,428,429} and hippocampal positioning,\textsuperscript{218} and synaptic potentiation, such as LTP/LDP\textsuperscript{430–432} and Ca\textsuperscript{2+} signaling.\textsuperscript{433–435} Any of these changes could potentially contribute to the complex set of symptoms or etiology underlying mood and affect disorders. Despite limited numbers, it is important to note that abGCs are well positioned in the hippocampus to mediate emotional responses,\textsuperscript{408} and due to their hyperactivity, well equipped to make a disproportionately larger impact than mature granule neurons nearby.\textsuperscript{310,436}

It is important as well to recognize that our results, while they suggest GR activation in abGCs induces anxiety-like behaviors, they only actually show that inactivating or reducing GR signaling in abGCs can attenuate cort-induced anxiety-like behaviors. This means that it is possible that GR is not the pathway responsible for fearful responses, but that somehow blocking its activity will eliminate this cort effect on behavior. In other words, GR activation in abGCs does not drive this behavior, but somehow it indirectly supports the cells or actions that are significantly more responsible. One potential explanation could be if reducing GR signaling disrupts the MR/GR balance, and aberrant MR signaling enhances resiliency.\textsuperscript{35} Additional evidence to suspect MR involvement comes from studies demonstrating that raised cort levels could rapidly enhance glutamate release and excitatory transmission in hippocampus through pre- and post-synaptic non-genomic MR.\textsuperscript{437,438} Another possibility could be GR activity in glial cells or even other limbic structures perpetrate these maladaptive responses and GR activity in newborn neurons functions to maintain supporting factors. A third potential explanation could
be that GR deficiency in abGCs impairs overall ability to detect novelty, thus, the animal would not register being in an unfamiliar environment, suppressing fearful responses. This idea was proposed by Dranovsky & Leonardo (2012), suggesting that GR activity in abGCs functions to assign a stress value to a contextual cue (novel paradigm) the way the amygdala assigns an emotional value to a sensory cue; the latter increases the strength of the memory trace, while the former may increase the anxiety-like response. Nonetheless, it is important to realize our behavioral results could just be caused by an overall reduction in GR signaling in the DG, and may not be specific to abGCs. At this point, we can only speculate.

My results challenge the current neurogenesis hypothesis of affective and anxiety disorders; namely, that decreased neurogenesis results in depression or anxiety. We show our experimental group had decreased neurogenesis induced by cort, however they did not exhibit a depressive or anxious phenotype. This suggests that specific GR-regulated mechanisms in abGCs are capable of suppressing anxiety-relevant behaviors. How this is accomplished warrants further investigation.

4.4 GR signaling in abGCs: implications for stress-related brain disorders

The research presented in this thesis addresses the cell-autonomous role of GR in adult neurogenesis, the functional and cellular impact of chronic cort exposure, and the functional significance of not only newborn neurons, but also GR activity within newborn neurons. Human neuroimaging studies have implicated hippocampal dysfunction in mood and anxiety disorders, and these human psychopathologies are also associated with a dysregulated HPA axis, impaired GR signaling, and aberrant cort levels as well, suggesting the relevancy of this research is not restricted to animals. Additionally, patients with psychotic mood dysregulation demonstrated significantly improved mood when treated with GR antagonists. Altogether, data presented here has implications for stress-related brain disorders, including generalized anxiety, PTSD, social anxiety, suicidality, panic disorders, and obsessive-compulsiveness, and demonstrate a model of stress resilience in spite of suppressed neurogenesis.

First, our results suggest stress- and cort- effects on neurogenesis are indirect and challenge the hypothesis that reduced neurogenesis can cause anxiety or depression. Indeed, this causal link was not found in many studies, antidepressant efficacy did not correlate with increased neurogenesis, and increasing neurogenesis did not produce any anxiolytic or anti-depressant–like behavioral effects. Our results suggest redirecting attention from reduced proliferation or differentiation to GR-regulated proteins or pathways in abGCs that may mediate or provide relief from anxiety-like responses. Whereas too many people suffering from anxiety-related disorders do not receive relief from pharmacological treatment or relief is delayed, subdued, or even temporary, understanding the etiology or underlying mechanisms of symptom relief will allow for the development of more targeted pharmaceutical therapies, as well as better prevention and intervention strategies. Though we found no causal link between a depressive phenotype and our chronic cort treatment or an effect of reduced GR signaling in abGCs, it is also important that these results are taken into consideration for understanding the etiology of depressive symptoms (perhaps not GR regulated) or targets for anti-depressants. In general, studying the relationship between abGCs and stress regulation could be clinically relevant to determining antidepressant efficacy and clarifying levels of resilience to stress. Our results not only provide a model for directly studying the
effects of stress (and cort) on adult hippocampal plasticity, but also advance the understanding of the underlying mechanisms of mood and affective disorders.

