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Glucocorticoid receptor knockdown and adult hippocampal neurogenesis

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Shoot for the moon. Even if you miss, you'll land among the stars.

Les Brown

Voor mijn ouders

ACTH	adrenocorticotrophic hormone
ADX	adrenalectomy
AGLV	advanced generation lentiviral vector
AVP	argentine-vasopressin
BBB	Blood-Brain Barrier
CA	cornu ammonis (part of hippocampal formation)
CamKII	Ca^{2+}/calmodulin dependent protein kinase
CMV	cytomegalovirus
CNS	central nervous system
CORT	corticosterone
CRH	corticotrophin releasing hormone
DBD	DNA binding domain
DCLK	double cortin like kinase
DCL	double cortin like
DCX	double cortin
DG	dentate gyrus
EGFP	enhanced green fluorescent protein
GC	glucocorticoid hormone (corticosterone, cortisol)
GCL	granular cell layer (of the dentate gyrus)
GFAP	Glial Fibrillar Acidic Protein
GR	Glucocorticoid Receptor
GRE	glucocorticoid response element
HPA axis	hypothalamo-pituitary-adrenal axis
LBD	ligand binding domain
LV	lentivirus
mRNA	messenger RNA
miRNA	micro RNA
mm-shGR	mismatch-shRNA
MMLV	Murine Maloney Leukemia Virus
ML	Molecular Layer
MR	Mineralocorticoid Receptor
mRNA	messenger RNA
NeuN	Neuron-specific Nuclear marker
NPC	Neuronal Progenitor Cell
Ns-1 PC12	Neuroscreen-1 Pheochromocytoma 12
PI	post- injection (time)
pm-shGR	perfect match shRNA against the GR
POMC	pro-opiomelanocortin
PTSD	post-traumatic stress disorder
PVN	Paraventricular nucleus
RISC	RNA induced silencing complex
RNAi	RNA-interference
SD	standard deviation
SEM	standard error of the mean
shGR	shRNA against GR, [shRNA] mouse model
siRNA	short interfering RNA
shRNA	short hairpin RNA
SGZ	sub granular zone (of the dentate gyrus)
SR	stratum radiatum
Syn	synapsin I
VSVg	Vesicular stomatitis virus glycoprotein

1

General introduction

OUTLINE

1.1 SCOPE OF THESIS

The research in this thesis is aimed at the elucidation of the role of the glucocorticoid receptor (GR) in hippocampal neuroplasticity and functioning. To achieve this, we have developed a novel method to specifically knockdown GR in a discrete cell population of the mouse brain.
In this thesis I report silencing of GR expression selectively in a population of neuronal progenitors and immature neurons of the dentate gyrus, using RNA-interference (RNAi) delivered by a lentiviral vector. Characterization of these cells resulted in the discovery that GR knockdown causes a striking modulation of hippocampal neurogenesis and remodelling of hippocampal circuitry. Functional studies further revealed consequences of GR knockdown for contextual memory performance and behavioural coping strategies during stressful conditions. The results demonstrate the feasibility to apply RNAi in discrete cell populations for study of the action mechanism of glucocorticoids underlying control of neuroplasticity and behaviour.

1.2 THE STRESS RESPONSE IN THE BRAIN

The organism strives to maintain a physiological balance called homeostasis. When this balance is disrupted by a challenge (stressor), the organism responds by behavioural and physiological adaptations, resulting in coping and recovery [1-5]. For example, an animal needs to react instantly when it is hunted by a predator and needs to decide on the best strategy for survival. This situation is often referred to as a "fight or flight" response and results in enhancement of systems that are directly crucial for survival, and repression of systems temporarily redundant [6]. At the same time, physiological and behavioural adaptations are promoted in preparation for future events. This can imply for example, that the animal needs to learn about the situation to prevent its repeated exposure to the endangering environment. Together, these adaptations are called the stress response.

The perception of the stressor is the key trigger that initiates the stress response. Central to the stress response therefore is the brain, because it determines what is threatening and, therefore, potentially stressful [7]. Generally, stressors can be divided into two classes, physical stressors and psychological stressors. Physical stressors, such as e.g. infections, tissue damage, blood loss, are usually homeostatic challenges sensed by the somatic, visceral and circumventricular pathways which activate aminergic cells in the brain stem [8]. Psychological stressors are external challenges that contain species- and individual- specific characteristics. They are processed by limbic brain areas, including the amygdala, hippocampus and prefrontal cortex [8;9]. These limbic areas mediate the cognitive and emotional processing of psychological stressors, thereby appraising the challenge and assessing its stressfulness. Both the brain stem and the limbic brain areas communicate to the hypothalamus which integrates the stressor-specific information [10].

1.2.1 The HPA axis

Subsequently, the hypothalamus organizes the adaptive response and communicates to peripheral organs by 1) activating the sympathetic nervous system and subsequent secretion of catecholamine's such as adrenalin. They are responsible for the immediate physiological changes. These include increased heart rate and cardiac output, diverting blood to the skeletal muscles and elevating blood glucose levels, processes crucial for the fight or flight response [6]. On the other hand, the sympathic nervous system suppresses the reproductive and digestive systems which are at that time non-relevant to survival. 2) Activating the hypothalamo-pituitary-adrenal (HPA) axis and subsequent secretion of glucocorticoid hormones (GCs; cortisol in man and corticosterone in rodents) (for review see [3;11]). This neuroendocrine system is responsible for more slow-acting adaptations which modulate and fine-tune the physiological changes initiated by the sympathetic nervous system. Physiological changes include inflammatory and immunity responses, metabolism and attention and information storage.

Under basal (non-stressed) conditions, the HPA axis activity is limited, resulting in the pulsatile release of low amounts of GCs from the adrenal cortex. This ultradian pattern of secretion has pulses with larger amplitude which define the circadian rhythm [12]. If activated by a(n acute)

stressor, the circadian rhythm is overridden and stress-induced HPA axis activity results in a rapid rise in hypothalamic corticotrophin releasing hormone (CRH) and vasopressin, activation of pro-opiomelanocortin synthesis and release of adrenocorticotrophin (ACTH) from anterior pituitary corticotrophs, which ultimately -after several minutes- leads to the secretion of GCs into the bloodstream [13].

1.2.2 Genomic and non-genomic actions of glucocorticoids

The lipophilic glucocorticoid hormones enter target cells by penetrating across the cell membrane. At the cellular level, GCs control the stress response through binding to two types of steroid receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR or NR3C1: nuclear receptor subfamily 3, group C, member 1; encoded by a gene on chromosome 5 in humans and chromosome 18 in mice) [14;15]. The steroid receptors belong to a superfamily of ligand-inducible, highly conserved nuclear hormone receptors.

They have a similar structural organization consisting of different domains that are implicated in their different action mechanisms (see Fig 1.1): A/B) an N-terminal regulatory region, (most unique part, only 4% homologous between GR and MR) and contains a ligand-independent activation function (AF-1) [16], C) a DNA binding domain (DBD), which has a homology of 94% with MR. It contains two zinc fingers of which the first is necessary for binding transcription factors [17]. The second zinc finger domain encodes for receptor dimerization and GRE-mediated transactivation [18]. The DNA binding domain further contains a nuclear localization signal (NLS1). D) A hinge region that is thought to link the DBD and the Ligand Binding Domain (LBD), E) a LBD. Along with the DBD, the LBD contributes to the dimerization interface of the receptor, and binds co-activator and co-repressor proteins. In addition, the LBD domain contains a second nuclear localization signal (NLS2) and a second ligand-dependent transcription activation function (AF-2). Both activation functions interact with co-regulator proteins and mediate the effects of the receptors on gene transcription. And F) the C-terminal part of the protein is about 60% homologous between GR and MR [16].

MR and GR are localized in the cytosol bound to chaperone and co-chaperone proteins [19], and upon activation by binding GCs they undergo a conformational change and translocate without their chaperones to the nucleus. Here they control the expression of glucocorticoid-responsive-genes. GCs are thought to influence about 20% of the expressed human genome by activating GR [20]. The genomic effects of GCs on these targets are noticeable within an hour after a pulse and last for days, weeks or even permanently [21]. However, using micro- array analysis, responsive gene patterns were measured within a time window of 1-5 hours after GC pulse [22].

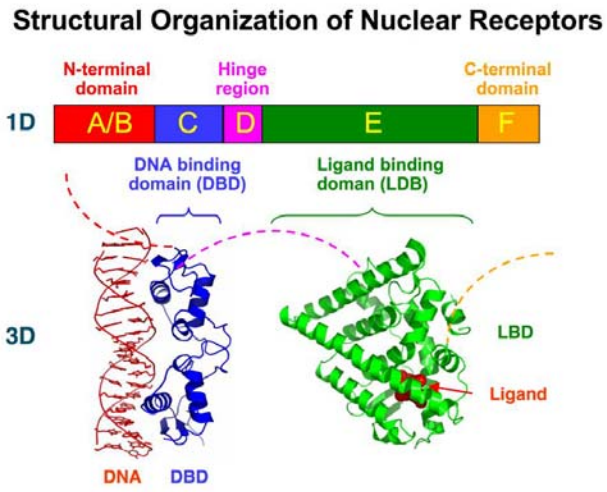

Figure 1.1 Structural organization of nuclear receptors like the GR. Top *– Schematic 1D amino acid sequence of a nuclear receptor.* ***Bottom*** *– 3D structures of the DBD (bound to DNA) and LBD (bound to hormone) regions of the nuclear receptor.*

The GC actions are mediated through two major mechanisms: 1) both receptor types can function as a dimer, by directly binding DNA at either positive or negative glucocorticoid response elements (GREs), in the promoter region of target genes. This transactivation mechanism is prominent in GC control of energy metabolism and cognitive processes, and occurs 3-5 hours after receptor activation [22]. Or 2) Only GR functions as a monomer, by modulating the activity of other transcription factors via protein-protein interactions and thereby inhibiting transcription [23-26]. This transrepression mechanism is prominent for glucocorticoid control of stress reactions and occurs predominantly during the first hour after GR activation [22].

Besides the genomic effects of MR and GR, more recently there has also been a breakthrough with the discovery of non-genomic steroid actions [27-30]. Di and Tasker (2003) discovered that in the PVN GR-like receptors mediate the release of endocannabinoids that block excitatory transmission towards CRH neurons. Karst and Joels (2005) demonstrated in the hippocampus rapid actions mediated by MR on the presysnaptic release of glutamate deducted from the enhanced mEPSCs [30-32]. Non-genomic MR-mediated actions are thought to improve attention, vigilance and appraisal processes, in addition to rapid GR-mediated HPA negative feedback [21;33-35].

1.2.3 Tissue-specific signalling pathways of GCs and their receptors

As previously mentioned, GCs exert their pleiotropic functions on a variety of different organ systems. In fact, it appears that GC-responsive target genes are to a great extent cell type specific [36]. Therefore, in addition to the central control of GC secretion, mechanisms are necessary to regulate GC signalling in order to fine-tune their different physiological actions. These specific

modes of GC signalling are particularly apparent in the dynamic and complex environment of the brain, one of the prime targets of GCs.

After secretion from the adrenals, bioavailability of GCs in the bloodstream can for example, be modulated by binding to plasma proteins, such as corticosteroid-binding globulin (CBG). In addition to regulating bioavailability and metabolic clearance of GCs in the bloodstream, CBGs have a role in tissue-specific GC release [37]. Furthermore, at the level of the cell membrane, passive diffusion of lipophilic GC molecules or their active transport can influence uptake into the cell. This is particularly relevant in the brain, where GC entry is regulated by the blood-brain-barrier. In the blood-brain-barrier, the multidrug resistant P-glycoprotein plays an important role in exporting synthetic GCs. Within the cytoplasm of target cells, enzymatic processes called "pre-receptor ligand metabolism" by 11β-hydroxysteroid dehydrogenase type 1 and 2 are yet another mechanism that can affect intracellular GC availability in a tissue- and cell type specific manner [35;38].

In addition to these pre-receptor regulation modes, the dual receptor system plays an important role in refining GC signalling. According to the MR:GR balance hypothesis, MR and GR function in complementary fashion and mediate genomic GC actions on distinct, yet overlapping sets of genes [3;11;39;40]. These complementary and sometimes opposite effects serve to coordinate the basal functions in sleep-related and daily events (MR), and in coping with stressful events (GR) [11]. There are several different possibilities how GC action through MR and GR can coordinate divergent functions under basal and stressful conditions. These can be divided into 3 groups.

1) Receptor-specific characteristics. Both MR and GR are characterized by their difference in ligand-binding capacities. GR has a tenfold lower affinity for GCs ($K_{d\ cort} \approx 5$ nM) than MRs and, as a consequence, the majority of GRs only become substantially occupied at elevated levels of hormone (i.e. at the ultradian and circadian peak or, following a stressor) [41-43]. This difference is especially relevant when receptors are co-localized in the cell, as it results in a MR: GR ratio in which physiological fluctuations in GC level will range from a situation of predominant MR activation when the organism is at rest and at the circadian nadir, to concomitant MR and GR occupation after stress or at the ultradian and circadian peak [44-47]. Another characteristic of both receptors is -when co-localized- their ability to homo- or hetero dimerize [48]. This implies also that relative receptor concentrations determine the proportion of receptor dimerization [19]. Homodimers are formed anytime and hetero-dimers are predominantly formed with high GC levels in response to stress. In addition, receptor expression levels ("Amount", discussed below) and activity levels ("function") are important for subtle differences in functioning. On the one hand, this is dependent on receptor-splice variant characteristics, as both MR and GR exist in several different isoforms due to mechanisms such as alternative mRNA splicing and further post transcriptional modifications [35;49;50]. Different isoforms of receptors are not only expressed in tissue specific manner, they are also associated with different transcriptional efficacies [35;50]. On the other hand, receptor expression and activity levels can also be influenced by GCs themselves. Overload of GCs for example can lead to a diminished expression of GR mRNA and protein, and

can even lead to receptor insensitivity, called "GR resistance" [8;51;52]. In fact, recent evidence points to an effect of parental care on the epigenetic regulation of hippocampal GR mRNA and GR splice variant expression [53].

2) Differential expression patterns of MR and GR. Although both receptors are constitutively expressed, the different localization of receptors naturally underlies differences in GC signalling. In fact, while GRs are almost ubiquitously expressed in the brain (but with very low levels in CA3, brainstem and suprachiasmatic nucleus), MRs are highly abundant expressed in the limbic system such as neurons in the hippocampus, amygdala, dorsolateral septum and parts of the prefrontal cortex. Even within the hippocampus, both steroid receptors are expressed heterogeneously in different subfields. While the MR is expressed in the entire cornu ammonis (CA1-4) and the Dentate Gyrus (DG), GR expression is predominantly in CA1, CA2 and the DG, with much lower levels in CA3 [14;54;55].

In addition to differential expression in tissues and anatomically determined areas, also between different cell types there can be differential MR: GR expression patterns. In contrast to the cornu ammonis, the DG, for example, is a highly heterogeneous subfield consisting of different cell types (see Box 2). In general, in the DG all mature cells, both neurons and astroglia, express GR but only granule neurons seem to express MR as well [56;57]. The differences in expression between tissues and cell types can be explained by the expression of different splice variants of the steroid receptors [35;58]. These splice variants or isoforms are associated with altered biological activity, which can play a role in its ligand-sensitivity [49].

At even smaller scale, cell populations of specific origin or age can give rise to differences in MR and GR expression. Again, the DG is a prime example as it contains different cell populations that arise from both a different origin (embryonic vs adult neurogenesis) and a distinct age or developmental stage. For example, both neuronal progenitor cells and immature adult born granule neurons lack MR, while GR is expressed in about 50% of the cells (see Figure 1.2) [57]. GR expression in adult born neurons develops in a dynamical pattern during the four-week maturation period. Also with increasing age of the mouse it seems that both GRs and MRs become expressed at higher levels in immature neurons with increasing age of the mouse, suggesting lifetime alterations in steroid sensitivity [57].

For these differences in expression not only transcriptional processes may be responsible. Recently, microRNAs have been found that control levels of gene expression in the post-transcriptional stage. For the GR, miRNA124a was observed to down-regulate GR protein levels in neural cells [59]. Expression of miRNA124a is restricted to the brain. Endogenous miRNA124a up-regulation during neuronal differentiation of a neural cell line *in vitro* was associated with a decreasing amount of GR protein levels. This observation may imply a potential role for miRNAs in the regulation of cell type-specific responsiveness to GCs, as may occur during critical periods of neuronal development. In two other studies, miRNA124a was indeed shown to regulate proper neuronal differentiation of neuronal progenitor cells *in vitro* and *in vivo* [60;61].

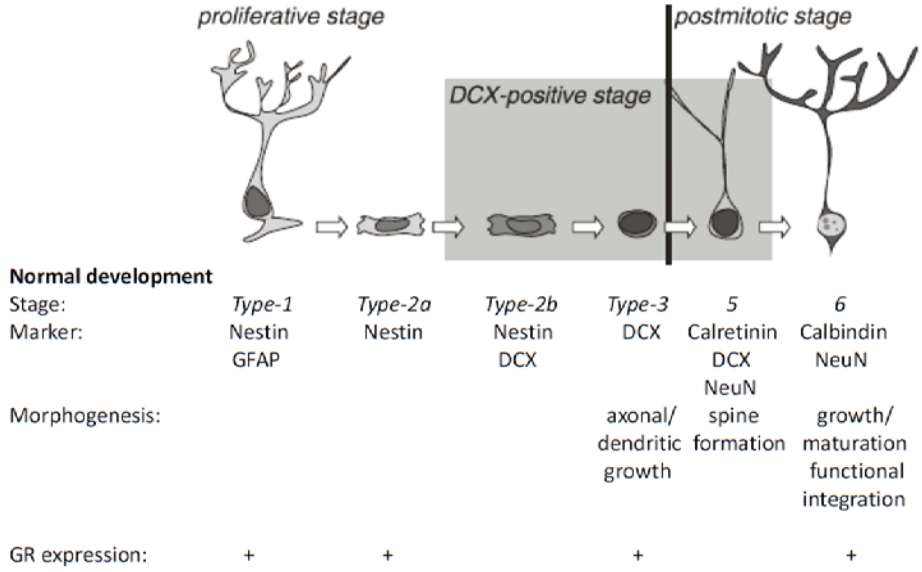

Figure 1.2 Proposed development of newborn neurons in the dentate gyrus. *Six stages of neuronal development in the adult hippocampus can be readily identified on the basis of morphology, proliferative ability, and expression of markers such as nestin, GFAP, DCX, calretinin, calbindin and NeuN. Development originates from the putative stem cell (type-1 cell; stage 1) that has radial glia and astrocytic properties. Neuronal development then progresses over three stages of putative transiently amplifying progenitor cells (type-2a, type-2b and type-3 cells; stages 2–4), which appear to be increasingly determined to the neuronal lineage because in vivo no overlap with any glial markers has been found in these cells, to an early post-mitotic stage (indicated by the 'one-way' sign). This transient early post-mitotic period is characterized by calretinin expression (stage 5). GR expression varies during the proposed stages of development during adult hippocampal neurogenesis. Distinction of cells as stem cells, transiently amplifying progenitor cells and lineage-determined progenitor cells is hypothetical and remains to be proven in vivo. Figure modulated from references* [57;62-64].

3) Cellular context of MR and GR. Receptor signalling can be variably controlled by differential expression patterns of co-activators/ co-repressors [65]. These transcriptional co-regulator proteins are enzymatically active proteins that reorganize the chromatin environment after recruitment by the ligand activated nuclear steroid receptor and thereby influence gene transcription. The ratio of co-activators and co-repressors expressed in the cell has been proposed to determine the nature and magnitude of the GR-mediated transcriptional response, particularly at sub-saturating levels of GCs [66].

In addition, proteins that control the translocation of steroid receptors to the nucleus can influence gene transcription in a cell type specific manner. Recently, such a control mechanism has been described for GR signalling, involving the microtubule-associated protein DCL, a protein that is specifically expressed in neuronal precursor cells in the DG and crucial for GR translocation to the nucleus [67].

Another type of cellular context in MR and GR signalling may be the differential sensitivity of GC-responsive target genes for steroid-receptor mediated transcriptional regulation. Although both

steroid receptors recognize the same response elements, or GRE's in the DNA, subtle differences in GRE- nucleotide sequence or number of GRE's may lead to preferential MR- or GR- mediated transcriptional transactivation.
Finally, there may be a higher order control of receptor interaction with the genome, relating to the spatial organization of the cell nucleus during cellular differentiation and growth [11;68].

Taken together, pre-receptor differences in GC bio-availability, and the cellular context combined with the dual steroid receptor system enable a precise, balanced and coordinated regulation of a variety of tissue-specific GC functions [4;44;48]. The role of GC receptors is particularly important in the local signalling pathways. GCs are a circulating ligand, and it therefore is the local receptors which ultimately initiate and translate the massage of GCs into actions in the specific cells and tissues.

1.3 ROLE OF HIPPOCAMPAL GR

A further understanding of brain mechanisms underlying the stress response and GC signalling requires identification of the processes occurring at multiple levels of complexity; from molecular, cellular and circuitry levels to the behavioural level. In the brain, GCs and several known glucocorticoid-responsive-genes influence these processes; including neurochemical processes, structural neuroplasticity, neurogenesis, motivation, emotions and cognitive performance. In addition, GCs target the HPA axis itself, exerting a negative feedback loop via their steroid receptors in the pituitary and the hypothalamus, with modulatory influences from the hippocampus, controlling HPA activity and preventing an overproduction of GCs [9;11;14;41;42;69].
As both MRs and GRs are highly expressed in the hippocampus, this brain structure is sensitive to circulating GCs. In addition, a wealth of information is known about the function of this region at the multiple levels of complexity. In fact, recently the hippocampus is more and more acknowledged in the pathophysiology of a number of neurological disorders. Moreover, different subfields are highly accessible for pharmaca, which enables manipulation of GR. Therefore, in this thesis I decided to focus on the hippocampus and in following section I will further discuss GC signalling and GR function in the context of the hippocampus and its DG subfield.

1.3.1 The hippocampus

The mammalian hippocampus is phylogenetically one of the oldest parts of the cerebral cortex. This well preserved and complex structure can be divided into two major regions that are interlocked with each other; the DG subfield, and the cornu ammonis (Figure 1.3) [70]. The cornu ammonis can be further subdivided into 4 pyramidal cell subfields that are designated as CA1, CA2, CA3 (and CA4 in humans).

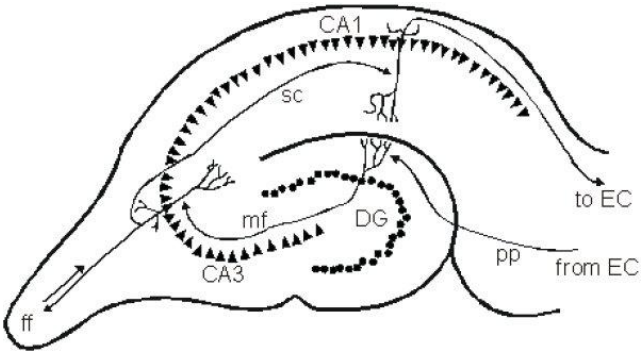

Figure 1.3 Hippocampal neuroanatomy. *Orientation of the dentate gyrus (black dots) and cornu ammonis (black triangles) and their connections with the trisynaptic circuit. Abbreviations: CA1-3 = cornu ammonis 1-3; DG = dentate gyrus; EC = entorhinal cortex; pp = perforant pathway; mf = mossy fibers; sc = Schaffer collaterals; ff = fimbria fornix. (Adapted from [71])*

The neurons of the different hippocampal subfields are interconnected via the excitatory trisynaptic circuit [72]. The glutamatergic input from the superficial layers of the entorhinal cortex enters the hippocampus via the Perforant path to the DG. This connection is the first of the trisynaptic circuit. Next, between the DG and CA3 is the unidirectional Mossy fiber path. This path connects the axons of the dentate granule neurons with the dendrites of the CA3 pyramidal neurons. The third connection is the Schaffer collateral path between the CA3 and CA1. The processed information then is projected back from the CA1 to the deeper layers of the entorhinal cortex. The hippocampus also receives input from several other connections, for example, from its contralateral part and several other brain regions (e.g. limbic system, fore brain, PVN and pituitary). These connections are often characterized by their inhibitory features.

Parallel with the central position of the DG in the trisynaptic circuit, is its unique neuroanatomy. This characteristic subfield consists of a trilaminar structure. The outer layer, the molecular layer, is relatively cell free. It comprises the dendrites of the dentate granule cells and axons originating from the performant path. The second layer or granule cell layer (GCL), is composed of densely packed granule cells, which have small spherical cell bodies (8-12 µm in diameter) and lack basal dendrites. The inner part, also referred to as polymorphic layer or hilus, contains besides the granule cell axons, also mossy cells, various types of interneurons, and astrocytes [70;73]. The GCL consists of two parts: the suprapyramidal (upper) blade and the infrapyramidal (lower) blade. Although they differ slightly in granule cell morphology (dendritic length and spine number) [74;75], both can be subdivided into 3 layers; the outer third, lining the molecular layer, the middle third and the inner third [76]. There is a fourth layer lining the inner third of the GCL and the hilus: the subgranular zone (SGZ). This two-nucleus-wide band contains neuronal progenitor cells (NPC's). The NPC's of the DG are, together with the NPC's of the lateral ventricular wall, unique to the brain. They are able to divide- even in the adult brain and therefore underlie the phenomenon of adult neurogenesis (see box 1).

Box 1: Historical perspective of the study of adult neurogenesis

"In the adult centres, the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated". This statement by Ramon y Cajal (1913) highlights what was one of the central dogma's of neuroscience: that neurogenesis –the birth of new neurons- was restricted to prenatal and early postnatal development, and that the adult mammalian brain was unable to facilitate this process. However, in the 1960's Joseph Altman and colleagues showed first evidence of the phenomenon in the brain of adult rats [77;78]. Although these results were initially not accepted by the scientific community, results were repeated and proved the neuronal phenotype of dividing cells in the hippocampus [79].

An important contribution to the study of neurogenesis is the increasing level of sophisticated tools and scientific methods. Cell division for example can be visualised using BrdU, a substance that incorporates into de DNA of dividing cells. By varying the paradigm and the examination time points after injection, this simple technique allows quantitative analysis of proliferation, differentiation and survival [80]. Analysis of adult born neurons can since recently also be performed using retroviral genetic marking, since retroviruses also exclusively enter the target cell during mitosis. In combination with the analysis of the expression of specific cellular markers the result is more specific [81]. Developing neurons express distinct markers during their maturation process [62]. For example, for immature newborn neurons doublecortin (DCX) is regularly used, while for mature neurons the specific adult neuronal marker of nuclei NeuN is mostly used.

It is now known that neurogenesis occurs in different species of rodents [82;83], primates [84] and even humans [85-87]. Although newborn neurons have been observed to functionally integrate in the neuronal circuitry, their precise function remains still elusive. Multiple studies have linked adult neurogenesis with functions of the hippocampus, including cognition, emotion, and pattern separation, as well as with the development of psychopathology and recovery from brain damage [88-91]. In addition, adult hippocampal neurogenesis has been found to be bi-directionally regulated by a wide array of factors such as stress, age, environment, hormones, neurochemicals and behaviour (see for an excellent review [80;92;93]).

Neurogenesis is the continuous process of development of new functional neurons from neural progenitors. The GCL of the DG therefore is built "from the inside out".

The process of neurogenesis takes about four weeks, during which newborn daughter cells mature through several stages including proliferation, selection, differentiation, migration and functional integration (see figure 1.2). These developmental stages have each their distinct physiological and morphological properties [94-97]. It is important to keep in mind that following this definition not only cell proliferation, but also cell survival, neuronal cell fate determination (differentiation) and correct incorporation of the newborn neurons are equally important processes.

It has been estimated that several thousands of new cells are generated daily [98-100], but only about 50% of them will survive and ultimately functionally integrate into neuronal circuits. There they remain for several months [77], receiving synaptic inputs [101;102], expressing a neuronal marker [83], extending dendrites and axons [103] and exhibiting electrophysiological properties similar to mature dentate granule neurons [95;98;103-108].

Recently, more and more evidence arises that these adult born granule cells may contribute to hippocampal function.

1.3.2 GC modulation of neurogenesis and neuroplasticity[1]

As the hippocampus is involved in cognitive processes such as learning and memory, it continuously needs to deal with new stimuli, process them, store them and adapt to them. It is now generally accepted that this is facilitated at the cellular level by underlying plastic processes [110]. During such processes, cells, connections between cells and circuitry are remodelled. The connections between (groups of) cells can for example become strengthened or weakened in an activity- dependent way by long-term potentiation (LTP) or long term depression (LTD). Such processes prepare the neurons within a network for their repeated use and facilitate their efficacy in communication. Other forms of (structural) neuroplasticity include the remodelling of elaborate dendritic trees, formation of new synapses (synaptogenesis) and the growth of new neurons (neurogenesis) [111].

GCs are able to modulate hippocampal neuroplasticity, thereby influencing hippocampal behavioural and neuroendocrine output [11;112;113]. A conspicuous feature of GC actions on cellular activity in the hippocampus is the apparent lack of effect when neurons are studied under basal conditions: resting membrane potential and membrane resistance do not show steroid dependence [11]. Only when neurons are shifted from their basal condition, e.g. by the actions of neurotransmitters, do GC effects become visible. This is illustrated by the way in which GCs affect neuronal excitability in the CA1 subfield. Calcium currents, accommodation and serotonin responses are large in both the absence of GCs (ADX) and when MRs and GRs are concomitantly activated. By contrast, these cell properties are small with a predominant MR activation, pointing to a U-shaped dose dependency. Due to these effects on CA1 excitability, hippocampal output is expected to be maintained in a relatively high tone with the predominant MR activation and reduced when GRs in addition to MRs are activated.

Although GC effects for the DG do not seem to follow such a U-shaped dose dependency, the DG, more than any other area in the brain studied so far, requires GC hormone levels to be within in the physiological range [5;44;114;115]. Full ablation of GCs by ADX results within 3 days in reduction of synaptic transmission by LTP [116;117], loss of neuronal integrity (Wossink et al., 2001) [118] and apoptosis of dentate granule cells [119]. Substitution with low doses of GCs, which preferentially occupies MR, can at least fully prevent apoptosis [117]. MR occupation is associated therefore with a neuroprotective effect and an enhanced excitability. However, less clear is the role of the GR in DG physiology. Acute stress and a single injection with high dose dexamethasone (agonist) result in increased apoptosis [120;121]. The effects of acute stress though, are largely normalized within 24 h [121], indicating that the impact of a single stressor is probably limited. Prolonged exposure of animals to high GC concentrations presumably makes dentate granule cells more vulnerable to delayed cell death by excitotoxicity [5].

In line with their growth inhibiting functions, GCs have also been shown to inhibit the proliferation and differentiation of neuronal progenitors, and also the survival of young neurons

[1] This section is partly adapted from [109]

[122;123]. As neuronal progenitor cells (NPCs) have been found predominantly in the direct vicinity of blood vessels [124], they are easily reached and influenced by circulating GCs. GC effects in NPCs are likely to be mediated directly through GR and also indirectly through MR or affecting other mediators of neurogenesis [5], such as growth factors [125-128], cell cycle inhibitors [129;130] and altered glutamate signalling [89;131-136].

The context, time course, duration, and concentration of GCs and the exposure to stressors are essential factors affecting neurogenesis. Removal of circulating GCs following adrenalectomy (ADX) increases cell proliferation and neurogenesis in young adult and aged rodents [82;133;137;138]. This can be reversed by treating ADX animals with a low dose replacement of corticosterone [139;140]. Similar effects on increased cell proliferation and adult neurogenesis were found using other methods of inhibiting HPA axis activity, such as blockade of CRF-1 and V1b receptors [89;141].

In contrast, excess levels of GCs, due to stress or treatment with exogenous GCs, results in structural changes in the hippocampus and a decrease in cell proliferation and neurogenesis both *in vitro* and *in vivo* [11;13;142-147]. These changes, including cell proliferation, cell survival and neuronal cell fate, can all be reversed after brief treatment with GR antagonists like mifepristone [148-151].

In addition to the concentrations of GCs, also the duration and time frame are influencing its effects on cell proliferation and neurogenesis. Temporarily increased levels of GCs after a single stressor in adult rats only mildly and reversibly suppresses proliferation [121], while repeated or chronic stress leads to a more prominent and sustained suppression of neurogenesis [121;152;153]. These experiments typically involve exposure of animals to a variety of mild stressors over a period of several weeks. Stressors include food and water deprivation, temperature changes, restraint and tail suspension [154-156].

However, severe, repeated or chronic stress during sensitive developmental stages leads to a more prominent and sustained suppression of neurogenesis [121;152;153] and can even persist permanently into adulthood beyond restoration of basal HPA axis activity [139;157-159]. Given the differences in the developing and adult brain, an increase in GCs during early postnatal life may therefore have profoundly different effects from those in adulthood and might even lead to an increased sensitivity to GCs.

Furthermore, the nature of the stressor and also the context in which the stressor operates are crucial in determining the effects on neurogenesis. Because under certain circumstances such as learning [160], exposure to an enriched environment [161;162], or voluntary physical exercise such as running [47;163-167], elevated GC levels are associated with enhanced neurogenesis [89;168-170]. Intriguingly, if animals were housed in isolation, the effects of stress and exercise on neurogenesis would be worse than if animals were socially housed [21;171;172]. This so-called glucocorticoid paradox is also shown in rodents where elevated GC levels due to caloric restriction causes increased longevity, whereas elevated corticosterone due to chronic stress does the opposite and enhances vulnerability to disease [21;173].

Thus, in addition to the intensity and duration of the stressor, the nature and context of exposure to the stressor determine whether the outcome is positive or negative. While these observations appear contradictory, a possible explanation of this glucocorticoid paradox may be the manner in

which an organism perceives the specific contexts as stressful, neutral or even pleasurable. It is thought that psychological variables such as predictability and controllability can determine the impact that otherwise identical stressors have on the organism, and are known to lessen or even protect against the negative consequences of stress on brain, body and neurogenesis [174-178]. Although the precise mechanism behind this phenomenon is still unknown, it may partly be explained by the processing of psychological but not physical stressors by the hippocampus (see paragraph 1.2) [8;179].

1.3.3 GC modulation of cognitive performance

Half a century ago, first indications of hippocampal function were observed by physicians studying patients like "patient H.M.". In patient H.M., large part of his medial temporal lobes, including the majority of his hippocampus, were removed in an attempt to stop his severe epileptic seizures. This resulted in severe anterograde amnesia [180]. The patient could not form long-term memory of new events while other types of memory and his general intelligence were intact. Later, studies in both animals [181] and humans [182] have revealed the involvement of the hippocampus in spatial and declarative memory. Since then, much more research on the intriguing functions of the hippocampus has revealed a wealth of information.

It is now known that hippocampal-dependent spatial learning and memory can be separated into distinct phases [183]. Based on lesion studies, computational modelling and physiological evidence, these phases have been attributed to the different hippocampal subfields. It is thought that the CA1 subfield plays a role in consolidation and retrieval processes, and cue related memory, whereas the DG is thought to be more important in the encoding of contextual and spatial information: spatial pattern separation [73;184-188]. The CA3 area plays a crucial role in rapid learning and pattern completion [187].

The hippocampus is particularly involved in the appreciation of (novel) experiences, labelling of declarative memories in respect to context and time and in the organisms' reaction to novelty and its spatial environment. The hippocampus exerts this function by integrating and processing spatial and contextual information of an organisms' environment, with information about the motivational, emotional and autonomic state of the organism [189]. This is in line with the theory that the hippocampus processes psychological stressors. In fact, the ventral part of the hippocampus is tightly linked to the amygdala, a limbic brain structure with a function in organizing fear related behaviours and anxiety. This may explain why emotionally arousing memories are among the strongest [190]. As a consequence, hippocampal function can also be tested in emotional tasks such as contextual fear conditioning [191;192].

There is profound evidence that GCs modulate the memories for these events [113;193;194]. Therefore I will focus here on how GCs and their receptors affect the cognitive functions of the hippocampus. The effects of GCs on hippocampal functioning are dependent on the concentration, timeframe, duration, and context of GCs and stressor modality.

As explained in paragraph 1.2.3, the concentration of GCs determines which receptor is activated. Basal GC levels activate predominantly the MR, which is involved in the acquisition and retrieval

phases of memory. MR activation is also important for reaction to novel information as well as determination of behavioural strategy [195;196]. Experimental removal of even basal levels of GCs by adrenalectomy results in a time-dependent impairment of acquisition of spatial learning and contextual fear conditioning [149;197-199]. This is thought to be contributed to – at least in part- by the DG, as lack of circulating GCs causes loss of dentate granule neurons (see paragraph 1.3.2) [5;119]. In general, a reduction of GR expression or function is associated with decreased memory consolidation [149;200]. The cognitive deficit can be reversed with replacement corticosterone therapy [149;201], although this is only effective if the DG is not completely disappeared [149;202].

In contrast, higher levels of GCs activate the GR, which is required for the consolidation of spatial memory [203-206]. After acquisition, administration of GCs facilitate memory consolidation in MWM under low stress (25° C water) but not high stress (19° C water or predator exposure) conditions, suggesting that moderate stress levels of GCs are beneficial [207;208]. In general, stress- mediated activation and over-expression of GRs are associated with enhanced memory consolidation [149;209]. This is a beneficial situation, as a mild/acute stressor for example, can create a situation of increased arousal, enhanced cognitive capacities and emotional salience enabling the organism to appropriately respond to the stressor and ensure survival.

Chronic stressors, excess of GCs and continuous GR activation are correlated -just as lack of GCs and GR activation- with maladaptive effects on emotion and cognitive performance [2;149;210]. Age-related increases of GCs in humans also are correlated with cognitive decline [211]. The detrimental effects on spatial memory in mice can be reversed by the application of selective and competitive GR antagonists [149;212]. This can also explain the improvement in neurocognitive function and mood following antiglucocorticoid treatment of patients suffering from psychotic depression [213;214] and age-related cognitive decline secondary to elevated GCs [211].

Strikingly, high levels of GCs and stress seem to improve the memory of the fearful event in contextual fear conditioning [149;209]. Although this is dependent on genotype [194;215], and probably also of hippocampal region [216]. However, for these “beneficial” effects not only the concentration but also the timeframe in which they occur is essential. Only when high levels of GCs are present during or immediately following the aversive event, they enhance long-term retention of learning. But when stress and high GCs are applied before the cognitive tasks they have been shown to impair acquisition, consolidation and retrieval [183]. In addition, GCs augment consolidation of fear memory extinction rather than decreasing retrieval or consolidation [149;217].

It seems thus that the timeframe and concentration of GC exposure determine a healthy adaptive stress response. GR-mediated transactivation enhances the storage of newly acquired information, while facilitating extinction of behaviour that is no longer relevant [44;149;149;218-221].

Duration is also an important parameter. A short duration of alterations in GC concentration is generally overcome more or less easily. In fact, a rapid onset of stress-induced GC rise is characteristic for a healthy individual, as long as the GC response is turned off effectively. More chronic elevations or chronic stress therefore are regarded as detrimental. This becomes especially clear in sensitive periods during development. Early life stressors are associated with long-term changes in brain function and behaviour, which can even remain into adulthood, a

phenomenon called developmental programming [222].

The impact of GCs further also depends on the context and the stressor modality. For example, GR activation within the learning context is required for consolidation of spatial information [223;224], whereas GR activation or additional stressors applied before acquisition training or retention testing and which are not related to the learning context may impair rather than improve acquisition and retrieval of spatial memory [218;225]. In respect to stressor modality, it is known that hippocampal lesions cause a prolonged stress response to psychological stressors [8;51;226;227], but not to physical stressors [8;227]. This is explained in the ways these different types of stressors are processed in the different brain regions (see paragraph 1.2) [8;179].

Thus, GCs and their receptors clearly play a vital role in modulating an array of cellular processes. These are underlying the functions of the hippocampus in emotion and cognitive performance.

1.4 GCS IN (PATHO-) PHYSIOLOGY: IMPLICATIONS FOR HIPPOCAMPAL FUNCTION

The hippocampus not only has an important function in emotion, cognitive performance and behavioural adaptation to stress. Recently there is growing evidence that the hippocampus is also a key structure in the pathology and course of several neuropsychiatric diseases and other neurological disorders. There are indications that the structure of the hippocampus is affected as well as hippocampal function. In depression [228;229] and in post traumatic stress disorder (PTSD) [3;230-233], a reduction in hippocampal volume, associated with disturbances in mood, cognition, and behaviour are commonly reported. Typically, the frequency and the duration of the untreated illness, instead of the age of subjects, predicts a progressive reduction in volume of the hippocampus [234;235]. In addition, malfunctioning of the hippocampus is observed in aging and dementia [236], and a variety of other diseases such as Cushing's disease, diabetes, schizophrenia and epilepsy [111].

In the following section, I will review the current evidence of how hippocampal dysfunction is associated with disease and how the stress system might be involved. To this end, I will illustrate two mechanisms or theories; 1) the stress theory, and 2) the neuroplasticity theory[2].

1.4.1 The stress theory

In previous paragraphs I have shed light on the functions of GCs and their receptors in neuroplasticity and hippocampal function. It was illustrated how GCs and their receptors function -with respect to adaptation- in a U-shaped-dose relation. This implies that too high levels of GCs are as detrimental as lack of GC signalling, and that there is a certain optimum in the middle, where levels of GCs are contributing to cellular integrity and stable excitability in the

[2] This section is partly adapted from [109]

hippocampus favourable for behavioural adaptation [237]. Although lack or excess of GCs are not directly life threatening, on the long run these conditions can have serious consequences. There is strong evidence that in genetically predisposed or otherwise vulnerable individuals, chronic stress, HPA axis hyperactivity is a primary, causal factor in the pathogenesis of neuropsychiatric disorders, such as depression [4;232;238-240].

Depression is a serious multifactorial disorder with a complex clinical nature [241]. The symptoms of depression fall into three primary categories, including changes in mood/ emotion (e.g. sadness, anhedonia, irritability), basic drives (e.g. eating, sleeping), and cognitive disturbances (e.g. memory loss, indecisiveness, guilt) [242]. The diversity of symptoms suggests that multiple neuronal (limbic) circuits are likely to be involved, such as the prefrontal cortex, hippocampus, amygdala, and nucleus accumbens [3;11;13;41;243]. All these structures are modulated by GCs [244;245] but in investigations of the neural substrates, especially the hippocampus received a lot of attention. It is connected to multiple other brain regions and underlying several of the emotional and cognitive symptoms seen in neuropsychiatric disorders [246]. In addition it is very sensitive to GCs. In fact, the disturbances in mood, cognition, behaviour and hippocampal atrophy coincide with abnormal levels of GCs in both humans [7;90;247]. Vice versa, chronic stress and elevated GCs in animals lead to hippocampal dysfunction and other symptoms of depression [6;154;155;248]. Major stressful or traumatic events seem to precede or even trigger depressive episodes, and about 50% of the depressive patients display hypercortisolemia, which appears to exist prior to the onset of clinical symptoms of depression [4;231;249].

Typical observations done in depressed patients with a hyperactive HPA axis are: reduced GR function as tested in the dexamethasone (DEX) suppression or the DEX-CRH test [230], elevated amplitudes of cortisol secretory periods [250;251], an increased frequency of adrenocorticotropic hormone (ACTH) secretory episodes [252], and several other aberrations at different levels of the neuroendocrine system [230;233;253;254].

There appears to be a direct correlation between the severity of symptoms and circulating cortisol levels [255;256]. This conclusion is strengthened by observations in patients receiving exogenous GCs, such as prednisolone. Particularly when given at high doses for extended periods of time, these produce symptoms that include depression, hypomania, insomnia, cognitive deficits and psychosis [257;258]. Also, patients suffering from elevated GC levels secondary to Cushing's disease illustrate the link between GCs and depression as they often suffer from anxiety and depression and in some cases from psychosis and suicidal thoughts [259].

These symptoms of HPA hyperactivity can typically be reversed with antidepressant (AD) treatment in both humans and animal models [260]. Moreover, some ADs have direct effects on the GR [261] and potential novel ADs, as galanin, modulate HPA axis activity and enhance GC secretion, suggesting a tight interaction with the GR/GC system [262;263]. Interestingly, short-term treatment (4 days) with GR antagonist mifepristone has been successfully applied to treat/ameliorate depression with psychotic features in clinical trials. It was found that mifepristone reduced depressive symptoms in a subset of severely depressed patients with highly elevated GC levels [254;260]. However, only high doses of mifepristone are effective [255], and these doses are often

associated with adverse drug effects, although not uniformly across patient populations. These adverse effects include fatigue, anorexia and nausea.

In spite of all the evidence, a direct causality in between HPA axis hyperactivity, hippocampal dysfunction and depression is still circumstantial. Also unclear is the underlying mechanism. Still, GR function seems altered in depression. There are two theories for a possible mechanism.

1) The glucocorticoid cascade hypothesis explains how a tightly regulated system -the HPA axis- can spin out of control through a cascade of events and eventually leads to disease. Chronically raised levels of GCs, as for example occurs during chronic stress, can trigger this cascade and become maladaptive as the continuous stress response becomes more damaging than the initial stressor itself. Energy resources become depleted, oxidative damage increases, immune responses are suppressed, physiological and behavioural adaptations become compromised and then inevitably enhanced vulnerability to additional challenges and disease is produced [6;111;264;265]. Since the elevated GC concentrations downregulate the GR in central feedback sites leading to further disinhibition of the HPA axis, the condition is further aggravated in a feedforward vicious cycle.