In addition to its relevancy to stress-related disorders, our results have implications for diseases marked by hypercortisolism, such as Cushing’s disease, as well as patients receiving exogenous cort treatment for various conditions, such as rheumatoid arthritis, sports injuries, and other inflammatory diseases. We show that chronic exposure to elevated cort can suppress neurogenesis, induce anxiety-like behaviors, and enhance fear conditioning. Specifically reducing GR activity in newborn neurons appears to block this effect on anxiety-like responses. While this may be relevant for understanding symptoms and medication side effects, this information may also lead to the development of more targeted treatment options or therapies with reduced side effects. Overall, understanding cell-specific GR signaling and functional contributions of adult neurogenesis will better inform us of pathways involved in brain plasticity, and help develop better therapeutic strategies when these pathways are dysfunctional.

4.5 Future directions

The GR\textsuperscript{NPCKO} model discussed in this dissertation highlights the stress-adaptive role of both newborn neurons and GR signaling, addressing long-lingering questions in the field regarding functional relevance. This in turn incites further queries into underlying pathways and other aspects of behavior that may be affected by additional experimental adjustments. Some of the underlying mechanisms that could be studied include the GR-independent pathways that cort induces suppression of neurogenesis and whether the functional consequences of GR-deficiency on behavior is mediated differentially along the dorsal-ventral axis of the hippocampus. Hen and colleagues have demonstrated with irradiation\textsuperscript{251} and optogenetic controlling\textsuperscript{317} that newborn neurons in the dorsal DG appear to play a role in contextual memory processing, while neurogenesis in the ventral DG plays a role in emotional processing. This is thought due to the dorsal region projecting to associational cortical regions, while the ventral region projects to frontal cortex, amygdala, and hypothalamus\textsuperscript{457–460}. It would be an interesting future experiment to either impair or temporally-control GR activity separately in dorsal versus ventral abGCs and characterize the behavior differences that may be attributed to each region.

Other immediate future directions include re-examining depressive-like symptoms and memory processes that may be affected by additional experimental adjustments. First, I would suggest adjusting the cort-dosing and/or treatment length to an amount that does produce depressive-like behaviors in the FST in an ecologically valid manner. I would then use this protocol to re-examine the influence of GR-signaling in abGCs. Additionally, I would also incorporate treatment with antidepressants and since these should reduce immobility in the FST, investigate whether our GR\textsuperscript{NPCKO} model blocks these effects. This may help elucidate the underlying mechanisms in which antidepressants attenuate depressive symptoms. In our experimental design, mice in the FST were able to see one another during testing. I would suggest testing mice in a bordered/closed apparatus such that the behavior or presence of other mice did not influence their own behavior. Also, it would be interesting to examine these behaviors in a different mouse strain, such as Balb/c mice. These mice have higher emotional and stress reactivity\textsuperscript{370,372} and apparently do not have the GR polymorphisms found in many mice of the C57 family\textsuperscript{399}. Having at least one allele of this GR polymorphism correlated with a lower stress response but increased anxiety-like behaviors\textsuperscript{399}. Any or all of these changes may reveal a role of abGCs in depressive-like symptoms via GR signaling.
In re-examining memory processes, I would better optimize the contextual fear discrimination protocol so that it was clear my control mice were successfully and consecutively discriminating for at least 3 days. This may involve changing the amplitude of the shock, the time between exposures to each context, or even the degree of dissimilarity between contexts. Much of the testing done in this experiment was performed at the end of the day. Thus, it would be interesting to see if behavior is different if testing is conducted in the morning, which is likely so due to the proposed dependence of adult neurogenesis on circadian cues\textsuperscript{461,462} and fluctuating hormone levels\textsuperscript{463}. Moreover, if possible, I would recommend testing all of the behaviors during the mice’s dark cycle, the time in which mice are actually awake and active, although this may make it difficult to compare behavior results across the literature. It is worth noting that most behavior testing in the literature occurs during the mice’s light cycle, when they prefer to sleep, thus, it would not be unsubstantiated to criticize that behavioral differences found, ours included, are confounded by sleep deprivation or disturbances.

Aside from changes to the experimental design, it would be worth testing GR\textsuperscript{NPCKO} mice in other tests of pattern separation or spatial memory, and examining fear extinction as well. Mice did not receive contextual fear conditioning on day 10 of our contextual fear discrimination protocol. Interestingly, on day 11, discrimination ability appears to decrease for control mice (freezing was similar in both contexts), whereas it did not for GR\textsuperscript{NPCKO} mice (freezing remained different in each context). Although there was not a significant genotype effect when comparing across all groups, there is a potential difference in fear-associated memory extinction that warrants further investigation. Whereas both GR and adult neurogenesis are implicated in memory processing, future research would benefit from thoroughly examining their roles and how they interact in these cognitive realms.
Table 4.1: Summary analysis highlighting studies that have studied how manipulations of GR function affect behavioral, cognitive, and physiological response.