2) The MR:GR balance hypothesis focuses on aberrant receptor functions as the primary cause of enhanced vulnerability or resilience. It is proposed that once the balance in actions mediated by the MR and the GR is disturbed, the individual is compromised in the ability to maintain homeostasis if challenged, for example by experiencing an adverse life event. This may lead to a condition of neuroendocrine dysregulation and impaired behavioural adaptation as risk factor for the precipitation of depression [3;11;39]. While GR over-expression or enhanced receptor function is correlated with post traumatic stress disorder (PTSD) [266], several lines of evidence have suggested that impaired GR function, is a primary, causal factor in the pathogenesis of depression [230;267].
The MR:GR balance hypothesis refers to the limbic circuitry e.g. hippocampus, and amygdala frontoparietal cortex, where both receptor types are abundantly expressed [8;11;14;39;51;52]. In this limbic circuitry psychosocial stressors are processed. Via limbic MR, GCs modulate appraisal of novel experiences and influence the selection of the appropriate behavioural response. If during the stress response the rising GC concentrations activate GR, the storage of the experience is promoted in preparation for the future. MR therefore organizes the stress response, which is terminated via GR. The rapid effects are mediated by the membrane MR, while the genomic MR variant is crucial for integrity of the hippocampus and a stable excitatory transmission in the limbic circuitry, which is suppressed via GR, if transiently raised by stressors [237;268].

The MR:GR balance can be altered by (1) genetic predisposition, resulting in a vulnerable phenotype with an altered behavioural pattern and altered HPA axis response to stressors [11;269]. Hence, GR variants exist that provide either higher sensitivity or resistance to the GR [270]. Recently, also MR gene variants were identified that enhance the expression of this receptor in

hippocampus and are associated with resistance to depression [271]. (2) it has been shown that early life experiences themselves also can interfere with long lasting changes in steroid receptor expression by an epigenetic mechanism [21;272-274]. Of particular importance is the quality of maternal care. Offspring of high licking and grooming mothers invariably has a high GR and MR expression in hippocampus. In addition to genetic predisposition and the impact of stressful early life events, the susceptibility to the disease state is further enhanced by (3) a subsequent challenge, such as a later life psychological stressors which are particularly potent if occurring in a repeated fashion under conditions that there is no prediction and no control possible over the psychosocial challenge [268;275].

Thus, the cumulative exposure to genetic and adverse early cognitive inputs leaves a signature in developmental programming of limbic (and hippocampal circuitry) in anticipation of later life conditions. This signature is characterized by dysregulation of the neuropeptides CRH, vasopressin and opioids as well as the GC hormones and its receptors. If these later life conditions do not match with the expectancy, vulnerability to disease is increased [222]. Therefore, the condition of uncontrollable, repeated stressors supposedly has the most devastating effect in well-groomed pups. The brain effects of genetic input combined with the effect of factors released by early and later life experiences is often called the “three hit hypothesis” [21].

1.4.2 The neuroplasticity theory

The neuroplasticity theory explains how hippocampal dysfunction, due to changes in neuroplasticity and neurogenesis, is underlying disease. According to this theory, a decrease in hippocampal neurogenesis is related to the pathophysiology of depression while enhanced neurogenesis is necessary for the treatment of depression [90;91;247;276-279]. However, thus far there is no evidence that the reduction of neurogenesis is causally related to the aetiology of depression [245;280], rather in rodents neurogenesis appears induced by chronic antidepressant treatment (see below).

Nevertheless, decreased neurogenesis could affect neuronal function in the hippocampus in different ways [244]. One way in which impaired neurogenesis could lead to depression is by weakening the mossy fibre pathway in the hippocampus. As the mossy fiber synapses are involved in controlling the dynamics of excitation and inhibition within CA3 [281], a decreased dentate gyrus-CA3 connectivity could result in a downward spiral leading to impaired learning and decreased possibility of coping with a complex environment, further impairing neurogenesis. In fact, this hypothesis is strikingly similar to what is observed in depressive patients: they show aversion to novelty and withdrawal from activities and challenges which traps them in a vicious circle [244;245;278;282].

Less speculative are the preclinical indications that adult hippocampal neurogenesis is necessary for mediating some of the behavioural effects of antidepressants. Remarkably, the delayed therapeutic actions of all major classes of marketed ADs (which take two to four weeks to develop) [283] coincides with the timescale of hippocampal neurogenesis and neuroplasticity [242;284].

It is notable that the induction of cell proliferation and neurogenesis is contingent upon chronic but not sub-chronic (acute) SSRI treatment [248;285-289]. Moreover, the unique physiological properties of adult-born dentate granule neurons, in terms of their location within the hippocampal neuronal circuitry and their functional plasticity, suggests adult neurogenesis as a potential common pathway associated with the functional effects of antidepressants [94;245]. Mature adult-born neurons may also contribute to the behavioural effects of SSRIs. This is in line with the observations that enhancing neurogenesis is necessary to exert antidepressant-like effects in animal models [280;287;290-292].

1.4.3 GCs, neuroplasticity, and hippocampal function in health and disease: A convergence of mechanisms?

The above described hypotheses are not mutually exclusive, nor do either of them completely fit reality. The stress theory of depression for example does not fit all patients, as only 50% of them suffer from HPA hyperactivity. On the other hand, the neurogenesis theory has a flaw as well as some studies point out that AD- behavioural effects can also be achieved in the absence of neurogenesis [293;294].

Depression is of course a very complex disorder and it is certain that factors other than stress and neurogenesis are involved. However, it is likely that stress and neurogenesis interacting together in modulating hippocampal function and underlying disease is a more appropriate hypothesis to model the situation, rather than either of the theories alone.

There are several lines of evidence for this hypothesis. As outlined in paragraph 1.4.1, several classes of ADs, with distinct modes of action, often restore HPA function in both humans [4] and animal models [43] while also boosting neurogenesis [295;296]. A recent study has shown that from a group of rats exposed to chronic stress, only a subset responded behaviourally to treatment with the antidepressant SSRI [297]. Interestingly, neurogenesis was restored to normal levels only in the behaviourally identified responders. In fact, the correlation between HPA axis functioning and AD effects is reinforced by the observation that distorted HPA axis diurnal rhythms prevented ADs to stimulate cell proliferation and hippocampal neurogenesis in rats [298]. These consistent observations support the possibility that reducing stress/ HPA activity and increasing neurogenesis is a common pathway through which ADs exert their behavioural and therapeutic effects on depressive symptoms [152;245;299;300].

Although the precise mechanism is not clear [244], it is thought that stress, GCs and their receptors are involved by the regulation of neurogenesis and neuronal plasticity and thereby affecting hippocampal function [282;301-303]. This hypothesis needs further study, since it is mainly based on rodent studies.

Box 2 Context of GR research in animal models

Since *in vivo* expression and functional studies are not feasible in humans, and the possibilities with *in silico* and *in vitro* studies are restricted, research has focused on experimental animals. Of course, with the use of experimental animals the complexity of human nature cannot completely be mimicked. The advantages however, are twofold. On the one hand, the similarity of rodents to humans in for instance "the stress response" makes it possible to investigate the function of specific genes by manipulating them artificially. This gives also fundamental information for the human situation.

Apart from these fundamental objectives, animal models can also be used as disease models which reflect core features of the respective human disorders. This enables to investigate the underlying mechanisms of human diseases and validation of possible drug targets for these diseases. A better understanding could allow the design of better treatment strategies with specific molecular target sites and fewer side effects. For this purpose, the human benefits are weighed against the animals' suffering by ethical committees.

As for a number of neurological and behavioural disorders/ syndromes, there is not a single gene responsible, but a complex multi-genetic background. This is especially true for stress-related neuropsychiatric diseases. Therefore mouse models based on single gene manipulations unlikely can be expected for truly mimicking this phenotype. However, such models can be used to study parts of it, such as specific symptoms or traits, so called "endo-phenotypes" [299;304;305]. This type of research is often performed in mice (Figure 1.4).

Figure 1.4 A fearless mouse... *A mouse model in which the smell was impaired by genetic manipulation, lost its display of anxiety behaviour to its predator (only when the cat is silent)* [306].

1.5 THE GLUCOCORTICOID RECEPTOR AS SUBJECT OF RESEARCH: ANIMAL MODELS

As discussed previously, the GR is a key regulator of the HPA axis, neuroplasticity, hippocampal function and also implicated in the pathogenesis and course of stress-related-disorders. In addition, drugs targeting the GR are used widely in clinic. GR agonists such as prednisolone are applied because of their powerful anti-inflammatory and immunosuppressive effects, while mixed progesterone- and GR antagonists such as mifepristone are used for example for abortion. Because of this important role, the GR has been a subject of research for decades. Most of this

research has been done in animals. By selective breeding for example, several strains of mice and rats have been generated with different stress-responsiveness, neuroendocrine, neurogenic, physical and behavioural phenotypes that are heritable and stable [307]. However, often the underlying molecular mechanisms leading to the differential phenotypes are complex and poorly understood. Another approach is therefore selectively targeting the different known elements and genes of the stress system and investigating the consequences. By manipulating GR *in vivo* - either pharmacologically or genetically-, the capacity to stress adaptation and sensitivity for stress-related-disorders can be investigated at the level of neurophysiology, cognition, emotion and motivation. In this section, I will describe the various animal models for the study of GR and then summarize their major cellular, HPA axis and behavioural changes (see for review: [113]).

1.5.1 Pharmacological models

As previously described (see paragraph 1.3) the HPA axis can be activated in rodents to different degrees; ranging from mild (e.g. handling, needle stick, novel environment) to moderate (e.g. swimming in MWM) to severe stressors (e.g. acute or chronic restraint). In addition, a distinction can be made between different types of stressors: physical or psychological stressors. The last condition is most severe as the individual has no control over the situation, prediction of an upcoming event, uncertainty and fear. Using these different types of stressors, investigators have been able to dissect the role of the GR in stress- associated HPA axis functioning [113]. For example, as tested during the conditioned emotional response (fear conditioning).

A more precise way of controlling stress-associated GC levels is in classical pharmacological substitution experiments. By adrenalectomy, the endogenous source of GCs is removed and hormone levels can be accurately manipulated by substitution with exogenous ones. Because some mice still have a residual GC secretion after adrenalectomy due to accessory adrenal tissue, their MR rather than GR is occupied. This is therefore a good model for investigating GR in the context of basal MR activation. Subsequent alterations in GC dose can for example be achieved by the implantation of corticosterone pellets [308], systemic injections [207] or local injections in the brain [309]. In such animal models, different types of stressors can be applied to see how it reacts.

However, hormone depletion by adrenalectomy has some disadvantages. Apart from leading to GC depletion, it also results in the removal of mineralocortioids and catecholamines and the replacement of GCs only allows a crude assumption of receptor occupancy [310]. In addition, agonists and antagonists may have a certain level of unspecificity, and therefore they may also target other nuclear receptors. Moreover, they often poorly penetrate the brain as they are not able to pass the blood brain barrier. RU38486 for example, needs therefore to be administered in 10^6 higher dose systematically than in the brain to achieve the desired effect [149].

1.5.2 Genetic models

Another approach to correlate altered steroid signalling and stress with physiological and behavioural changes involves the use of animals with a genetic GR manipulation. Several mouse

lines have been generated in which GR expression or function is altered throughout the body (see for reviews [23;113;266;311]): 1) GR antisense mice, 2) two different conventional knockout approaches, 3) partial knockouts, 4) mice with disrupted GR dimerisation, 5) mice with a chimeric ER/GR receptor, 6) GR over-expression mice, and 7) Polymorphic GR mice.

1) GR antisense mice (AGR mouse line). The first published genetic model of glucocorticoid disruption involved the introduction of antisense GR cDNA into the mouse genome and is known as the antisense GR mouse [312;313]. A 1.8 kb fragment of the GR cDNA was inverted and placed under the control of the neurofilament promotor. This strategy was designed to reduce expression of endogenous GR in the nervous system. However, inconsistent expression of the transgene induced differing amounts of reduced GR expression in neural (e.g. 50–70% decrease in the GR expression) and non-neural (e.g. 30–50% reduction in liver and kidney) tissue, limiting the interpretation of the data. GR reduction in these mice resulted in changes in energy balance and lipid metabolism. Similar to the human situation, GR antisense mice show an impaired negative feedback loop of the HPA axis with a blunted circadian rhythm and lack of response to the Dexamethasone Suppression Test. The HPA hyperactivity becomes apparent under stressful conditions [313;314], but can be reversed by antidepressant treatment [315;316]. At the behavioural level, the GR antisense mice were intensively studied [316] and found to present a reduced anxiety behaviour as well as several cognitive deficits for hippocampus-dependent memory tasks, such as the Morris Water Maze.

2) Conventional GR knockouts. To investigate the effects of loss-of-function for the GR, two conventional knockout animals have been produced: Exon 2 targeted GR^{Hypo}; [317] and Exon 3 targeted GR^{Null}; [318]. The GR^{Hypo} mice were developed by inserting a PGK-Neo cassette into Exon 2 of the GR gene, a region involved in transactivation, while the GR^{Null} mice were developed using mutant mice containing loxP sites surrounding Exon 3, a region involved in DNA binding. It has been reported that most of the GR^{Hypo} mice and all of the homozygous GR^{Null} mice died in the first hours of life from severe lung atelectasis [317;319], demonstrating an essential function of the receptor for survival. The surviving fraction of mice, 5-10%, display the characteristic insensitivity to GCs and an impaired negative feedback regulation of the HPA axis leading to extreme elevations in both plasma-ACTH (15-fold) and -corticosterone (2.5 fold) levels [317;320]. At the behavioural level, these mice displayed hippocampus- dependent memory deficits in several tasks. These mice were further investigated for the presence of aberrantly truncated GR proteins to explain the phenotype of the survivors. Analysis showed that GR^{Hypo} mice on an outbred strain have a truncated GR with a ligand-binding domain that can bind the synthetic glucocorticoid dexamethasone [320]. So, GR^{Hypo} mice may have some remaining GR function that could limit interpretation of findings, particularly when differences in action are not found.

3) Partial knockouts. Heterozygotes of both conventional GR knockout models survive into adulthood and have been convenient as these mice models aimed to model human disorders,

may mimic more naturally the situation of patients with affective and stress-related disorders as receptor expression reduction is more likely than a full knock out [321]. Heterozygotes have a ~50% reduction of GR protein expression in the brain and have been used to study a variety of physiological, endocrine and behavioural factors [318;322]. Typically, these mice under normal circumstances resemble wild type controls [318]. Only when subjected to stress, $GR^{+/-}$ mice show a genetic predisposition for depressive-like behaviours and depression-like neuroendocrinological abnormalities. Importantly, these mice show hippocampus- dependent deficits in spatial memory when tested in the Morris Water Maze.

4) Mice with disrupted GR dimerization. As described in paragraph 1.2.2, glucocorticoid binding to GR can induce cellular changes through dimerization-dependent and independent actions. To investigate these two types of GR activity on a variety of cellular processes, a GR mutant with a point mutation in Exon 4 was developed ($GR^{Dim/Dim}$) [323]. Using a knock-in strategy replacing the endogenous GR gene, a point mutation (A458T) was introduced in the dimerization domains of the gene. A458T, had previously been shown to disrupt D loop formation causing a loss of GR dimerization and direct DNA binding [26]. Interestingly, the resulting $GR^{Dim/Dim}$ homozygous mice are born at the normal Mendelian ratios from heterozygote to heterozygote pairings and did not show signs of increased mortality. Consequently, $GR^{Dim/Dim}$ mice are unable to control GRE-driven genes by direct, cooperative binding of the receptor to the DNA, but able to indirectly influence gene transcription by modulation of other transcription factors via protein-protein interactions. As these mice did not die after birth, it appears that the transrepression mechanism is important for survival. In addition, based on their neuroendocrine profile, it appears that the mechanism of protein-protein interactions is important to some but not all aspects of GR-mediated negative HPA axis feedback at the level of the hypothalamus [324]. On the behavioural level, these mutant animals displayed selective GC-dependent deficits in spatial memory in the Morris water maze (MWM; a hippocampus- dependent task) [325], but no alterations in anxiety-related behaviour.

5) Mice with a chimeric ER/GR receptor [208]. In this transgenic mouse line the DNA-binding domain of the GR gene is replaced by the homologous part of the estrogen receptor. The gene still contains the GR-ligand-binding domain. As a result ER/GR transduces deleterious GC signals into beneficial estrogenic ones as estrogen is associated with enhanced hippocampus-dependent spatial memory performance which can counteract the deleterious effects of GCs.

6) GR over-expression mice. To complement the loss-of-function studies, an YGR mouse model has been generated in which GR is over-expressed. This was achieved by introducing two additional copies of the full length GR gene using a yeast artificial chromosome [326]. These mice over-express GR mRNA by 25% and GR protein by 50%. Phenotypically, they display a strong suppression of the HPA axis which reflects an increased GR negative feedback control in the HPA system [318;326]. These over-expressing mutants provide an interesting framework to study the effects of increased GR activation on stress-mediated adaptations.

7) Polymorphic GR mice. This mouse model (GR^{Qn}) was generated by divergent genetic selection of two strains of mice [327]. High Stress and Low Stress strains with different allele frequencies of GR were selected for an altered corticosterone response to stress. These mice, when tested, showed an altered stress-response and increased anxiety-type behaviours.
The above described mouse models with systemically altered GR expression, clearly demonstrate the pronounced effects on the HPA axis and behaviour, but one disadvantage is that they are not very specific for the brain. In fact, these systemic mutants have a number of peripheral changes in metabolism and immune function. Although, not fully characterized, these peripheral changes make it more difficult to derive specific correlative conclusions about the role of GR in stress and nervous system function. Therefore more refined animal models are necessary with targeted GR expression in the brain.

1.5.3 Brain-specific genetic modifications

Instead of a constitutive loss or gain of genetic function, new advanced techniques allow temporal and spatial control of gene expression in the adult central nervous system (CNS). These innovations allow conditional gene disruption in specific anatomical regions at chosen time points. For example, the Cre/Lox recombination system enables the selective disruption of a gene conditionally induced by tamoxifen [328]. These approaches can also be used to investigate GR in specific cell types without affecting its activity in other cells of the organism. In addition to the "confounder" of gene effects in respective other cells, this allows investigating gene function in adult animals without the drawback of developmental illnesses and genetic compensation/ adaptation mechanisms. To more precisely define the role of GR in the CNS, several mouse models have been generated (see for reviews [23;113;266;311]): 1) The nervous system specific GR knockout mouse, 2) the forebrain-specific GR knockout, 3) the forebrain-specific inducible GR knockout, and 4) the forebrain-specific GR over-expression mouse.

1) The nervous system specific GR knockout (GR^{NesCre}) [319]. In these mice GR expression is deleted specifically throughout the nervous system in both neurons and glia. The Cre-loxP model starts with two types of genetically altered mice. In one, the exon 3 of the GR gene is flanked with loxP sites. Mating these mice with second type containing nestin- Cre recombinase, results in offspring with deletion of GR in all CNS neurons and glial cells. GR^{NesCre} mice have normal survival but exhibit a Cushing's syndrome-like phenotype. These mice have altered fat deposition with lowered weight gain, osteoporosis, and immunological abnormalities. At neuroendocrine level GR^{NesCre} display a strong activation of the HPA system with markedly elevated levels of circulating corticosteroids following a preserved but blunted circadian rhythm due to the lack of the negative feedback normally exerted at the level of the hypothalamus via GR. Investigation of GR downstream MAPK targets revealed a down-regulation of p-ERK1/2, Ras, Raf-1 and Egr-1 with potential implications for stress responsiveness and fear-based learning and memory [329]. Indeed, inactivation of brain-GR reduced anxiety behaviour in two tests. In cognitive studies, the absence of GR signalling in the brain of the GR^{NesCre} mice exhibited a mild memory deficit in the MWM task,

therefore these mice possibly have some cognitive deficits [319;330]. Unfortunately in GR^{NesCre} mice GR is deleted in the PVN, a site of major negative feedback inhibition of the HPA axis, leading to severe hypercorticism and wasting, confounding behavioural analysis. Furthermore, in the GR^{NesCre} mice, and all the above described systemic GR mouse models, GR is deleted early in development. This makes it difficult to separate effects resulting from alterations that occur during development from those resulting from an acute requirement for GR.

2) The forebrain-specific GR knockout (FBGRKO). To test the effects of acquired GR disruption in adult mice, Boyle et al (2005) developed forebrain-specific GR knockout (FBGRKO) mice [331]. This mouse model was again produced by mating two types of mice: mice containing a floxed GR Exon 1C through 2, with mice containing Cre recombinase expressed selectively in the CamKII promoter. Using this strategy, promoter elements at the normal translation start sites for GR are deleted progressively from the age of 3 weeks till 6 months in neurons (and glia?) of the forebrain. This results in GR knockout in regions as hippocampus, cortex, basolateral nucleus of the amygdala (BLA) and nucleus accumbens, but intact GR expression in the PVN, thalamus and central nucleus of the amygdala (CeA) [332]. Truncated GR were not detected [333].
Mice exhibited hyperactivity of the HPA axis, impaired negative feedback of the HPA axis upon acute psychogenic but not physical or unpredictable chronic stressors, increased depression-like behaviour, and decreased anxiety-like behaviours in specific tests [8;331;332]. In this mouse model, the depression-like behaviours, but not the anxiety-like behaviour can be reversed by chronic antidepressant treatment (tricyclic imipramine).
The additional spatial specificity (i.e. forebrain only) and temporal aspects of deletion (i.e. deletion after 3 months of age) make the FBGRKO mice a particularly interesting model to investigate the role of extra-hypothalamic sites of GR on basal and stress-induced HPA axis activity as well as the role of GR in limbic modification of behaviour in the absence of non-specific developmental changes.

3) The forebrain-specific inducible GR knockout ($CaMKCreER^{T2}$) [328]. To achieve cell-type specific GR gene inactivation, this mouse model was produced by mating brain-specific Cre mice with GR^{flox} mice. Subsequently, in offspring the Cre/ LoxP receombination system was advanced by the tamoxifen-inducible protein consisting of the Cre recombinase and the mutated ligand binding domain of the human oestrogen receptor to achieve ligand-dependent Cre activity. The unliganded form of the CreERRT2 fusion protein resides in the cytoplasm and, upon tamoxifen binding, translocates into the nucleus and mediates site-specific recombination of the LoxP-flanked DNA sequence. Phenotypically, these mice showed spatially restricted loss of GR protein expression in neurons of the adult forebrain, including the hippocampus, upon tamoxifen treatment. Also, it was observed that these mice display an increase in basal morning corticosterone levels 6 weeks after tamoxifen treatment [328].

4) Forebrain GR over-expression mouse [334]. In this mouse model (GR^{Ov}), GR was over-expressed by introducing a transgene containing the CamKII promoter driving expression of the GR cDNA. GR^{Ov} mice exhibit about 78% over-expression of GR in the forebrain (including the cortex, hippocampus, CeA, BLA and nucleus accumbens) as well as the PVN, and possibly includes ectopic expression of GR within groups of neurons not normally expressing GR in the CNS such as the suprachiasmatic nucleus (SCN) [335]. Importantly, GR over-expression excludes the cerebellum, thalamus and anterior pituitary gland as well as all peripheral organs. GR^{Ov} mice show increases in CRHmRNA in the CeA and in expression of various neurotransmitter transporters [334]. GR^{Ov} mice offer the opportunity to investigate the role of increased GR in important limbic areas with the caveat that PVN over-expression of GR might make it difficult to disentangle hypothalamic vs. extra-hypothalamic GR modulation of HPA axis drive. Phenotypically, these mice presented increased levels of anxiety and despair in a number of specific tests.

The above discussed variety of mouse models give clear insights into GR functioning in the CNS and brain. GR seems indeed implicated in emotion and cognitive performance. However, the diverse models have gained conflicting results regarding their role in hippocampal functioning. And still, there is not much known about the function of GRs in individual cells and discrete locations of the brain. This requires studies in more detail. With the advancement of refined molecular tools, it has become now possible creating animal models in which the functions of genes can be investigated in discrete brain regions and cell types. Therefore, the aim of my PhD project was to investigate the function of the GR specifically in the adult born dentate granule neurons of the hippocampus in respect to stress- related behaviour, neuronal networks and neurogenesis. The strategy of choice was an animal model in which GR expression could be reduced both selectively in place as well as time.

1.6 RNA-INTERFERENCE TECHNOLOGY

1.6.1 Biological function

RNA-interference (RNAi, see Box 1.3) is a natural process triggered by double stranded RNAs (dsRNAs) [336] that cells use to turn down, or silence, the activity of specific genes. The phenomenon is highly conserved, as it is thought to have evolved about a billion years ago, before plants and animals diverged. The process exists in a wide variety of organisms, including single-celled organisms, fungi, plants [337], worms [338], mammals [339] and even humans [340]. In cells, RNAi has been implicated in temporal and spatially restricted gene regulation, imparting roles in brain morphogenesis and neuronal cell fate (reviewed in Davidson et al., 2007) [341].

Box 3 Discovery of RNA-interference

The first discovery of the RNA-interference (RNAi) mechanism was by a lucky accident and occurred in petunias. Dutch researchers aimed to deepen the purple colour of petunias, by injecting the gene responsible into the flowers. But they were surprised by the results. Instead of a darker flower, the petunias were either variegated or completely white (Figure 1.5)! This phenomenon was termed co-suppression, since both the expression of the existing gene (the initial purple colour), and the introduced gene (to deepen the purple) were suppressed. Co-suppression has since been found in many other plant, fungi and animal species. It is now known that double stranded RNA is responsible for this effect: RNA interference. In 2006, Fire and Mellow were awarded the Nobel Prize for describing the phenomenon which is among one of the major discoveries in cell biology. Currently, this biological mechanism is applied as scientific tool for investigating the function of genes. This has become even more important since the sequenced human genome has revealed the presence of a staggering number of 30.000 genes. In addition, RNAi is being tested for possible applications in gene therapy. By the ability to knock down the expression of disease genes, RNAi is a promising new cure for a number of diseases previously no treatment was available for, such as cancer, viral infections, prion diseases and neurodegenerative disorders like ALS.

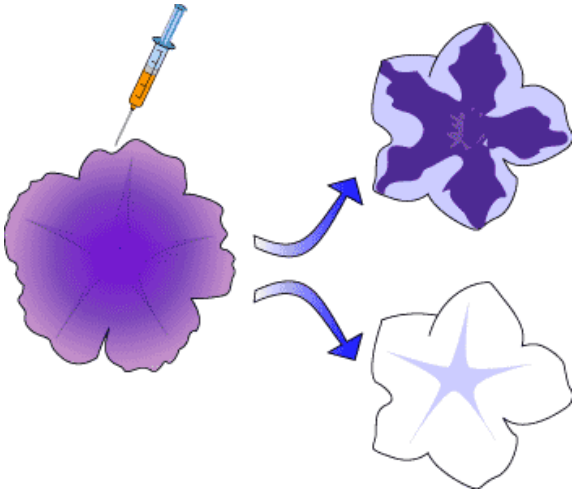

Figure 1.5 Discovery of RNAi phenomenon in purple petunias. *A variegated petunia, upon injection of the gene responsible for the purple colouring in petunias, the flowers became variegated or white rather than deeper purple as was expected.*

In addition to functioning in endogenous gene regulation (for example as an epigenetic mechanism during development), RNAi may originally have evolved to prevent or control genetic instability by silencing repetitive genes and transposons. Transposons are genetic elements in a double stranded RNA form, which can wreak havoc in the DNA by jumping from spot to spot on a genome, sometimes causing mutations that can lead to cancer or other diseases. The RNAi mechanism is triggered by the transposons and mediating their break down. Adding up to these cellular functions of RNAi, it is highly likely that RNAi has also evolved as a cellular defence mechanism against invaders such as RNA viruses. When they replicate, RNA viruses temporarily exist in a double-stranded form. Like transposons, this double-stranded intermediate would trigger RNAi and inactivate the virus' genes, preventing an infection [342].

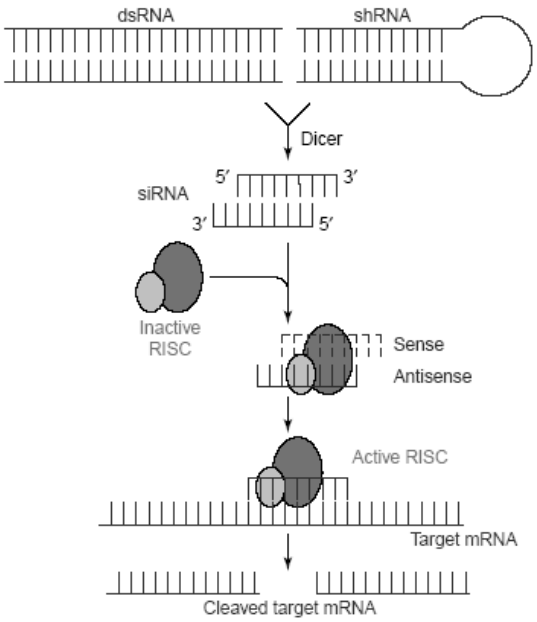

Figure 1.6. Mechanism of RNA-interference. *Upon entering a cell, the double-stranded RNA molecules that trigger RNAi are cut into small fragments by the RNAse called Dicer. The small fragments then serve as guides, leading the cell's RNAi machinery to mRNAs that match the genetic sequence of the fragments. The machinery then slices these cellular mRNAs, effectively destroying their messages and shutting off the protein expression of the corresponding gene. Figure by* [343].

1.6.2 Mechanism of gene knockdown

RNAi works in highly specific fashion by destroying the molecular messengers (mRNAs) that carry information coded in genes to the cell's protein factories (figure 1.6). In mammalian cells, the process starts when small interfering RNAs (siRNAs) are produced by enzymatic processing from double stranded RNAs (dsRNAs) by the RNase III class endoribonuclease Dicer. The newly formed siRNAs, usually about 21 base pairs in length, associate with Dicer, and other factors to form the RNA-induced silencing complex (RISC). As only one strand of the RNA is needed, once RISC has been associated, the non-functional "passenger strand" is discarded, whereas the other "guide strand" is retained and further directs the sequence specific gene silencing by disintegrating the mRNA of the target.

The cellular RNAi machinery can be triggered endogenously by a variety of dsRNA sources, such as micro RNAs (miRNAs), transposons or viruses. It can also be triggered exogenously by the delivery of short hairpin RNAs (shRNAs, basically processed pre-miRNAs) or short interfering RNAs (siRNAs, or "mature" miRNAs) which can be made homologous to the target mRNA and then guide its sequence specific degradation (by the hydrolysis of complementary strands).

The inhibitory RNAs can be designed specifically against the sequence of the target gene mRNA by a set of "design rules". For reliable results, the specificity of RNAi must therefore be well

considered for its applications as a biological or therapeutical tool. The silencing can sometimes be non-specific or resulting in off-target effects on other genes, when siRNAs bind to and regulate unintended mRNA targets. Engineered siRNAs, shRNAs and miRNAs utilize endogenous RNAi machinery and can therefore at high doses cause toxicity independent of the sequence [341]. Also, dsRNAs can, when introduced into mammalian cells, lead to an interferon response resulting in cell death and global gene silencing, but this can be circumvented by directly delivering siRNAs [339].

1.6.3 Application of RNAi in functional genetic analysis and gene therapy

After its discovery, the RNAi mechanism was rapidly used as a tool to investigate gene function (functional genetic analysis). The strength of RNAi as a research tool has an enormous potential impact on medicine. Knocking down a gene's activity yields a wealth of information about its functions in cellular pathways and could lead to new therapy targets. But prior to the discovery of RNAi, the process was laborious and could take months, especially in transgenic (knockout) animal models, of which the development is laborious, costly and cumbersome.

Investigating genes optimally requires control over gene expression in place and time. Place, because for example genes may have different functions in different cell types or tissues, and time because genes may have different functions in development. Careful control over gene expression may therefore limit unwanted side effects and compensation mechanisms. Use of RNAi -together with a suitable delivery approach- enables this and therefore is preferred over other strategies of gene manipulation (see Table 1.1).

Pharmacological: (ant-) agonists + Rapid action + Systemic or local delivery + In almost any model organism ± Short term, reversible approach ± More or less selective ligands available - Slow development	**Immunological: antibodies** + Rapid action + highly specific + In almost any model organism ± Systemic delivery only ± Short term, reversible approach - Slow development
Genetical: transgene and gene targeting approaches + Long term effect, irreversible + Inducible, reversible systems possible - Involves removal of part of the genes on both alleles - Developmental disruptions, side effects - Confounding compensation mechanisms and adaptations - Time consuming and costly - Preferably in mouse	**Post-transcriptional: RNAi** + Highly sequence (even allele) specific + Time, location and cell specific delivery + Inducible and transgenesis systems possible + Relative fast and easy technique + Reversible and irreversible approaches + In almost any model organism ± Partial knockdown gene function - Only inhibition of gene expression possible - Possible off-target effects

Table 1.1. Comparison of different techniques for the manipulation of the GR gene, its mRNA and protein products. *See for in vivo applications of the pharmacological and genetical approaches for GR also §1.5.*

Other advantages of the technique are its superb sequence specificity (even alleles), the possibility of inducible, reversible and permanent approaches and the partial knockdown of gene function, which mimics natural circumstances more than in full knockout approaches. In fact, partial inhibition of target gene function is also closely mimicking the approach of pharmacological inhibition for validating genes by antagonists [344].

RNAi can be used as a research tool to silence selected genes quickly and easily, investigating their function and possibly this may lead to new drug targets. An effective knockdown of exogenous as well as endogenous genes has been demonstrated in several mammalian organs (e.g. liver, lung, spleen, kidney, brain, pancreas and skeletal muscle; reviewed in [344]). Moreover, RNAi has been applied in cultured cell systems, organotypic slice cultures and different animal models. In animal models, knocking down genes underlying disease can for example induce a disease phenotype, in which the underlying molecular aspects can be studied.

Another line of research implementing the RNAi phenomenon characterizes endogenous miRNAs expression patterns as they in fact could be underlying the molecular basis of disease. MiRNAs are involved in translational repression or mRNA degradation and can thereby lead to subtle (individual) differences in gene expression at protein level. The group of Uchida for example, has shown that the differential expression pattern in specific brain regions of miRNA-18a targeting the GR at protein level, are underlying the phenotypic differences in stress vulnerability in two rat strains; Fisher 344 rats and Sprague-Dawley rats [307].

In addition to the application of RNAi as a research tool, RNAi can be used as a gene therapy in medicine. Diseases that can be blocked by down regulating the activity of one or several responsible genes are the most promising targets for RNAi-based therapies. Cancer, for example, is often caused by overactive mutations in onco-genes, and quelling their activity could halt the disease. Several pharmaceutical companies are currently testing RNAi-based therapies for various forms of cancer [345].

Viral infections are important potential targets for RNAi-based therapies as well. Reducing the activity of key viral genes would cripple the virus, and numerous studies have already hinted at the promise of RNAi for treating viral infections. In laboratory-grown human cells, investigators have stopped the growth of HIV, polio, hepatitis C, and other viruses. RNAi-based therapies against HIV and other viruses are expected to soon enter clinical trials [346].

Recently, the RNAi technology has also been used to limit prion-disease like scrapies [347-349]. This was tested using lentiviral-mediated delivery in the oocytes of both goats and cows. Also in a mouse model of scrapies, successful lentiviral-mediated RNAi knockdown of the diseasing prion protein was obtained. Mice not only survived longer, but also their behavioural deficits and neuronal damage could be reduced using RNAi as treatment [347]. As the strategy was successful, in future it could be used to generate transgenic prion-disease resistant live hood stocks.

However, one of the most promising and appealing applications of RNAi in therapy is probably in neurodegenerative disorders; severe diseases where previously no cure for existed. Engineered RNAi molecules for example have been tested as novel therapeutics for treating these

neurological disorders in mouse models for Huntington's disease, Alzheimer's, Spinocerebellar ataxia type I, and Amyotrophic lateral sclerosis, among others (see for a review [341;342;350-352]).

Thus, in relatively short time, RNAi has proven to be a very potent and selective research tool with lots of possibilities, applications, and advantages. We therefore selected this technology for knocking down GR protein expression in adult born dentate granule cells of the hippocampus of adult mice.

1.7 DELIVERY OF RNAI IN THE BRAIN

As discussed in the previous paragraph, spatial and temporal control of gene expression is *the* approach for studying gene function properly in animal models. RNAi enables this, and has several advantages over the more classical pharmacological and genetic approaches. However, effective delivery of RNAi molecules (miRNA, shRNA, siRNA) into target cells and tissues is critical for successful RNAi application. In this section I will describe the difficulties associated with siRNA delivery in the brain and an approach to target the GR in such a way that its protein expression may be manipulated in a cell type specific manner.

1.7.1 Delivery difficulties

A major difficulty in targeting genes by RNAi is the delivery. Delivery of compounds such as siRNA molecules needs to be performed in such a way that tissue damage is prevented, while the compounds can reach their target. Especially delivery into the brain is complex. The brains intricate anatomical divisions, molecular complexity and the fragile nature of its cellular populations make interventions very complicated [342]. In addition, the brain is inaccessible for compounds (> 500 dalton, without lipid solubility or transport systems) such as siRNA molecules because of the Blood-Brain-Barrier (BBB). This neuroprotective, membranic structure acts primarily to protect the brain from chemicals in the blood, while still allowing essential metabolic function.

Overcoming this delivery hurdle, mechanisms for siRNA delivery in the brain involve going either "through" or "behind" the BBB. For RNAi delivery locally in the brain, the last strategy is most optimal. Strategies for siRNA delivery behind the BBB include for example the application of naked or chemically modified RNAi molecules, plasmid transfection and viral transduction by local intra-cerebral injection using a stereotact (Fig 1.7). Using the stereotactic injection-strategy it is possible to deliver RNAi molecules or their vectors at any target site in the brain as small as a hippocampal subfield, such as the DG, with a minimum of damage [94;208;347;353]. Using cannulae/ implanted pumps even continuous injections can be given [354].

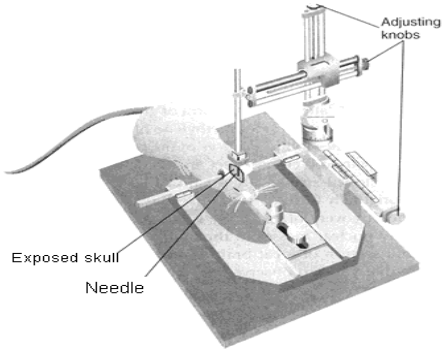

Figure 1.7 Stereotactic delivery in the mouse brain. *Using a stereotact, precise and localized injections can be placed in the brain. Using the frame, the injection needle can be placed at the specified coordinates with a precision of 0.05 mm.*

Another difficulty is the short, temporary, timeframe of RNAi induced by transient transfection of naked siRNAs requesting continuous or repeated delivery. Moreover, the high doses that are needed to achieve the knockdown in the brain go together with the off-target and non-specific side effects. Chemically modified or plasmid-based RNAi is often insufficient as well. These problems can be circumvented using viral vectors that genomically express shRNAs. Long-term RNAi may for instance be achieved with lentiviruses [355-357], adenoviruses [358] and adeno-associated viruses [359-362].

1.7.2 Lentiviral transgenesis

Viral vectors are a well established means for (long-term) delivery of shRNAs into the brain. In general, viral vectors are commonly used to deliver genetic material into cells both *in vitro* and *in vivo* without severely affecting cell viability and physiology. Especially lentiviral vectors can deliver a significant amount of genetic information such as transgenes or shRNAs, into the DNA of the host cell [363], without inducing an immune response or cytotoxicity [364]. Moreover, lentiviral vectors are able to transfect cells that are normally difficult to manipulate [355] and are generally simple and inexpensive to produce. In addition, the stable integration of transgenes into the genome of the target cells can be -depending of course on research questions and experimental set up- a convenient characteristic. It results in a model of long term and irreversible transgene expression, and as the genes are incorporated into the DNA, the transgenes are passed on to the progeny when the cell divides. Because of this feature, combined with the many other advantages, lentiviral vectors have been intensely used in both *in vitro* and *in vivo* research models.

Lentiviral vectors are derived from Human Immunodeficiency Virus 1 (HIV-1), which belong to the *Retroviridae* family, and are characterized by a long incubation period. Because they originate from pathogenic viruses, the major emphasis in the construction of these vectors has been on their safety. The general strategy has been to use as few genetic elements of the lentiviral genome as possible by deleting specific genes (required for replication and pathogenic properties)

and to make them replication incompetent and self-inactivating, while still enabling strong transduction efficiencies.

Another safety concern characteristically for retroviruses is their unpredictable, random integration site of their RNA-based genome into the DNA. The site of integration may cause problems, when the provirus disturbs the function of cellular genes and lead to activation of onco-genes. This can promote the development of cancer and leukemia. This however is unlikely to happen during the short duration of animal experiments.

1.7.3 Transduction of adult born dentate granule neurons

Although the dentate gyrus has been shown to be the most susceptible brain region to the gene knockdown effect of intracerebal-ventricular delivery of naked siRNAs [344], cell type specific delivery in the brain is a challenge. With the advancement of technology, only recently a few animal models with more or less cell type specific delivery have been described. Most of these models use gene targeting strategies in which the targeted genes are expressed or suppressed under de control of a cell type specific promoter [98;365;366]. In these transgenic animals, the genes are targeted from embryonic development. Other recent strategies have used retroviral labelling of dividing NPCs in adult animals [95;103;367;368].

A major advantage of lentiviral-based shRNA delivery systems is that they, in contrast to other retroviral vectors, such as MMLV, can efficiently infect both actively dividing, non-dividing post-mitotic, and terminally differentiated cells such as neurons and muscle cells [353;355;369;370]. Lentiviral vectors are therefore valuable tools for permanent and stable gene silencing in neurons at different stages of development, such as differentiating adult born dentate granule neurons.

In order to deliver shRNA molecules in adult born dentate granule neurons, we have chosen for a third generation lentivirus. This delivery system contains two expression cassettes. On cassette contains a hairpin sequence encoding siRNA precursor. This typically uses the type III class of RNA polymerase III promotor sequences, such as e.g. H1, to drive constitutive expression of the hairpin. The other cassette is a RNA polymerase II transcription unit directing stable expression of a marker protein such as green fluorescent protein (GFP). This marker is widely used to permanently label living cells *in vitro* and *in vivo.* This makes it possible to track transduced cells and their progeny for analysis of gene knockdown.

1.8 RATIONALE AND OBJECTIVES

In the previous sections I have described the stress response and the effects of GCs on the different tissues and cell types of the brain. I focused on the profound effects of GCs in modulating cellular properties, circuitry and behaviour in the hippocampus. Subsequently, I briefly laid out the present evidence for the possible role of GCs and hippocampal GRs in health and disease. Based on recent evidence suggesting that 1) neurogenesis may be a substrate for certain types of hippocampal function (see Box 1), and 2) adult born dentate granule neurons express GR

(see paragraph 1.2.3) this culminated in the ***hypothesis*** that

hippocampal GRs may affect hippocampal function by modulating neurogenesis.

The underlying mechanism is not clear and needs thorough investigation. This not only will gain fundamental information about the biology and cellular processes involved; knowledge of the pathogenic mechanisms underlying disease may provide novel targets for therapy. In the case of stress-related-diseases this is particularly important as chronic stress and all its associated pathologies play an ever increasing role in Western society. In addition, current therapies targeting the HPA axis are not very specific as they affect all cells of the body. More knowledge about the cell type specific functions of HPA axis parts may be the basis for the development of more specific and refined drugs with fewer side effects.

Therefore, the main objective of this thesis was investigating the role of the GR in adult born dentate granule neurons of the hippocampus in relation to neuroplasticity and cognitive performance.

In the last sections of this chapter, I explained that although GR has already been thoroughly investigated in a variety of animal models, thus far there has been a lack of cell type specific models for GR. I reasoned that this was caused by a lack of refined techniques. I introduced then the new RNAi technology and proposed a lentivirus-mediated cell type-specific delivery strategy for targeting adult born dentate granule neurons in the hippocampus.

Therefore, the second objective of my PhD project was to investigate the applicability of such a new, precise method: lentiviral-shRNA injections in the dentate gyrus.

1.9 CHAPTER OVERVIEW

Chapter two is the first experimental chapter. In this chapter I will show the results of the optimization phase of the experiments. shRNAs directed against the GR (shGR) were designed, tested and selected in an *in vitro* system. A neuronal cell line was used for testing of different types and doses of shRNA constructs and their controls. Then a selected shGR construct and its mismatch control was further optimized by dose-response tests and tests for the time frame of the GR knockdown. Finally, these constructs were successful built into a lentiviral vector.
In ***chapter three***, the results of optimization of delivery in the mouse hippocampus by different types of lentiviral vectors are shown. We investigated the transduction efficiency and GFP expression patterns of neuron-specific and non-cell type specific viruses. We show for the first

time that the lentivirus transduces a specific subpopulation of DCX+ neuronal progenitor cells and immature, adult born dentate granule neurons. This observation is fundamental for the *in vivo* study of genes in a cell type-specific manner.

In ***chapter four,*** the functional effects of LV-shRNA mediated GR knockdown in the DG are described in several experiments at the cellular level. One week after GR knockdown, we observed altered differentiation and migration of newborn dentate granule neurons. These observations were strengthened by evidence for altered structural plasticity and physiological properties of matured dentate granule neurons 5 weeks after GR knockdown. This evidence points to a critical role of GR in the fate determination of newborn dentate granule neurons.