<table>
<thead>
<tr>
<th>GR manipulations</th>
<th>technique</th>
<th>tissue affected</th>
<th>anxiety</th>
<th>depression</th>
<th>neurogenesis</th>
<th>spatial memory</th>
<th>HPA regulation</th>
<th>cort levels</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO &lt;10% of hippoc neurons</td>
<td>Cre/lox: CamKIIa Cre + GR&lt;sup&gt;loxP&lt;/sup&gt;</td>
<td>all limbic areas</td>
<td>↓ mild</td>
<td>↑</td>
<td>n.s.</td>
<td>n.s</td>
<td>↓</td>
<td>↑</td>
<td>393,394</td>
</tr>
<tr>
<td>KO &lt;10% of hippoc neurons</td>
<td>CreERT2/lox: CamKIIa CreERT2 + GR&lt;sup&gt;loxP&lt;/sup&gt;</td>
<td>all limbic areas</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s</td>
<td>↑</td>
<td>395</td>
</tr>
<tr>
<td>KO</td>
<td>Cre/lox: Nestin Cre + GR&lt;sup&gt;loxP&lt;/sup&gt;</td>
<td>all brain</td>
<td>↓</td>
<td>↓</td>
<td>n.s.</td>
<td>↓ mild</td>
<td>↓</td>
<td>↑</td>
<td>239</td>
</tr>
<tr>
<td>antisense KD</td>
<td>antisense RNA inhibition</td>
<td>all tissue</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s</td>
<td>↓</td>
<td>↑</td>
<td>391</td>
</tr>
<tr>
<td>antisense KD</td>
<td>antisense RNA inhibition</td>
<td>all tissue</td>
<td>n.s.</td>
<td>⇝</td>
<td>n.s.</td>
<td>n.s</td>
<td>↓</td>
<td>↑</td>
<td>390</td>
</tr>
<tr>
<td>KD of exon 2, function reduced</td>
<td>gene targeting</td>
<td>all tissue</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s</td>
<td>↓</td>
<td>↑</td>
<td>392</td>
</tr>
<tr>
<td>antisense KD</td>
<td>antisense RNA inhibition</td>
<td>all tissue, mostly neuronal</td>
<td>↓</td>
<td>↑</td>
<td>n.s.</td>
<td>n.s</td>
<td>↓</td>
<td>⇝</td>
<td>240</td>
</tr>
<tr>
<td>KD: GR heterozygous mice (GR&lt;sup&gt;-/+&lt;/sup&gt;)</td>
<td>homologous recombination</td>
<td>all tissue</td>
<td>X</td>
<td>X</td>
<td>n.s.</td>
<td>⇝</td>
<td>↓</td>
<td>⇝</td>
<td>249</td>
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<tr>
<td>KD</td>
<td>lentiviral RNA interference</td>
<td>dentate gyrus</td>
<td>n.s.</td>
<td>n.s.</td>
<td>↑</td>
<td>↓</td>
<td>n.s</td>
<td>⇝</td>
<td>218</td>
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<tr>
<td>OE by 2fold</td>
<td>yeast artificial chromosome</td>
<td>all tissue</td>
<td>⇝</td>
<td>⇝</td>
<td>n.s.</td>
<td>⇝</td>
<td>↑</td>
<td>⇝</td>
<td>249</td>
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<tr>
<td>OE by 2fold</td>
<td>yeast artificial chromosome</td>
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<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s</td>
<td>↓</td>
<td>↓</td>
<td>403</td>
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<tr>
<td>OE</td>
<td>transgene CamKIIa-HA-GR</td>
<td>all limbic areas</td>
<td>↑</td>
<td>↑</td>
<td>n.s.</td>
<td>n.s</td>
<td>⇝</td>
<td>n.s</td>
<td>398</td>
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<tr>
<td>polymorphism</td>
<td>mice selected for high/low cort response</td>
<td>all tissue</td>
<td>↑</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s</td>
<td>↓</td>
<td>↓</td>
<td>399</td>
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<tr>
<td>Gr&lt;sup&gt;dim&lt;/sup&gt;: impaired dimerization</td>
<td>Cre/lox: point mutation</td>
<td>all tissue</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s</td>
<td>↓</td>
<td>n.s</td>
<td>33</td>
</tr>
<tr>
<td>Gr&lt;sup&gt;imm&lt;/sup&gt;: impaired dimerization</td>
<td>Cre/lox: point mutation</td>
<td>all tissue</td>
<td>⇔</td>
<td>n.s.</td>
<td>n.s.</td>
<td>↓</td>
<td>n.s.</td>
<td>↑</td>
<td>400</td>
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<td>chimeric GR receptor with ER effects</td>
<td>intracerebral infusion</td>
<td>DG of dorsal hippocampus</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s</td>
<td>n.s</td>
<td>401</td>
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<tr>
<td>chimeric GR receptor with ER effects</td>
<td>intracerebral infusion</td>
<td>DG of dorsal hippocampus</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>↑</td>
<td>n.s</td>
<td>n.s</td>
<td>402</td>
</tr>
</tbody>
</table>

KO: knockout; KD: knockdown; OE: overexpression; n.s.: not shown; ER: estrogen receptor; hippocampus


175. Hodes, G. & Brookshire, B. Strain differences in the effects of chronic corticosterone exposure in the hippocampus. *Neuroscience* **269**–280 (2012).


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