In ***chapter five***, the functional effects of LV-shRNA mediated GR knockdown in the DG are described at the behavioural level. In this experiment, 5 weeks after LV-shRNA treatment, mice were subjected to a context and cue fear conditioning test to measure their fear-related memory capacities and coping strategies. In GR knockdown mice we observed a specific memory consolidation impairment. In addition, we found plasma corticosterone concentrations were similar between GR knockdown and control mice.

This thesis will end with ***chapter six***. In this final chapter a synopsis of all major findings is given. The application of lentiviral-mediated RNAi for the generation of new and more selective animal models is discussed. In addition, the outcome of GR knockdown for the fate of newborn dentate granule neurons is evaluated to assess the role of GR in modulating neurogenesis and hippocampal functioning. Furthermore I will discuss the possible consequences and implications of the new insights gained in the present study.

2

IN VITRO VALIDATION OF GLUCOCORTICOID RECEPTOR SILENCING BY RNA-INTERFERENCE

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ABSTRACT

In this study we describe the design and optimization of effective RNA-interfering constructs targeting the glucocorticoid receptor (GR). To achieve potent knockdown of the GR, we have designed four different sequence-specific short interfering RNA constructs. These constructs were cloned into pSuper vector in a short hairpin format. Subsequently, pSuper-shRNA constructs were transfected into a neuronal cell line and assessed for their potency to down-regulate GR protein levels. Using Western Blot analysis we determined the efficacy of the different constructs compared to sham, empty vector and corresponding mismatch shRNA. We found four effective pm- shRNAs, one (pm-GR3) with high potency to yield more than 90% GR protein knockdown, whereas the 3 others were less potent (pm-GR2 ~ 60%, pm-GR1 ~ 46% and pm-GR4 ~ 25% respectively). Pm-GR3 was subsequently cloned into a lentiviral vector and its potency was verified, gaining > 70% GR protein knockdown. Using shRNA constructs it was possible to specifically down regulate GR expression both via plasmid- and lentiviral vectors in a neuronal cell line. Therefore, this lentiviral vector may be a useful tool to knockdown GR in specific cell populations in the brain.

INTRODUCTION

Glucocorticoid receptors (GRs) mediate a wide array of cellular and physiological processes and are central in adaptation to stress and maintaining homeostasis [11]. They exert these functions by regulating the expression of numerous downstream glucocorticoid-responsive genes, thereby mediating a wide array of cellular and physiological processes. This fundamental role has made GR a target in many studies of functional genomic analysis. However, although ubiquitously and constitutively expressed, GR's tissue- and cell type specific actions have thus far been difficult to unravel [35]. This is particularly true in the central nervous system, where its complex anatomical organization underlies the pleiotropic actions of glucocorticoids [44;371].

Abrogating gene function is still one of the primary means of examining the physiological significance of a given gene product [344]. However, traditional pharmacological and transgenic animal models generally do not have enough resolution to investigate gene function at the level of a small brain region. We therefore chose to apply a new strategy: RNA-interference (RNAi), to investigate the function of the GR in specific neuronal subfields. RNAi is able to knockdown gene expression by degrading mRNAs of target genes. This phenomenon has since its discovery in purple petunias (see also CHAPTER 1, box 3) [337] and *Caenorhabditis elegans* [338], been observed in a variety of other organisms, including mammals [339]. Initially acknowledged as a cellular surveillance system, RNAi rapidly became a powerful tool to investigate gene function.

Gene silencing by RNAi is triggered by the cytoplasmatic presence of small double stranded RNAs. These small non-coding RNAs are enzymatically processed by RNase III class endoribonuclease Dicer, yielding ~ 21 pb short interfering (si) RNAs. Newly produced small duplex siRNAs then associate with Dicer and other factors, and compose the RNA-induced silencing complex (RISC). Once RISC has been associated, the non-functional "passenger strand" is discarded, whereas the other "guide strand" is retained. The guide strand anneals to fully complementary target mRNA and further directs the sequence-specific gene-silencing.

Dependent on the sequence complimentarity of the small non coding RNAs, RNAi results either in translational arrest or full mRNA degradation. MessengerRNA degradation by hydrolysis of the target sequence is typically caused by full complementary short interfering and short hairpin RNAs (siRNAs, shRNAs respectively) of exogenous (viral) origin [372]. In contrast, endogenously originating microRNAs (miRNAs) generally have lower sequence compatibility, leading to translational arrest. Although RNAi is a natural occurring process of gene regulation, in recent years it was noticed, that the varying levels of sequence complimentarity can have different effects. In fact, semicomplimentary RNAi sequences could underlie not only different levels target gene silencing, but also render RNAi ineffective or even underlying interference with other genes, resulting in off-target effects.

Much attention therefore has focused on understanding how precisely the sequence of short RNA duplexes determines the efficiency and specificity with which RISC degrades mRNA [373;374]. The effectiveness of gene silencing appears to depend on the sequence specific and thermodynamic properties of siRNA [375]. This not only determines compatibility with the target but also loading of

the appropriate guide strand into the RISC complex. Based on this, a set of empirically based and rational "design rules" has been developed (See Figure 2.1) [373;374;376].

In this study we have applied these rules to design several RNAi constructs and their controls for GR knockdown. Towards this end, we optimized and selected potent and efficient shRNA constructs for functionally silencing GR at both mRNA and protein level. In addition, we incorporated these shRNA constructs in lentiviral vectors for effective neuronal delivery.

MATERIALS AND METHODS

Experimental setup

In this study we designed several perfect match (pm) short hairpin RNAs (shRNAs) constructs against the GR and their non-specific, mismatch (mm) constructs bearing two point mutations. The efficiency for downregulation of GR expression was subsequently assessed *in vitro*. Firstly, we tested the efficiency of shRNA constructs to knockdown endogenous GR in the rat Neuroscreen-1 (Ns-1) PC12 cell line. GR protein knockdown was assessed by Western Blot analysis. Selection of the most optimal shRNA construct involved dose-response curves and time course studies in comparison with mm-shRNA, empty vector, and mock transfection as controls. Selected shRNA constructs were checked for functional GR knockdown in a reporter gene assay and subsequently cloned into lentiviral vectors. These lentiviruses were then verified for their ability to knockdown GR protein expression.

Design of short hairpin RNA constructs

Four different pm-shRNA constructs (named pm-GR1-GR4) and their 2 nt-mismatch controls (named mm-GR1-GR4) directed against a consensus sequence of the mouse (*mus musculus*; GR1-4), rat (*rattus norvegicus*; GR1-4) and human (*homo sapiens*; GR3-4) glucocorticoid receptor (Nr3C1 at chromosome 18) [377] were designed from the Ensemble genome browser/ database (www.ensembl.org; gene ID: ENSMUSG00000024431 and transcript ID: ENSMUST00000025300). The design was done according to the 9 critera of Ui-Tei et al and Reynolds et al (Figure 2.1 and Table 2.1A-B) [373;374]. These pm sequences of GR- targeting shRNAs (NM_008173) were checked for theoretical specificity against the mouse transcriptome. BLAST search indicated perfect sequence homology with NR3C1 (GR) and limited sequence homology with a Zink finger gene (pm-GR2; 15/ 19 nucleotide homology), TATA box gene (pm-GR3; 15/ 19 nucleotide homology) or synaptotagmin (pm-GR4; 16/ 19 nucleotide homology). However, according to the design rules, our pm-shRNA constructs are expected not to influence expression of these genes as there is only partial overlap. In addition, none of the pm-shRNA sequences overlap with seed regions of any mRNA. To get insight into which extent a guide strand binding site is accessible, we inspected prediction of secondary structures by Sfold software [378] of the GR mRNA. Mismatch sequences were used as negative controls for pm-shRNAs to account for non-sequence specific effects.

Oligonucleotides were obtained from Isogen (Isogen Life Science, De Meern, The Netherlands) (See Table 2.2).

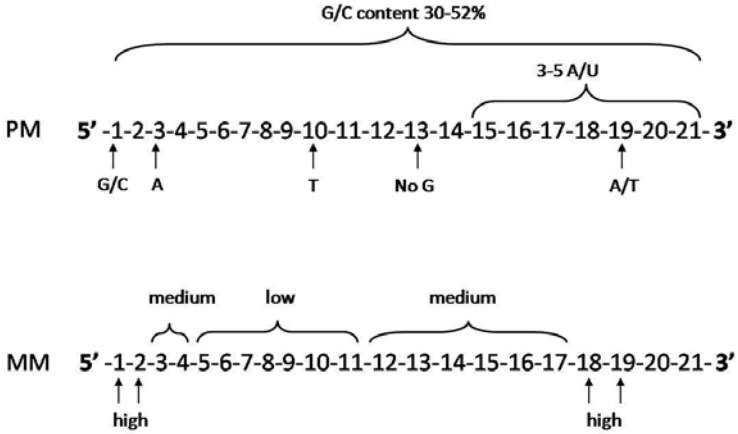

Figure 2.1 General criteria to design efficient shRNAs and their mismatch duplexes. *The criteria are based on guide (sense) strand.* ***PM)****. pm-siRNA design rules. The design rules can be divided into two categories; 1) Rules attributing the thermodynamic properties of the shRNAs, important for initial shRNA-RISC recognition; such as I) Use 21-nt RNA duplexes, II) 2-nt overhangs, III) No G/C rich regions (longer than 9 bp); G/C content 30-52%, IV) A/T richness in the 3' end of the Sense strand (last 7 bp), V) lack of internal repeats. 2) Rules that may affect critical shRNA-protein interactions, such as VI) T/A in position 19 of the Sense strand, VII) A in position 3 of the Sense strand, VIII) T in position 10 of the Sense strand, IX) No G in position 13 of the Sense strand, X) G/C at the 5' end of the Sense strand* [373;374]*.* ***MM).*** *Mismatch design rules* [376]*. Indicated areas give tolerance for the mismatch point mutation. Best positions for point mutation are 5-11. To generate effective point mutations, nucleotide substitutions should be A to C; T to A/G; G to T/C and C to A/G.*

Cell cultures

Ns-1 PC12 rat pheochromocytoma cells (Cellomics Europe) were used for Western blotting experiments and express GR endogenously. Ns-1 PC12 cells, were cultured at 37 °C at 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 5% fetal bovine serum (FBS), 10% horse serum, penicillin (20 U/mL), and streptomycin (20 µg/mL; all Invitrogen, Carlsbad, CA). The N1E-115 mouse neuroblastoma cell line was previously shown to express GR endogenously as well [67]. This cell line was used for reporter assay experiments. N1E 115 cells were cultured at 37 °C at 5% CO_2 in DMEM medium (4500 mg/l glucose, Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with Glutamine, penicillin (20 U/mL) and 2% FBS.

A. Name pm-shRNA construct	**pm design rules according to thermodynamic properties**				
	I Length 21 nt	II 2 nt overhangs	III %GC=30-52	IV Last 7≥5A/T	V Lack of repeats
pm-GR1	Y	Y	Y (48%)	N (4)	Y
pm-GR2	Y	Y	Y (48%)	N (4)	Y
pm-GR3*	Y	Y	Y (38%)	N (4)	Y
pm-GR4	Y	Y	Y (38%)	N (3)	Y

B. Name pm-shRNA construct	**pm design rules according to nucleotide type and position**				
	VI 19=A/T	VII 3=A	VIII 10=T	IX 13≠G	X 1=G/C
pm-GR1	Y	N	N	Y	Y
pm-GR2	Y	Y	N	Y	Y
pm-GR3*	Y	Y	N	Y	Y
pm-GR4	N	Y	N	Y	N

C. Name mm-shRNA construct	**mm design rules according to nucleotide type and position**			
	Point mutation 1 nucleotide type	Point mutation 1 position	Point mutation 2 Nucleotide type	Point mutation 2 position
mm-GR1	G → T	3	T → G	13
mm-GR2	A → C	3	T → G	13
mm-GR3*	A → C	3	A → C	13
mm-GR4	A → C	3	A → C	13

Table 2.1 Overview of the application of shRNA design rules to the four constructs *(see also figure 1).* ***A)*** *Design rules for pm-shRNA constructs according to thermodynamic properties.* ***B)*** *Design rules for pm-shRNA constructs according to nucleotide type and position, affecting shRNA-protein interactions. Y(es): design rule is applied, N(o): design rule is not applied. The four pm-siRNA constructs apply to 6-8 out of 10 design rules. * Selected for in vivo studies (see results section).* ***C)*** *Design rules for mm-siRNA constructs. Rules for both nucleotide mutations and nucleotide position have been applied. For both mm-siRNA and pm-siRNA constructs two additional rules are followed for length of the construct (19-25 bp) and the presence of a 3' dinucleotide overhang (reviewed in [379]).*

name construct	flanking region	shRNA sequence	hairpin	shRNA sequence reverse	flanking region
pm-GR1 sense	5′ gatcccc	cagactttcggcttctgga *passenger*	ttcaagaga	tccagaagccgaaagtctg *guide*	tttttggaaa 3′
pm-GR1 antisense	3′ ggg	gtctgaaagccgaagacct *guide*	aagttctct	aggtcttcggctttcagac *passenger*	aaaaaccttttcga 5′
mm-GR1 sense	5′ gatcccc	caTactttcggcGtctgga *passenger*	ttcaagaga	tccagaCgccgaaagtAtg *guide*	tttttggaaa 3′
mm-GR1 antisense	3′ ggg	gtAtgaaagccgCagacct *guide*	aagttctct	aggtctGcggctttcaTac *passenger*	aaaaaccttttcga 5′
pm-GR2 sense	5′ gatcccc	gcagcagaggattctcctt *passenger*	ttcaagaga	aaggagaatcctctgctgc *guide*	tttttggaaa 3′
pm-GR2 antisense	3′ ggg	cgtcgtctcctaagaggaa *guide*	aagttctct	ttcctcttaggagacgacg *passenger*	aaaaaccttttcga 5′
mm-GR2 sense	5′ gatcccc	gcCgcagaggatGctcctt *passenger*	ttcaagaga	aaggagCatcctctgcGgc *guide*	tttttggaaa 3′
mm-GR2 antisense	3′ ggg	cgGcgtctcctaCgaggaa *guide*	aagttctct	ttcctcGtaggagacgCcg *passenger*	aaaaaccttttcga 5′
pm-GR3* sense	5′ gatcccc	gaaagcattgcaaacctca *passenger*	ttcaagaga	tgaggtttgcaatgctttc *guide*	tttttggaaa 3′
pm-GR3* antisense	3′ ggg	ctttcgtaacgtttggagt *guide*	aagttctct	actccaaacgttacgaaag *passenger*	aaaaaccttttcga 5′
mm-GR3* sense	5′ gatcccc	gaCagcattgcaCacctca *passenger*	ttcaagaga	tgaggtGtgcaatgctGtc *guide*	tttttggaaa 3′
mm-GR3* antisense	3′ ggg	ctGtcgtaacgtGtggaGt *guide*	aagttctct	actccaCacgttacgaCag *passenger*	aaaaaccttttcga 5′
pm-GR4 sense	5′ gatcccc	ttaagcaagagaaactggg *passenger*	ttcaagaga	cccagtttctcttgcttaa *guide*	tttttggaaa 3′
pm-GR4 antisense	3′ ggg	aattcgttctctttgaccc *guide*	aagttctct	gggtcaaagagaacgaatt *passenger*	aaaaaccttttcga 5′
mm-GR4 sense	5′ gatcccc	ttCagcaagagaCactggg *passenger*	ttcaagaga	cccagtGtctcttgctGaa *guide*	tttttggaaa 3′
mm-GR4 antisense	3′ ggg	aaGtcgttctctGtgaccc *guide*	aagttctct	gggtcaCagagaacgaCtt *passenger*	aaaaaccttttcga 5′

Table 2.2 Sequences siRNA constructs and complete short hairpin format against GR. *In this table the four different 64-oligonucleotide constructs for perfect match and mismatch shRNA against GR are shown. Passenger shRNA sequences are comparable to the target GR mRNA sequences. The guide shRNA sequence is complementary to the passenger and incorporated in RISC. Capitals in mm-shRNA sequence indicate point mutation compared to pm-shRNA sequence. * Selected constructs for in vivo studies.*

Plasmid-shRNA transfections

The sense and antisense oligonucleotides of 64 bp long were annealed and cloned in between *BglII* and *HindIII* sites of the plasmid p-Super (The Netherlands Cancer Institute, Amsterdam, The Netherlands) [375]. Insertion of the oligonucleotides was confirmed by sequence analysis and positive clones were stored at -80°C. A day prior to transfection, $3x10^4$ cells per well were plated in a 24 well plate, and then incubated under normal growth conditions (37°C and 5% CO_2). For each well, the cells were transfected with 3 µg plasmid using using 6 µl Superfect transfection

reagent (Promega Corp. Madison, WI, USA). After transfection, cells were kept in DMEM containing 5% stripped FBS overnight. For the dose-response experiment, different total concentrations of DNA were kept constant using empty vector p-Super.

Protein extraction and Western blot analysis

Western Blot analysis was performed to verify shRNA-mediated GR protein knockdown in NS-1 PC12 cells. For this purpose, cells from two separate culture dishes per experimental group, were lysed in ice cold 0.5× radioimmunoprecipitation assay (RIPA) buffer (20 mM triethanolamine, 0.14 M NaCl, 0.05% deoxycetant, 0.05% SDS, 0.05% Triton X-100) substituted with protease inhibitors (complete Protease Inhibitor Cocktail tablets; Roche Applied Science, Penzberg, Germany). Subsequently, the cell lysates were centrifuged for 30 minutes at 13.000 rpm at 4°C after which the supernatants were collected. Protein content was quantified using the BCA™ Protein Asay (Pierce Biotechnology, Rockefort, IL, USA) and from each sample, 25 µg was loaded onto a 10% SDS-PAGE gel [380]. After electrophoresis, the samples were blotted overnight onto an Immobilon P membrane (Millipore Corp., MA, USA) and processed as described (Vreugdenhil et al., 2007) [381]. Blots were blocked in 10 mm Tris-HCl (pH 8.0), 150 mm NaCl, and 0.05% Tween 20 containing 5% nonfat dried milk powder. GR was subsequently detected using the M-20 GR antibody (1:500, Santa-Cruz Biotechnology, Santa Cruz, CA, USA) as a primary antibody and goat-anti-rabbit IgA conjugated with horse raddish peroxidase (1:5000- 1:10.000, Jackson ImmunoResearch Laboratories, PA, USA) as a secondary antibody. Tubulin (monoclonal anti-α-tubulin antibody; Sigma, 1:1000- 1:5000) expression levels were used for normalization. Luminol sodium salt (Sigma®) substituted with p-Coumaric acid (Sigma®) was used as substrate for the peroxidase reaction. Western blot experiments contained two biological samples per treatment group. Grey levels of immunopositive bands were determined by analyzing relative optical densities using Image J software (NIH, Bethesda, MD).

Dual luciferase reporter assay

Construct pm- and mm-GR3 were screened for its functional *in vitro* knockdown efficiency in a luciferase reporter assay. In this assay, GR-dependent transcriptional activity was measured in transfected N1E-115 cells by using a Dual Luciferase (Promega Corp. Madison, WI)- based GC response element reporter gene assay as previously described [67]. Cells were co-transfected with either pm-GR3- or mm-GR3- p-Super plasmid, TAT3 (containing 3 different GRE's controlling the firefly luciferase expression) and PCMV (containing Renilla luciferase as internal control), as described above. 24 h after transfection, cells were treated for another 24 h with $1x10^{-7}$ M dexamethasone, a potent GR agonist. Results are expressed as mean GR transcriptional activity ±SEM of three independent experiments performed in duplicate.

Lentiviral vectors

p-Super vector GR pm-shRNA and corresponding mm-shRNA constructs were sub-cloned into a vesicular stomatitis virus G glycoprotein (VSV-G)-pseudotyped advanced generation lentiviral

vector (Invitrogen BV, Breda, The Netherlands) downstream of the H1 promotor (see figure 2.2). In addition, the lentiviral vector contained an EGFP transgene downstream of a cytomegalovirus (CMV) promoter [382]. Lentiviral vectors were produced in 293FT cells using the ViraPower Lentiviral Expression System following the manufacturer's instructions (Invitrogen BV, Breda, The Netherlands). Virus containing supernatant was harvested 48 hr after transfection. Lentiviral constructs were concentrated by two rounds of ultracentrifugation. The titers were measured by rt-PCR and verified by EGFP expression as previously described [67]. Titers of both viruses were comparable and ranged between $1x10^8$ and $1x10^9$ transducing U/ml. Virus suspensions were stored at -80°C until further use and were briefly centrifuged and kept on ice immediately before transduction of Ns-1 PC12 cells.

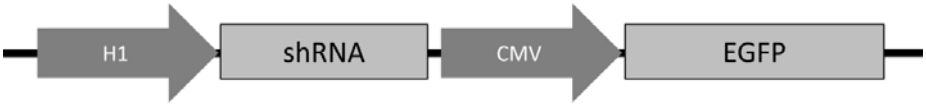

Figure 2.2 Schematic representation of the Lentiviral vector for shRNA delivery. *The lentivirus contains the shRNA construct expressed from a H1 promotor and in addition a visual marker; enhanced green fluorescent protein (EGFP), expressed from a CMV promoter.*

Statistics

Overall statistical analysis was performed using unpaired *t* test (two groups) or one-way ANOVA (three or more groups) using SPSS 15.0 and statistical significance was determined with Tukey's multiple comparison tests with $P < 0.05$.

RESULTS

Design of multiple shRNA constructs against the glucocorticoid receptor

In this study, we aimed to characterize efficient shRNA constructs targeting the glucocorticoid receptor. Firstly, we designed four different 21 nt long oligonucleotide sequences, targeting the murine GR and incorporated them into DNA vectors (see Table 2.2). As the GR gene consists of areas that are highly homologous to other members of the nuclear receptor subfamily 3, we selected a less conserved domain of the GR gene. As exon 2 contains unique sequences for the GR gene, we designed all the pm-shRNA sequences (and their mismatch controls) against this region (see figure 2.3).

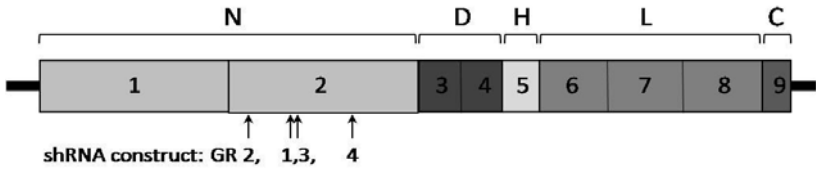

Figure 2.3 Schematic representation of the glucocorticoid receptor transcript and its shRNA targets. *Numbers indicate exons and abbreviations functional domains of GR: N) N-terminal domain including a ligand-independent transcription activation function-1, D) DNA-binding domain, H) hinge region, L) Ligand-binding domain, and C) C-terminus. Arrows indicate sequence areas of shRNA constructs targeting the murine transcript (according to Ensemble; ENSMUS&25300); sequence area GR1 (nt) 519-537; GR2 289-307; GR3 539-557; GR4 1041-1060.*

Testing of constructs

Plasmid delivery of shRNAs is a relatively easy strategy to introduce shRNA molecules into cells. We assessed therefore the efficiency of plasmid-based pm-shRNAs for GR protein knockdown in a Ns-1 PC12 cell line. These neuronal cells express GR endogenously and constitutively, and are in that sense comparable to the *in vivo* situation. Firstly, the four different plasmid-shRNA constructs were assessed for their potency to knockdown GR at the protein level. To this end, different plates of Ns-1 PC 12 cells were transfected with 3 µg of plasmids containing the different pm-shRNA constructs and two mm-shRNA controls. As a pilot experiment, two, four and six days after plasmid transfection, GR protein levels were determined by Western Blot analysis (data not shown). We observed that pm-GR3 is able to down-regulate GR protein levels by more than 70 % at an optimal time point of four days. Other constructs were less potent; pm-GR1 46%, pm-GR2 60% and pm-GR4 25% respectively (data not shown). These initial observations for pm-GR3 were strengthened by a similar experiment *in triplo*. Four days after shRNA-plasmid transfection, GR protein was significantly down-regulated by 95% (4.7% ± 1,8 STD, p=0.000), as compared to controls. In fact, mm-shRNA control did not differ from both mock transfection and empty vector pSuper controls (see Figure 2.4A). Therefore we selected pm-GR3 for further experimentation.

In a subsequent experiment, we optimized the transfection dose. From three different doses of pm-GR3; 1, 2, and 3 µg, efficiency for GR protein knockdown was measured *in duplo* four days after transfection. This resulted in GR protein knockdown of 63% ± 18.4, 83% ± 0.6, and 90% ± 9.3 respectively (Figure 2.4B). Although statistically not significant, a clear trend is observed suggesting a dose-response relationship in which the highest dose of pm-GR3 gave the highest GR protein knockdown. Actually, GR protein knockdown was significantly down-regulated at all pm-GR3 shRNA-plasmid doses compared to the three controls used (P= 0.000). Again, the mm-shRNA control proved an appropriate control as compared to both mock transfection and empty vector pSuper controls.

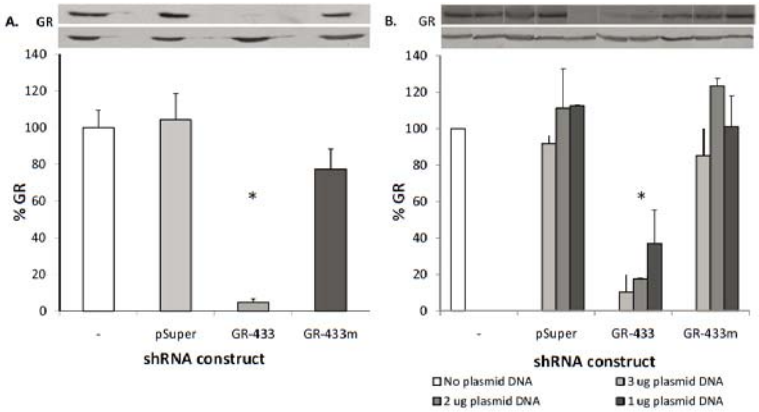

Figure 2.4. Selection of potent pm-shRNA constructs. A) *pm-GR3 was tested for its efficiency to knockdown endogenous GR protein in Ns-1 PC12 cells. Relative GR protein levels four days after treatment with 3 µg pm-GR3 in comparison with different controls (mm-GR3, mock transfection (-) and pSuper). Data were normalized by measuring α-tubilin levels. Asterisk indicates p=0,000. Representative Western blots are depicted on top of the graph.* ***B)*** *Dose response curve for plasmid-shRNAs. Relative pm-GR3-mediated GR knockdown in Ns-1 PC12 cells, as determined by Western Blot. Data were normalized by measuring α- tubilin levels. Asterisk indicates p=0,000. Representative Western blots are depicted on top of the graph. For further details: see Materials and Methods and text.*

In addition to assessing GR knockdown at protein level, we measured whether pm-GR3 could affect GR transactivation properties by performing a Dual luciferase reporter assay. In this assay, luciferase expression is driven by multiple GREs and luciferase activity is a measurement for GR trans-activitation properties. Dexamethasone (DEX)-activated GR was assessed for its capacity to induce transcription of a luciferase reporter gene. Inactive, non-ligand bound GR and mm-GR3 conditions were used as negative controls. As expected, four days after mm-GR3 treatment a robust DEX- dependent activation of luciferase expression (0.194 ± 0.016 L.U.) was observed (Figure 2.5). In contrast, pm-GR3 treatment did not show such a DEX-dependent luciferase expression (0.016 ± 0.003 L.U.). In fact, compared to mm-GR3/+DEX treatment this lack of luciferase induction corresponds to a significant 92% (p= 0.000) reduction of GR trans-activation properties. Moreover, RNAi-mediated inactivation of ligand-bound GR transactivation properties was comparable to non-ligand bound GR function (-DEX; see figure 2.5).

These results indicate that plasmid-based expression of pm-GR3 is effective at both downregulation of GR protein levels as well as ligand-activated downregulation of GR trans-activation properties.

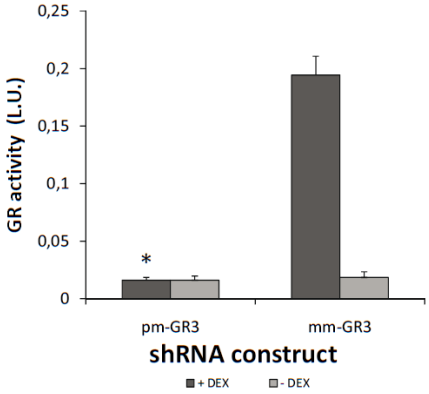

***Figure 2.5 GR transactivation properties in a dual luciferase assay**. pm-GR3 was tested for its ability to knockdown GR transactivation properties in a dual luciferase reporter assay. Dexamethasone was used to assess binding of ligand-activated GR to GRE's resulting in luciferase expression. Luciferase expression was controlled by renilla expression. Asterisk indicates p<0,05. For further details: see Materials and Methods and text.*

Effective lentiviral-shRNA-mediated GR knockdown

Lentiviral vectors are a well established means for long-term delivery of genetic information in a wide variety of cell types both *in vitro* and *in vivo* [383;384]. To investigate the efficiency of viral-expressed shRNA (see figure 2.2), we measured relative GR protein levels in Ns-1 PC12 cells four and six days after transduction. We observed pm-GR3-mediated downregulation of GR after four days (46% ± 1,70), albeit not significant (see Figure 2.6). However, six days after lentiviral transduction we observed a significant knockdown of GR protein as determined by Western Blot analysis (71%, p= 0.022). This indicates that pm-GR3 is a potent and selective shRNA for GR down-regulation after both plasmid and lentiviral delivery.

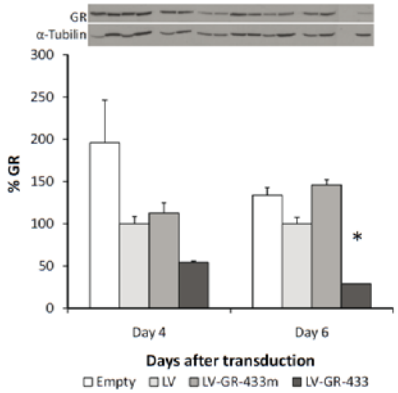

***Figure 2.6 Lentiviral-mediated knockdown of the GR in Ns-1 PC12 cells.** Time-dependent down regulation of GR protein after LV-pm-GR3 transduction of Ns-1 PC12 cells. Western blot: Data were normalized by measuring α-Tubilin levels. Asterisk indicates p= 0.022. Representative Western blots are depicted on top of the graph (for each representative bar in duplo). For further details: see Materials and Methods and text.*

DISCUSSION

In this study we have designed, selected and optimized shRNA constructs against the GR. The results of the experiments reported here show that we generated a potent pm-shRNA construct. In fact, construct pm-GR3 was capable of mediating GR knockdown in the Ns-1 PC12 cell culture by 70- 95% within 6 days after plasmid delivery. This GR protein knockdown was also potent enough to down-regulate ligand-activated GR transcription in a dual luciferase reporter assay. In addition to transient plasmid delivery, we showed that long lasting lentiviral- mediated delivery of shRNAs *in vitro* was successful as well to knockdown GR protein levels efficiently.

GR knockdown

Other studies have shown comparable levels of shRNA-mediated protein knockdown *in vitro* [344;354;362;385]. This partial knockdown is typical of RNAi, and is also occurring endogenously in most eukaryotic cells. Initially, RNAi leads to downregulation of target mRNAs, while protein downregulation follows gradually. Protein downregulation is dependent on the stability and half-life time of the corresponding proteins. In our study, GR protein knockdown was found optimal four days after plasmid delivery and six day after lentiviral delivery. This pattern is in line with the known half-life time of GR proteins. Although dependent on cellular conditions, the GR protein was found to degrade with a half-life of approximately 24 hours [386]. After four days this entails a 95% reduction of GR protein. In the presence of its ligand the GR half-life is even shorter [45] and thus optimal RNAi-mediated GR knockdown may be achieved sooner. The two days delay after lentiviral delivery can be explained by the fact that shRNAs from lentivirus are incorporated in the DNA of the host cell first and then transcribed from there. In contrast, shRNAs from plasmids are directly transcribed which may explain the shorter period needed to knockdown the GR protein levels by plasmid delivery. An alternative explanation for the 2 days delay with lentiviral transduction could be a probable difference in shRNA copy number expression, delivered by the two methods.

The extent of protein down-regulation further depends on the availability of splice variants and isoforms. The GR gene is host to two GR splice variants, GR-α and GR-β, transcripts which are generated by alternative splicing of the 3' UTR part [35;49;50;58;387]. These two splice variants share identical N-termini encoded by exons 2-8 and are distinguished only by their unique C-terminal ligand binding domain. Similar isoforms are known to be produced from alternative translation initiation sites and differ in their N-terminal region [35;49;50;58;387]. These GR-isoforms are expressed in various tissues of the mouse, and are underlying the tissue- specific functions of GR [18;24;35]. Because not much is known yet about the tissue-specific functions of these isoforms in the hippocampus, we chose to target the full-length GR mRNA -and thereby all splice variants and isoforms- by targeting exon 2 of the GR mRNA.

Effectivity of designed shRNA constructs

From the four different constructs initially designed, efficiency in GR knockdown was variable. Two pm-shRNAs (pm-GR2 and GR3) showed more than 50% GR knockdown, while two other constructs were less potent in silencing GR. This variability was expected, as the design and selection of efficient RNAi constructs is an empirically determined process requiring testing of multiple constructs. There are two possible explanations for this. The siRNA efficacy can depend on either siRNA-specific properties or on target mRNA properties.

Firstly, it is well known that the effectiveness of gene silencing depends on the sequence-specific and thermodynamic properties of RNAi constructs and their targets. Such properties can partly be estimated by theoretical design rules (See Fig 2.1 and Table 2.1a,b) [374-376]. The pm-shRNA constructs designed by us were, although 100% sequence compatible to the target mRNA, not completely in line with the design rules. In fact, constructs applied to only 6-8 out of 10 rules (see table 1a). A clear relationship can be observed between the effectiveness of the pm-shRNA construct and the extent to which these rules were applied.

Interestingly, both effective shRNAs; pm-GR2 and -GR3 shared the highest number of applied rules, i.e. 8 out of 10 (see table 1, figure 2.7), versus 6-7 out of 10 design rules for the less effective constructs (pm-GR1 and- GR4; see table 2.1, figure 2.7). Less effective shRNAs may therefore have a lower chance of being built into the RISC, as imposed by the design rules.

A second explanation for the observed variability may lie in the tertiary structure and accessibility of target mRNA. An open tertiary structure facilitates binding of shRNAs, while a closed, double-stranded structure shields shRNA binding sites and renders the targeted transcript inaccessible [341]. In line with the expectation, the target sequence in the GR mRNA of the least potent shRNA, pm-GR4, had the predicted most closed structure (10 out of 21), while pm-GR3, the most potent, has a more predicted open tertiary structure with only 8nt bound (see figure 2.7).

Specificity and controls

In RNAi studies, several types of controls have been used; 1) Scrambled shRNA with a random sequence [354;360;388;389], 2) Non-coding, random shRNA, without any (known) biological activity [390-392] 3) Non-specific shRNAs directed against a different (trans)gene (e.g. GFP or housekeeping genes) [393;394], and 4) Mismatch-shRNAs, bearing a few point mutations from the perfect match [356;395]. Of these different types of controls, mismatch-shRNAs are the most similar to the perfect match as they entail a complete sequence homology with the pm-shRNA constructs, except for two nucleotides. As RNAi can tolerate a few sequence mismatches, it is important to design these in the vicinity of the middle cleavage site [343]. We therefore applied this design rule at position 13 of the siRNA sequence.

Because of their similarity to perfect match sequences, mismatch-shRNAs may have similar seed regions and this should result in similar side effects. The differences observed between mismatch and perfect match treatment, are therefore plausible effects resulting from specific GR knockdown. This is the main reason why we chose for mismatch-shRNA constructs as negative controls in our experiments. As shown in this study, RNA-interference is a highly specific

mechanism as two nucleotides can make the difference between target mRNA degradation or control situation. Thus, RNA-interference is highly sequence specific. In fact, many experiments have shown that RNAi is capable of causing specific degradation of target mRNAs with as little as one base pair difference to other transcripts [339;342;396].

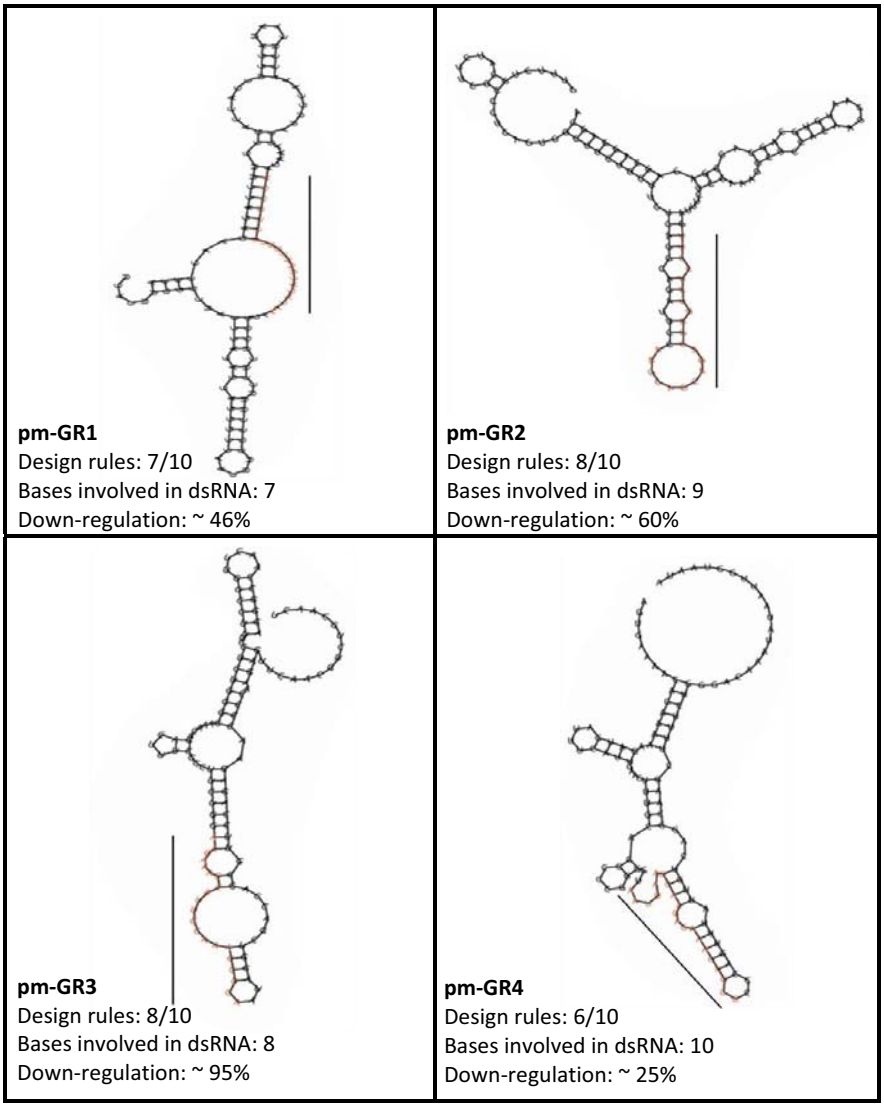

Figure 2.7 Theoretical model of mRNA tertiary structures and binding sites for different guide strand shRNA constructs*. Positions marked by a line indicate bases predicted to be involved in RNA binding.*

Off target effects

The use of RNAi as tool to manipulate gene expression in mammalian cells might result in side-effects. There are three types of these so-called off-target effects.

A first type of off-target effect is associated with an incomplete binding of shRNA molecules to other mRNA molecules potentially resulting in non-specific silencing of genes other than the specific target. It is thought that such binding occurs when parts of the 5' prime end of the guide-shRNA strands are similar to 7-8 nt long seed regions of endogenously expressed miRNAs [341;397;398]. Incomplete seed-region binding within the 3' untranslated region of an mRNA then results in translational arrest. The presence of seed regions is difficult to avoid given the small degree of similarity implicated in off-target gene regulation (Jackson et al., 2003) [398]. Whether or not the pm-shRNA constructs designed in our study are underlying such off-target effects is presently unknown and cumbersome to investigate. Possible approaches are bio-informatic analysis of potential seed regions, microarray analysis of non-specific gene silencing, and the application of multiple effective pm-shRNA constructs to exclude off-target effects [341].

However, in the design of the shRNA constructs we have used exon 2 of the GR transcript as target. Although GR belongs to a family of highly similar and evolutionary conserved nuclear steroid receptors (see also CHAPTER 1.2.2), exon 2 is known as the most unique part. BLASTing of passenger shRNAs confirmed a lack of any sequence complementarity to other nuclear receptors such as MR. In fact, BLASTing showed a limited sequence complementarity to a few other genes. As described above, RNAi is so sequence specific that such a partial sequence overlap is not expected to result in gene knockdown any other than the target. In addition to *in silico* predictions, it is also essential to use appropriate controls to rule out off-target effects while circumventing "more cumbersome" approaches. Therefore in our studies we used the mismatch-shRNA construct as a control. This construct is essentially similar to the perfect match-shRNA construct except for two nucleotides. Therefore it is expected that all differential effects observed from mm- and pm-shRNAs are due to specific GR knockdown only.

A second potential off-target effect associated with shRNAs and dsRNA > 30 nucleotides in length is an immune response (see for review [399]). These are typically characterized by PKR and Toll-like-receptor (TLR)- mediated interferon activation, infiltrations of granulocytes and increased apoptosis. These complex processes are part of the cellular defense system, which is capable of sensing and destroying exogenous particles of possibly pathogenic origin. It appears that these processes are also induced by delivery of shRNAs. Viral delivery, expression of shRNAs from pol III promoters and certain immunostimulatory sequence motifs have in some instances been associated with the innate immune response [341;397;399;400].

Although we did not test for the presence of an innate immune response after shRNA delivery, we avoided the presence of immunostimulatory sequence motifs during the design of the shRNAs. In fact, beyond viral delivery, genomicly incorporated shRNAs become endogenously expressed, which should circumvent the mammalian innate immunity [399]. Twenty-one base pair siRNAs, processed from shRNAs, are also known to circumvent the mammalian immune response [339;372;397].

During experimentation we also used specifically the mm-shRNAs to exclude this type of off-target effects. Moreover, it is unlikely that there are off-target effects due to immune responses in Ns-1 PC12 cells, as neurons are known to lack most types of TLRs (e.g. 1, 2, 4-10) [399]. Therefore this makes the possibility unlikely that Pol III and immunogenic shRNA sequences induce a TLR-mediated interferon response. Moreover, we used a lentiviral vector in our study. This type of vector is lacking its original genes and therefore not expected to express viral-dsRNAs, which are known to activate the single present TLR3 in neurons [399].

A third type of off-target effect is cellular stress caused by an overshooting RNAi response. Engineered shRNAs utilize the endogenous RNAi machinery and therefore can cause at high doses cytotoxicity independent of sequence [341]. Although not a primary aim, in our studies for GR protein knockdown we also have indirectly tested for cellular homeostasis by measuring the expression of the housekeeping gene α-tubilin. As expected, we did not find any changes in its expression relative to basal condition (sham), empty vector delivery and mm-shRNA treatment. Therefore it is plausible that the basal cellular machinery is not disrupted. Also in this situation our mismatch shRNAs are a proper control, as they are expressed from the same H1 promotor in comparable levels.

Delivery of shRNAs

Beyond the specificity and effectivity of shRNAs; delivery of shRNAs into target cells is an important hurdle. This is especially difficult in the mammalian nervous system as it consists of terminally differentiated cells. As neurons are also extremely sensitive to external influences, they are -*in vivo*- well protected by the blood-brain-barrier, again making delivery even more complicated. Therefore good shRNA delivery strategies are essential. In this study, we have shown extensive GR knockdown after both plasmid and lentiviral delivery in a neuronal cell line. Both approaches have their benefits and drawbacks. Short hairpin RNAs expressed from plasmids are transient in nature and therefore the knockdown effect is temporary. This may be an insufficient strategy for (long term) functional studies. Depending on the research question, long term viral-mediated shRNA expression can be an effective strategy for functional studies. The use of lentiviral delivery of shRNAs in the nervous system has already been described *in vivo* (see for review [341]).

Expression of shRNAs from the delivery vectors occurs by transcription from specific promoters. In our study and in most others studies, RNA polymerase III promoters have been used for shRNA expression. This type of promoters is relatively small while highly active and therefore ideal for continuous shRNA expression in a variety of cell types. shRNAs themselves have the advantage of being more stable than siRNAs [401].

Conclusion

In conclusion, we have identified a very potent shRNA construct: pm-GR3, to knockdown GR protein levels *in vitro.* Expressing this construct by both plasmid-based delivery and lentiviral delivery resulted in efficient GR knockdown in a mammalian neuronal cell line. This construct led

to GR protein knockdown as well as strong impairment of GR transactivation properties. Using the 2 nt- mismatch-shRNA as a specific control, we likely minimized off-target effects and differences in phenotypes may therefore be due to specific knockdown of the GR. The pm- and mm-GR3 lentiviral constructs may form excellent tools to study GR function in discrete neuronal cell populations in the brain.

3

LENTIVIRUS-MEDIATED TRANSGENE DELIVERY TO THE HIPPOCAMPUS REVEALS SUB-FIELD SPECIFIC DIFFERENCES IN EXPRESSION

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ABSTRACT

Background In the adult hippocampus, the granule cell layer of the dentate gyrus is a heterogeneous structure formed by neurons of different ages, morphologies and electrophysiological properties. Retroviral vectors have been extensively used to transduce cells of the granule cell layer and study their inherent properties in an intact brain environment. In addition, lentivirus-based vectors have been used to deliver transgenes to replicative and non-replicative cells as well, such as post mitotic neurons of the CNS. However, only few studies have been dedicated to address the applicability of these widespread used vectors to hippocampal cells *in vivo*. Therefore, the aim of this study was to extensively characterize the cell types that are effectively transduced *in vivo* by Vesicular Stomatitis Virus G glycoprotein-pseudotyped lentivirus-based vectors in the hippocampus dentate gyrus.

Results In the present study we used Vesicular Stomatitis Virus G glycoprotein-pseudotyped lentivirual vectors to express EGFP from three different promoters in the mouse hippocampus. In contrast to lentiviral transduction of pyramidal cells in CA1, we identified sub-field specific differences in transgene expression in the granule cell layer of the dentate gyrus. Furthermore, we characterized the cell types transduced by these lentiviral vectors, showing that they target primarily neuronal progenitor cells and immature neurons present in the sub-granular zone and more immature layers of the granule cell layer.

Conclusions Our observations suggest the existence of intrinsic differences in the permissiveness to lentiviral transduction among various hippocampal cell types. In particular, we show for the first time that mature neurons of the granule cell layer do not express lentivirus-delivered transgenes, despite successful expression in other hippocampal cell types. Therefore, amongst hippocampal granule cells, only adult-generated neurons are target for lentivirus-mediated transgene delivery. These properties make lentiviral vectors excellent systems for over-expression or knockdown of genes in neuronal progenitor cells, immature neurons and adult-generated neurons of the mouse hippocampus *in vivo*.

INTRODUCTION

The hippocampus is a brain structure that forms part of the limbic system and is involved in memory formation and spatial navigation. The Dentate Gyrus (DG) field, despite of being composed mainly by granule cells, is an heterogeneous structure [402]. Moreover, the subgranular zone (SGZ) of the DG, along with few other few areas of the adult brain, is characterized by the existence of ongoing neuronal generation known as adult neurogenesis [403;404]. All in all, these and other important observations have called for extensive attention on the study of the adult DG and its functions.

In this respect, one challenging task is to identify and employ genes and molecular mechanisms directly involved in hippocampal functions, such as neuronal plasticity and neurogenesis [405;406]. The ability to manipulate the genotype *in vivo* provides major opportunities for studying gene function in the mammalian nervous system and for developing novel therapeutic strategies [369].

Viral-mediated single-cell gene manipulation has proven to be one of the most successful approaches to study molecular mechanisms involved in adult neurogenesis in an intact brain environment [367;368]. With this aim, retroviral vectors have been extensively used in the study of neurogenesis due to their ability to transduce only replicative cells [103;367]. Also, lentiviral vectors have been extensively used to deliver transgenes to replicative and non-replicative cells, such as post-mitotic neurons of the CNS [383;384]. Among lentiviral vectors, Vesicular Stomatitis Virus G glycoprotein (VSV-G)-pseudotyped are the most widely used due to their very broad tropism and stability of the resulting pseudotypes. Moreover, they have received considerable attention since they have recently entered human clinical applications [384]. Interestingly, numerous reports have described on the use of lentiviral vectors on hippocampal neurons *in vivo* [357;363;369;407-411].

Aiming to demonstrate the usefulness of modified lentiviral vectors to deliver transgenes to the adult mouse hippocampus and extensively characterize the cell types that are effectively transduced *in vivo*, we used a previously described VSV-G-pseudotyped advanced generation lentiviral vector (AGLV) to express the enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus (CMV) promoter [67].

EGFP expression was analyzed one and five weeks after stereotactic injection to the mouse hippocampus and the local distribution of EGFP+ cells within different hippocampal sub-fields was compared. We identified the different cell types transduced in the DG using cell-lineage specific markers [62;295]. The distribution and location of EGFP+ cells were also analyzed and quantified in the DG and Cornu Ammonis 1 (CA1) fields for comparison.

We report that lentivirus-mediated transgene expression in the DG is restricted to a subpopulation of NPC and immature neurons present in the inner granule cell layer (GCL), while presumably more mature granule cells located in the outer layers are resistant to transgene expression.

These results reveal for the first time the existence of hippocampus sub-field and cell-type specific differences in lentivirus-mediated transgene expression. These properties make lentiviral vectors

excellent delivery systems for studies aiming to characterize the functions of hippocampal NPC and immature neurons, where *in vivo* gene manipulation is requested.

METHODS

Experimental setup

We investigated expression of EGFP and cell-type specific markers in hippocampal cells after transduction with AGLV [412]. In these vectors, EGFP expression was under the control of three different Polymerase II promoters, as described in the Results section [67;383]. Hereto, animals were divided into experimental groups of 5 animals each and intra-hippocampally injected into the DG or SR with one of the three types of lentivirus. One or five weeks after injection, brain tissue was processed for immunohistochemistry.

Cloning and Lentiviral vector production

Replication incompetent and self-inactivating Advanced Generation lentiviral vectors were produced and titrated as previously described [67]. All lentivirus batches used for experiments had comparable titers ranging from $1x10^8$ to $1x10^9$ transducing U/ml. Virus suspensions were stored at -80 °C until use and were briefly centrifuged and kept on ice immediately before injection.

Animals

Male C57Bl/6J mice (seven weeks old at injection, Janvier Biosciences, France) were housed 5/cage for one week before surgery as acclimatization. Thereafter, mice were single housed in filtertop cages, in a temperature and humidity controlled room with 12:12 dark- light cycle (light on at 08:00 A.M.). Mice had free access to food pellets and water. All efforts were made to minimize animal suffering and the number of animals used. All experiments were approved by the committee of Animal Health and Care, Leiden University, the Netherlands and the Netherlands ministry of VROM and performed in strict compliance with the European Union recommendations for the care and use of laboratory animals.

Stereotactic surgery

Stereotactic injections were performed essentially following previously described methods [367]. Animals were deeply anaesthetized by a mixture of Hypnorm (0.5 mg/kg/ml) and Dormicum (5 mg/kg/ml) and Milli-Q purified water (Millipore, Amsterdam, The Netherlands) at volume ratio of 1:1:2 (10 ul/g). Bilateral injections of lentiviral vectors into the Dentate Gyrus (AP: -2.00 mm, ML: +/-1.50 mm, DV: -1.90 mm, relative to Bregma) or the Stratum Radiatum (AP: -2.00 mm, ML: +/-1.50 mm, DV: -1.50 mm, relative to Bregma), were conducted using a small animal stereotact (900 series, David Kopf Instruments, Tujunga, CA) and an injection pump (Harvard Apparatus, Holliston, MA) with injection volume=1 μl, rate=0.4 μl/ min, connected to a Hamilton needle (5 μl, 30

gauche), and customized borosilicate glass micro-capillar tips of approximately 100 μm. After surgery animals were placed under a heating lamp until awakening and further monitored and weighted daily.

Immunohistochemistry

One or five weeks after injection, animals were sacrificed and brains were fixed by transcardial perfusion. Before the procedure the animals were deeply anaesthetized by IP injection of sodium pentobarbital (Nembutal 60 mg/ml, 0.1 ml). Animals were transcardialy perfused with 0.1M PBS for 10 minutes. Brains were removed and kept in 25 ml 4% PFA for one hour. Then, they were washed in 0.1M PBS and immersed in 15% and subsequently 30% sucrose solution for 3-4 days. Brains were blotted dry and snap-frozen for 10 sec in isopentane on dry ice and stored at -80˚ C until sectioning.

Serial coronal 20 μm-thick sections were obtained using a cryostat (Leica CM 1900, Leica Microsystems, Rijswijk, The Netherlands). All brain sections containing the hippocampus were collected and thaw-mounted on SuperFrost microscope slides and stored at -80˚ C until further use.

Immunofluorescent double and triple labelling was performed as described [381]. Primary antibody were from: Santa Cruz Biotechnology, Inc; Heidelberg, Germany (Doublecortin (C-18), used 1:200; Ki67 (M-19), used 1:100; GFAP, mouse monoclonal, used 1:1000); Chemicon-Millipore International BV, Amsterdam, The Netherlands (NeuN (A60), used 1:200), BD Biosciences, Breda, The Netherlands (Nestin, (556309), used 1:200) or Molecular Probes/Invitrogen, Breda, The Netherlands (GFP, chicken polyclonal, used 1:500). After 24 h incubation at 4 °C with continuous stirring, sections were incubated with correspondent Alexa488 or Alexa594-conjugated secondary antibodies (1:400, Molecular Probes/Invitrogen) for 2 hrs at RT in 100 μl 1xPBS/0.3% TritonX-100. Sections were counterstained with Hoechst 33342 when indicated, as previously described [381]. Sections were embedded with Aqua-Poly/Mount (Polysciences Europe, Eppelheim, Baden-Württemberg, Germany). Similar samples were processed in parallel excluding primary antibodies and used for comparison as negative controls (not shown).

Organotypic hippocampal slice cultures

Early postnatal rat hippocampal slices were produced an cultured as previously described [67]. Briefly, slice cultures were prepared from 4- to 6-day-old male Wistar rats (Charles River Laboratories, Inc., Frankfurt, Germany) using the modified interface culture method. At the time of the first medium change (day *in vitro* (DIV) 1), hippocampal slices were inoculated with 10 μl of the CMV-EGFP lentiviral vector stock. Slices were fixed 4 days later with 4% paraformaldehyde for 1 h at 4 °C and used for immunofluorescence studies.

In situ hybridization for EGFP mRNA

Perfused mouse brain sections were used for in situ hybridization with a 720 base pair long digoxigenin (DIG)-labelled EGFP riboprobe (antisense to NCBI gene ID DQ768212). The in situ

hybridization was performed essentially as described by Schaeren-Wiemers and Gerfin-Moser [413], with small modifications. Briefly, sections were fixed in 4% paraformaldehyde (PFA) for 5 minutes, treated for 10 minutes with 10 ug/ml proteinase K and 0.1% Triton-X100 in phosphate buffered saline (PBS, pH 7.4), followed by 10 minutes extra fixation with 4% PFA. Thereafter, sections were rinsed 3 times in PBS for 3 minutes. After acetylation for 10 minutes (0.25% acetic anhydride in 0.1 M triethanolamine), sections were washed 3 times in PBS for 5 minutes and prehybridized for 2 hours at room temperature in hybridization solution, containing 50% deionized formamide, 5x SSC, 5x Denhardt's solution, 250 µg/ml tRNA Baker's yeast and 500 µg/ml sonicated salmon sperm DNA. 150 µl of hybridization mixture containing 400 ng/ml DIG-labeled riboprobe was applied per slide, covered with Nescofilm and hybridized overnight at 68°C. The next morning slides were quickly washed in 2x SSC followed by 0.2x SSC for 2 hours, both at 68°C. DIG was detected with an alkaline phosphatase labelled antibody (1:5000, Roche, Mannheim) using NBT/BCIP as a substrate. After DIG in situ hybridization, slides were counterstained with 0.5% methyl green, quickly dehydrated in ethanol, cleared in xylene and mounted using Entellan.

Histological analyses and confocal microscopy

Quantification of EGFP+ cells and quantitative analysis of different classes of neuronal cells in the hippocampus of treated animals were performed using the optical fractionator sampling method, as described by Encinas and Enikolopov [81]. Briefly, every tenth hippocampal section was collected starting at the DG following the fractionator scheme, to ensure that each slice is 200 nm apart from the next slice within each collected set of approximately 11 slices (Fitzsimons et al., 2008) [67]. For quantification of EGFP+ cells, three sets of slices from at least three independently injected animals from each experimental group were used. Sections surrounding the injection site were routinely discarded. For quantitative analysis of neuronal cell-types other three sets of slices from at least three independently injected animals from each experimental group were used. Confocal images were acquired using a Nikon C1si Spectral confocal microscope, as described [67]. Expression of markers and cell-localization analyses were done counting more than 50 EGFP+ cells per animal. Co-localization was assessed through the entire z-axis of each cell, using an optical slice of 0.3–0.6 µm. Morphology was analyzed from three-dimensional reconstructions of series of sequential confocal images taken at 0.3–0.6 µm intervals in EGFP+ cells.

Image analysis

For EGFP+ cell-localization analyses within the DG or CA1 sub-fields, maximum intensity z-axis projections of series of sequential confocal images were constructed using ImageJ, as described [67]. Using these projections, EGFP+ cells were automatically identified and counted using Cell Profiler (http://www.cellprofiler.org) [414]. This procedure was validated by comparison to manual counting performed by an experienced operator using the optical fractionator method sampling scheme and unbiased stereology estimation of cell numbers as described by West and co-workers [415]. The "pipeline" used to automate cell counting was composed of the following Cell Profiler's modules, in the specified order: LoadSingleImage, ColorToGray, CorrectIllumination_Calculate,

CorrectIllumination_Apply, IdentifyPrimAutomatic. By using this pipeline we routinely found a strong correlation between the manual unbiased stereology method and the automated procedure (r=0.985, Pearson's correlation test performed with GraphPad Prism 4, GraphPad Software, Inc., La Jolla, CA). EGFP+ cells were individually pseudo-coloured to facilitated visualization and cell-localization maps were generated using Cell Profiler. Subsequently, based on a previously described manual method to study granule cell location within the GCL [416] the GCL was subdivided in four 2-cell-body-wide sub-layers using ImageJ (http://rsb.info.nih.gov/ij/) to generate a superimposed grid, guided by Hoechst 33342 staining of cell nucleus. These sub-layers were denominated: sub-granular zone (SGZ) and granule cell layer (GCL) 1 to 3, as described by others [76;94;368]. Then, the pseudo-coloured cell-localization maps generated with Cell Profiler were used to manually assign and count individual EGFP+ cells to the 4 sub-layers of the GCL of the DG. In all cases, EGFP+ cells present in the apex of the DG were excluded from the analyses. A similar procedure was used in experiments comprising EGFP+ cells in CA1.

For quantification of different cell-type markers in EGFP+ cells, total EGFP+ cells were automatically identified and counted using Cell Profiler from z-projected confocal images. From the same images, cells positive for each individual co-stained marker were also automatically identified and counted with Cell Profiler using the corresponding confocal channel. Cells positive for each marker analyzed were expressed as percentage of total EGFP+ cells. All image analyses procedures were performed in hippocampal slices from at least three independently injected animals as described above. In all cases, image analyses were performed by an operator blind to treatment.

Dendrite tracing and three-dimensional reconstructions.

Three-dimensional reconstructions of dendritic arbors and spine density analysis were performed using TDR3D software package (http://bioimaging.liacs.nl/tdr3dbase.html), using a simulated fluorescence process-based algorithm [417;418]. Briefly, three-dimensional reconstructions for morphological analyses were generated from series of confocal images of EGFP+ neurons taken at 0.3–0.6 µm intervals from at least three independently injected animals. All cells used for morphological analyses were positive for the neuronal marker NeuN (not shown). Quantification of dendritic protrusions and dendritic length was done with ImageJ (NeuronJ plugin).

RESULTS

Lentivirus-mediated EGFP delivery to the DG

In order to transduce cells present in the DG of the mouse hippocampus, we used a previously described AGLV system where the CMV promoter controls EGFP expression [67], further referred here as CMV-EGFP. This vector was infused by stereotactic injection into the DG (Figure 3.1). Under these experimental conditions we observed a marked restriction of EGFP expression to the hilar region and the SGZ and only few EGFP+ cells present in the GCL one week after injection

(Figure 3.1A). This spatial distribution is reminiscent of previous observations with murine Maloney Leukemia virus (MMLV)-derived retroviruses, transducing only dividing cells [95;103]. Notably, increased EGFP expression from higher lentiviral vector delivery titers did not result in a substantially increased proportion of EGFP+ cells located in the GCL, while the total numbers of EGFP+ cells were drastically increased, resulting in massive EGFP expression in the hilar region and the SGZ (Figure 3.1B).

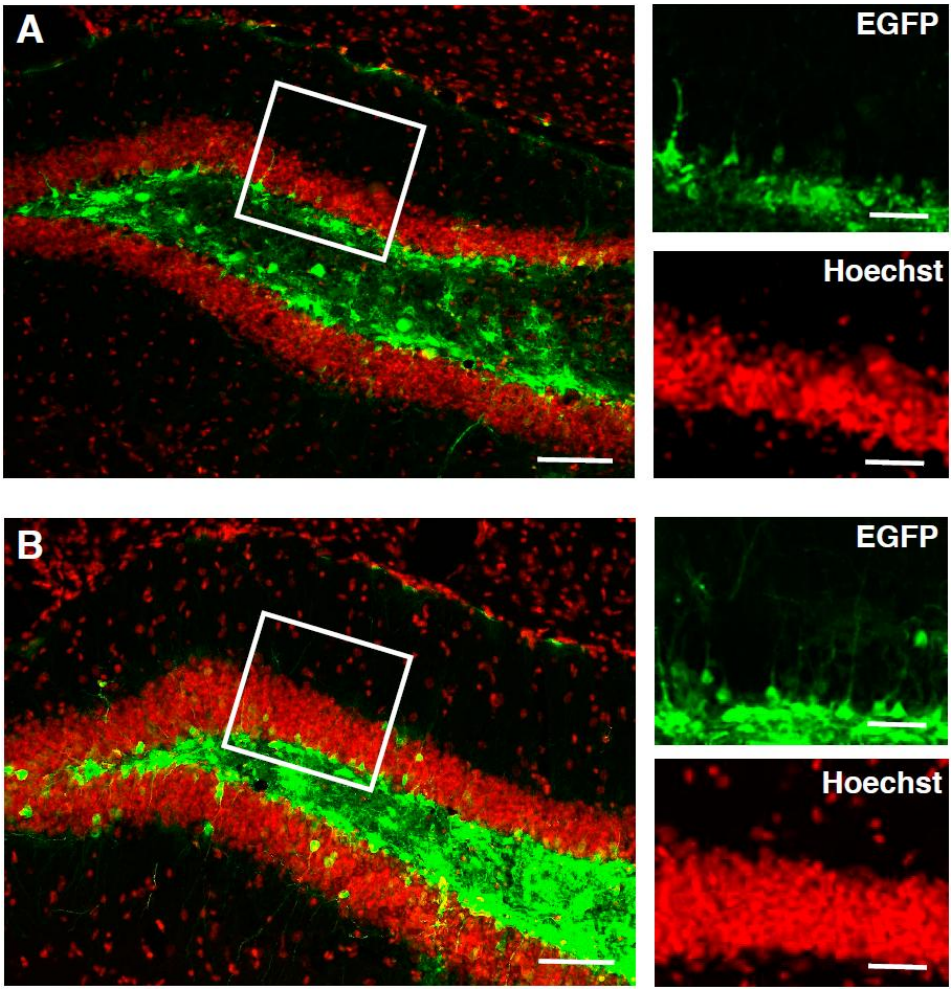

Figure 3.1 Lentivirus-mediated EGFP delivery to the DG. *Lentivirus-mediated delivery by stereotactic injections to the hilar region of the hippocampus does not results in substantial EGFP expression in the GCL, despite low* ***(A)*** *or high* ***(B)*** *EGFP expression, 1 week after injection. Each image shown is representative of 5 animals independently injected. Right panels represent the boxed area in the left panels of the figure. Scale bars: left panels 100 µm; right panels 20 µm.*

Spatial distribution of EGFP+ cells in the GCL after CMV-EGFP injection.

In order to account for the spatial distribution of the EGFP+ cells in the GCL of the DG, we subdivided the GCL in 4 different two-nucleus-wide regions, following the method described by Kempermann *et al.*, and extensively used by others [76;94;368](Figure 3.2). These four regions were designated SGZ, GCL1, 2 and 3 (Figure 3.2C). Thereafter, we applied semi-automated, software assisted, quantification of the percentage of total EGFP+ cells present in each of these regions. Although the numbers of total EGFP+ were variable among different injections, as described for MMLV-based retroviral vectors [103], the relative percentages of cells present in the different subdivisions of the DG were consistently reproducible. We found that one week after stereotactic injection, a large percentage of the cells reside in the SGZ (57±1 %, n=5 animals) and the innermost layer of the GCL, GCL1 (32±2 %, n=5 animals; Figure 3.2A, C and D). When the number of EGFP+ cells was assessed five weeks after stereotactic injection, we found that the larger percentage of EGFP+ cells still resided in the SGZ (27±4 %, n=5 animals) and the GCL1 (42±3 %, n=5 animals) with a significantly higher percentage of EGFP+ cells located into the intermediate third of the GCL (GCL2, 26±3 vs. 9±4 %, 5 and 1 weeks respectively, $p<0.05$ Student t test, n=5 animals each; Figure 3.2B and E). Notably, EGFP+ cells rarely reached the outer third of the GCL (GCL3) and the percentage of cells located in the GCL3 was not significantly different from the one observed one week after injection (5±3 vs. 2±2 %, 5 and 1 weeks respectively, n=5 animals each; Figure 3.2D and E).

Spatial distribution of EGFP+ cells in the GCL after CaMKII-EGFP injection

In order to assess the possibility that the distribution of EGFP+ cells in the GCL after lentivirus transduction may depend on the promoter used to control EGFP expression, we used two other previously described lentiviral vectors where EGFP expression is controlled by neuron-specific promoters, the Synapsin I (denoted here Syn-EGFP) and the CamKII (denoted here CaMKII-EGFP) promoters. These vectors promote different levels of EGFP expression in mature post-mitotic cortical neurons, presumably due promoter's specificity for different neuronal developmental stages [383]. All lentiviral constructs were produced with the same packaging system and pseudotyped with VSV-G protein to avoid possible differences in cell-type targeting due to the use of different pseudotyping proteins [419]. When the spatial distribution of EGFP+ cells was assessed one week after CaMKII-EGFP injection, we observed that this distribution was significantly different from that observed one week after CMV-EGFP injection (Figure 3.3). Injection of CaMKII-EGFP resulted in a significantly smaller percentage of EGFP+ cells present in the SGZ (12±2 vs. 57±1 %, CaMKII-EGFP and CMV-EGFP respectively, $p<0.05$ Student t test, n=5 animals each) and a concomitant larger percentage present in GCL1 (45±4 %, n= 5 animals) and GCL2 (36±3 %, n=5 animals). Nevertheless, GCL3 was still the layer with fewer cells, with only 7±2 % of the EGFP+ cells present in this particular layer (Figure 3.3 A-B).

These results suggested that, although the promoter used to control EGFP expression is relevant to obtain cell type specific (neuronal) expression, the outer neuronal layer of the GCL (GCL3) is not easily transduced by (VSV-G pseudotyped) lentiviral vectors.

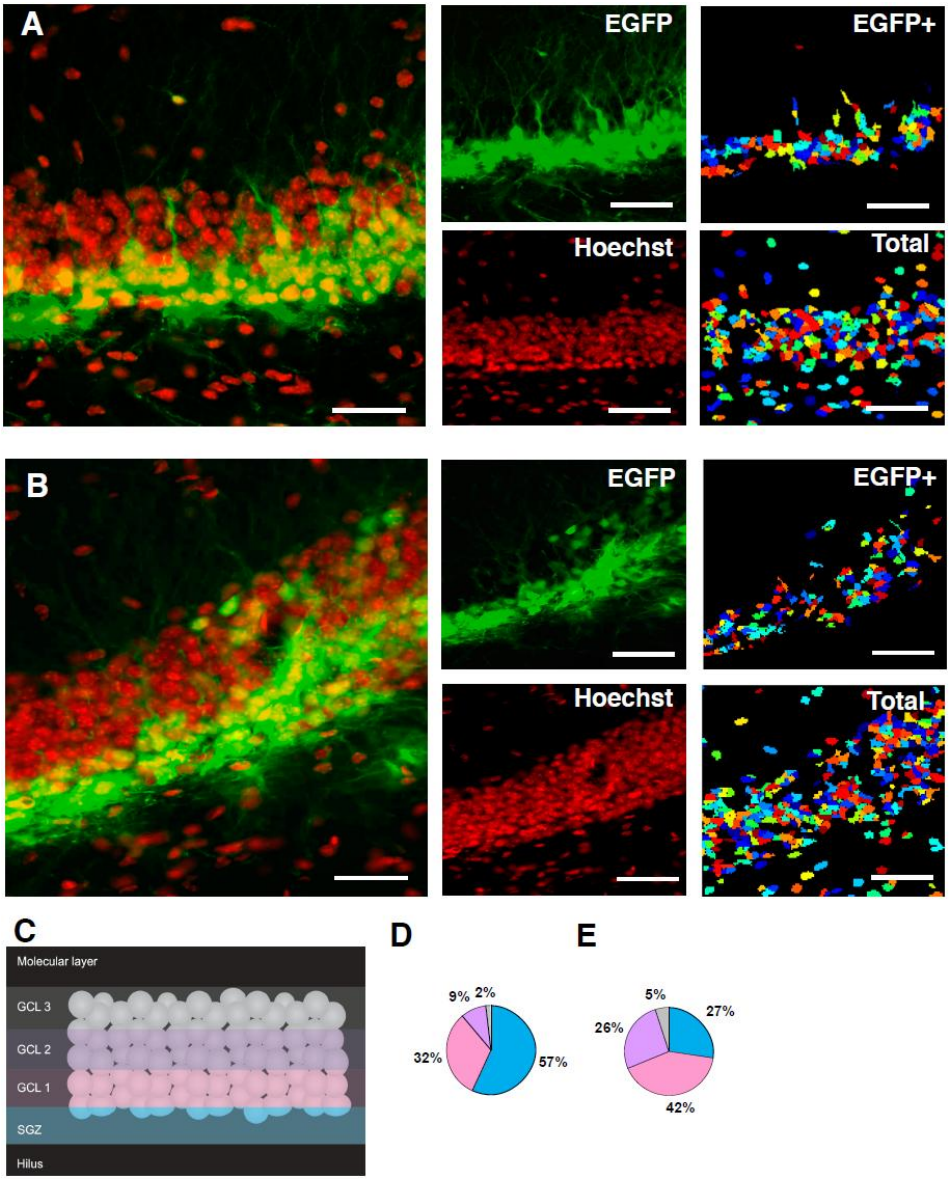

Figure 3.2 EGFP+ cell location after injection with CMV-EGFP in the DG. *Distribution of EGFP+ cells in the gcl 1* ***(A)*** *or 5* ***(B)*** *weeks after stereotactic injection of CMV-EGFP. The central panels represent the split confocal channels shown merged in left panels. Right panels depict pseudo-colored cell-localization maps, used for quantitative image analyses, generated with cell profiler showing the automatically identified EGFP+ and total cells. Scale bars: 20 µm. Each image shown is representative of 5 animals independently injected.* ***C)*** *schematic diagram depicting the subdivisions of the GCL used for quantitative image analyses, reproduced from* [94]*, with permission from the authors. Distribution of EGFP+ cells within the GCL 1* ***(D)*** *or 5* ***(E)*** *weeks after the stereotactic injection, normalized to the total number of EGFP+ cells. Each portion of the pie diagrams represents the mean percentage of EGFP+ within internal subdivisions shown in (C), indicating the distribution across the GCL, color-coded according to (C).*

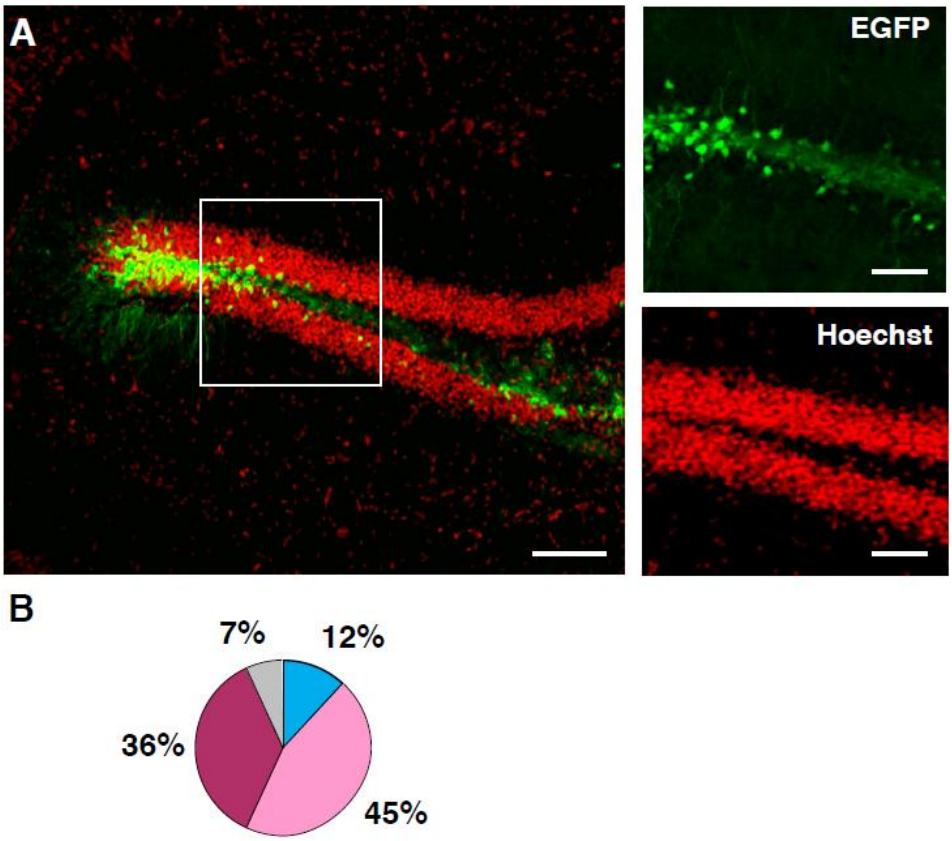

Figure 3.3, EGFP+ cell location after injection with CamKII-EGFP in the DG. *See legend on the next page.*

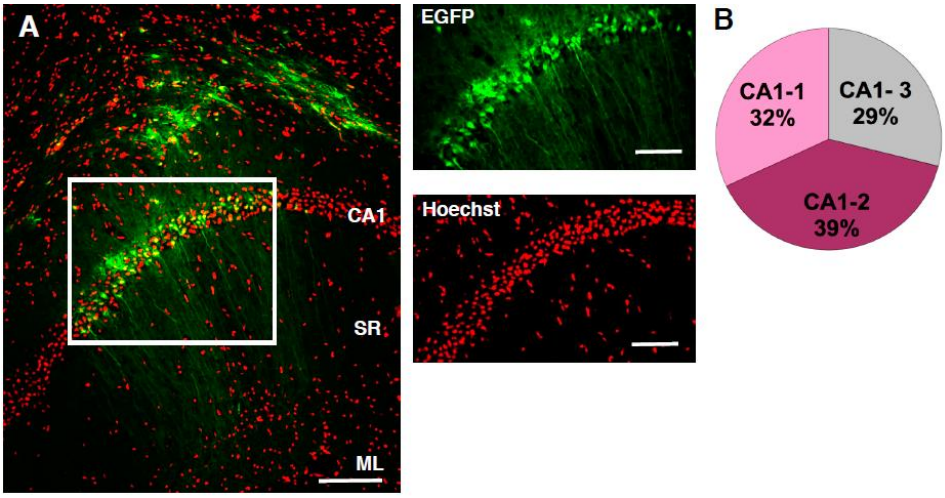

Figure 3.4 EGFP+ cell location in CA1 after injection with CMV-EGFP in the SR. *See legend on the next page.*

← ***Figure 3.3 EGFP+ cell location after injection with CamKII-EGFP in the DG. A)*** *Distribution of EGFP+ cells in the GCL, 1 week after stereotactic injection with CamKII-EGFP. Right panels represent the boxed area in the left panel of the figure. Scale bars: left panel 100 µm; right panels 20 µm. Each image shown is representative of 5 animals independently injected.* ***B)*** *Distribution of EGFP+ cells within the GCL 1 week after the stereotactic injection, normalized to the total number of EGFP+ cells. Each portion of the pie diagram represents the mean percentage of EGFP+ within internal subdivisions of the GCL, color-coded according to (3.2C).*

← ***Figure 3.4 EGFP+ cell location in CA1 after injection with CMV-EGFP in the SR. A)*** *Distribution of EGFP+ cells in CA1, 1 week after stereotactic injection of CMV-EGFP to the SR. Right panels represent the boxed area in the left panel of the figure. Scale bars: left panel 100 µm; right panels 20 µm. Each image shown is representative of 5 animals independently injected.* ***B)*** *Distribution of EGFP+ cells within the CA1, 1 week after the stereotactic injection, normalized to the total number of EGFP+ cells. Each portion of the pie diagram represents the mean percentage of EGFP+ within each internal subdivision of the CA1. SR: Stratum Radiatum; CA1: Cornu Ammonis 1; ML: Molecular layer; DG: Dentate Gyrus.*

To test this hypothesis, we utilized a lentiviral vector where the expression of EGFP was controlled by the Synapsin promoter (Syn-EGFP). This promoter has been shown to drive EGFP expression in earlier, presumably more immature, stages during neuronal development [383]. EGFP expression controlled by the Synapsin I promoter led to a pattern of distribution of EGFP+ cells in the GCL very similar to that obtained with CMV-EGFP, confirming that the promoter controlling EGFP is of relevance for the spatial distribution of EGFP+ cells in the GCL (Table 3.1). Nevertheless, a very small percentage of the EGFP+ was found to be in the GCL3, as observed with the other lentiviral vectors used in this study (Table 3.1).

	CMV-EGFP	Syn-EGFP	CaMKII-EGFP
SGZ	57 ± 1%	45 ± 1%	12 ± 3%
GCL1	32 ± 2%	41 ± 1%	45 ± 4%
GCL2	9 ± 1%	12 ± 2%	36 ± 2%
GCL3	2 ± 1%	2 ± 1%	7 ± 2% *

Table 3.1. Distribution of EGFP+ cells in the DG of animals transduced with three different lentivirus-based vectors. *Distribution of EGFP+ cells, expressed as percentage of total EGFP+ cells, with their soma within different domains of the DG (as defined in materials and methods) at 1 week post infection. Values represent mean ± SEM (n = 5 animals; *: significantly different vs CMV-EGFP; p<0.05, Student t-test).*

Spatial distribution of EGFP+ cells in the CA1 after CMV-EGFP injection.

These observations prompted us to speculate that the CMV promoter may not be highly expressed in mature neurons. To test this hypothesis we delivered CMV-EGFP to the CA1 region of the adult mouse hippocampus. One week after virus injection, we observed a strong expression of EGFP+ in the CA1 field. Typically, EGFP+ cells presented their somata in the CA1 region and extended long dendrites into the Stratum radiatum (SR), phenotypically resembling CA1 pyramidal cells (Figure 3.4A). These results confirmed that the CMV is capable of driving EGFP expression in mature post-mitotic neurons, as previously shown by others [420].

In analogy to the procedure applied for the GCL, we arbitrarily subdivided the CA1 layer in three identical width regions (CA1-1, CA1-2 and CA1-3) and accounted the distribution of EGFP+ cells in them. EGFP+ cells were homogeneously distributed across the CA1 layer of pyramidal neurons (Figure 3.4B), indicating that the irregular distribution of EGFP+ cells observed in the GCL of the DG reflects an inherent difference among granule cells in their permissiveness for lentivirus transduction.

To challenge this hypothesis we directed the stereotactic injection to the SR of the hippocampus (Figure 3.5), arguing that by doing so granule cells present in the outer layers (GCL3) will be directly exposed to the lentivirus, bypassing any possible physical barrier that may obstruct the free diffusion of the lentiviral suspension through the GCL when injected into the hilus. If the CMV-EGFP lentivirus would be able to transduce granule cells present in the outer shell of the DG, we should observe EGFP+ cells in the GCL3. Interestingly, we observed strong EGFP expression in cells present in the Molecular Layer (ML) and CA1 and even some EGFP+ positive cells in the GCL2 but none in the GCL3 (Figure 3.5A). Increased EGFP expression from higher lentiviral vector delivery titers did not result in a substantially increased proportion of EGFP+ cells located in the GCL, while the total numbers of EGFP+ cells were drastically increased (Figure 3.5B). These observations strengthened our conclusion that cells present in the GCL3 have inherent properties that make them less permissive to lentivirus-delivered transgene expression.

Transduction pattern of the CMV-EGFP lentivirus vector in the DG

In order to verify our hypothesis that the lack of transduction of GCL3 neurons is a consequence of inherent cellular properties and not of technical limitations of our delivery strategy we performed a series of experiments, presented collectively in Figure 3.6.

Correlational studies have demonstrated a large degree of discrepancy among transcript (mRNA) and protein expression levels in the mouse hippocampus [421]. Previous studies have used in situ hybridization to detect with high sensitivity the expression of lentivirus-delivered transgenes in the DG [363]. Therefore, we decided to use this technique to assess EGFP expression levels in the DG upon CMV-EGFP lentivirus delivery. As shown in Figure 3.6A, in agreement with our previous observations on protein expression using EGFP native fluorescence, one week after injection the EGFP in situ hybridization signal was mostly restricted to the hilus and the SGZ, demonstrating that the lack of EGP expression in the outer layers of the GCL is not a consequence of possible post-transcriptional regulation but more likely of lack of transgene expression.

To substantiate this conclusion we should be able to show that there are no major physical obstacles to reach the DG by stereotactic injection into the SR. To achieve this goal we used a fluorescently labelled transferrin-derived peptide (T12-Cy5, Prosensa BV, Leiden, The Netherlands) delivered by stereotactic injection into the SR (1 μl; 30 μM). Transferrin-derived peptides have been shown to increase delivery efficiency of molecular cargos to neuronal cells *in vivo* [422;423]. Following this approach, 48 h after injection we observed fluorescence distributed across the SR and ML fields, reaching the CA1 and DG (Figure 3.6B). A closer observation of the DG clearly displayed a fluorescence pattern with maximal intensity in the ML and gradually diffusing

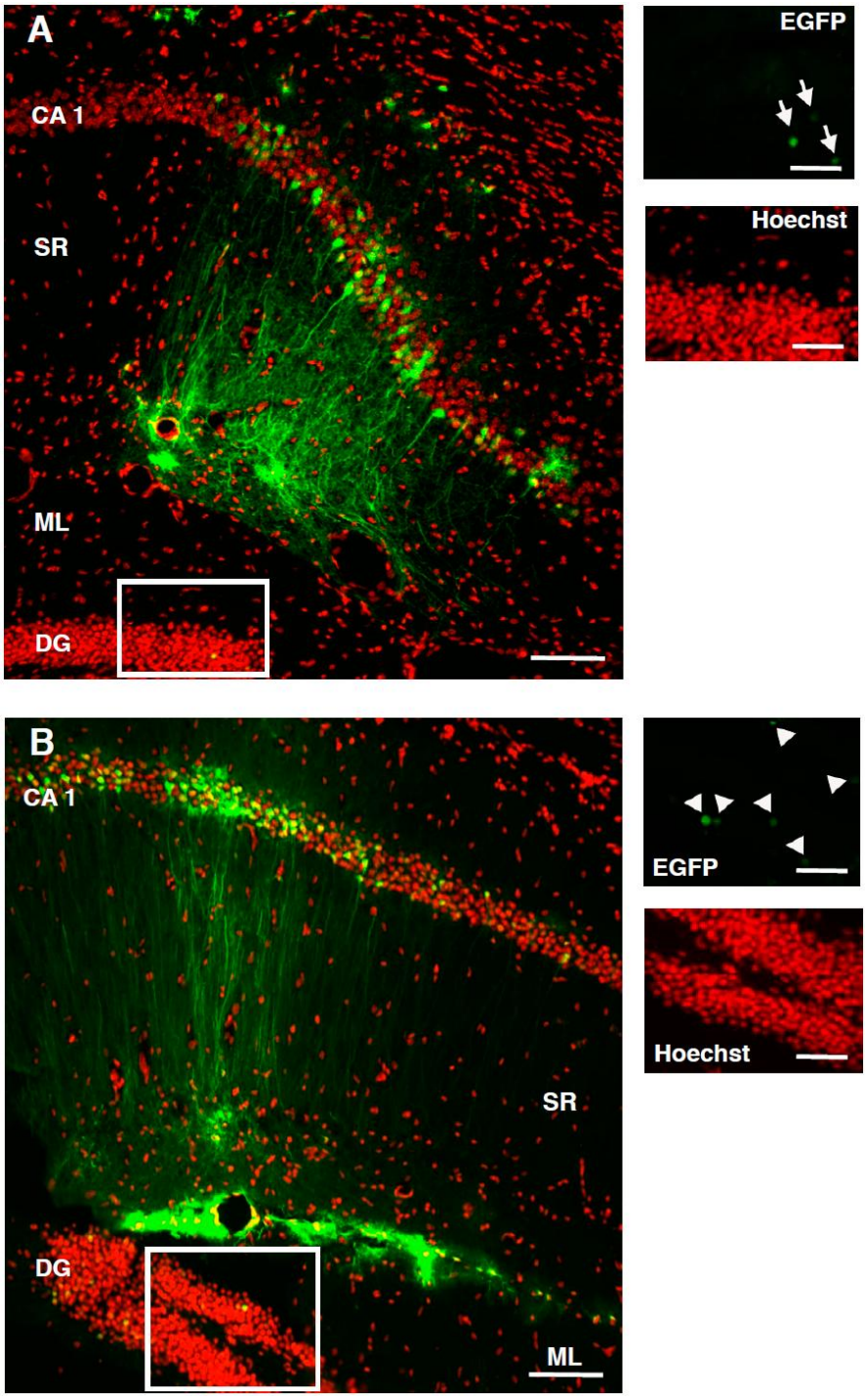

Figure 3.5 Lentivirus-mediated EGFP delivery to the SR. *See legend on the next page.*

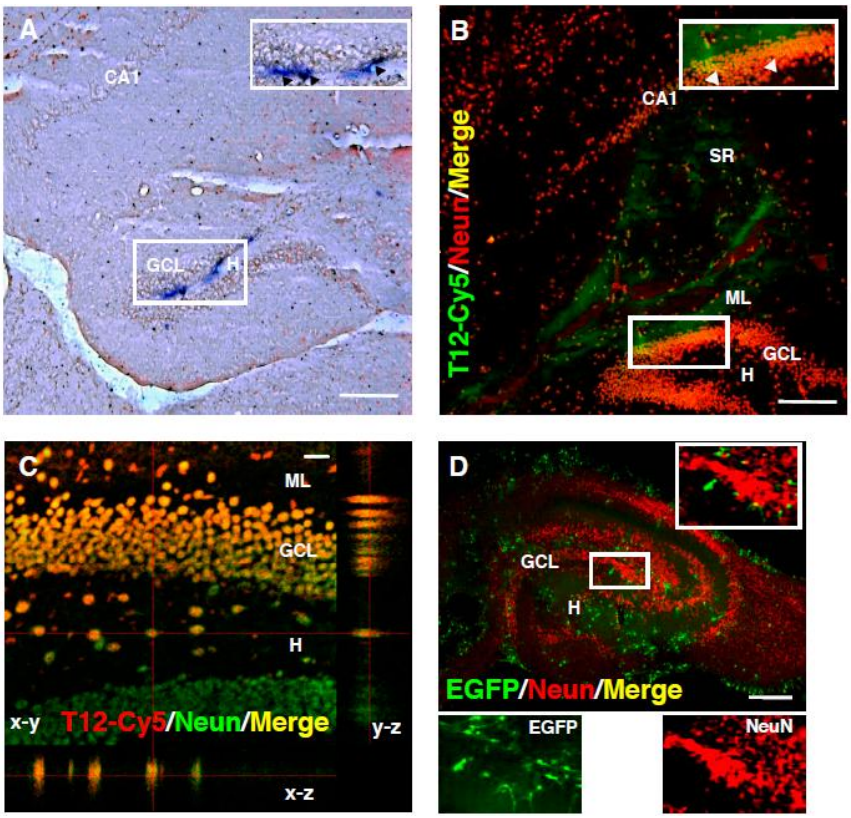

Figure 3.6 Transduction pattern of the CMV-EGFP lentivirus vector in the DG after delivery to the SR. A) *In situ hybridization for EGFP mRNA in the GCL, 1 week after stereotactic injection of CMV-EGFP to the SR. Inset: higher magnification view of the boxed area. Arrowheads indicate positive EGFP expression in the hilus and SGZ.* ***B)*** *Stereotactic injection to the SR of a fluorescently labeled transferrin-derived peptide (T12-Cy5, pseudocolored green). NeuN+ cells are shown in red. Inset: higher magnification view of the boxed area. Arrowheads indicate NeuN+ cells in the outer GCL positively transduced with the fluorescent peptide (yellow). Animals were sacrificed 48 h after the injection.* ***(C)****: Higher magnification confocal image showing co-localization (yellow) of T12-Cy5 (red) and NeuN (green) in cells located across the supra-pyramidal blade (top) hilus and infra-pyramidal blade (bottom) of the DG. The orthogonal projection on the y-z axis shows a gradient of peptide expression from the ML to the H with highest expression in cells located in the outer GCL.* ***D)*** *CMV-EGFP transduction pattern in DIV-5 organotypic hippocampal slice cultures. Inset: higher magnification of the boxed area. The split panels at the bottom show the corresponding EGFP and NeuN signals from the same area. Note the almost complete lack of co-localization. Each image shown is representative of 5 animals independently injected. Scale bars: A, B and D: 100 µm; C: 10 µm. CA1: Cornu Ammonis 1, SR: Stratum Radiatum; ML: Molecular layer; GCL: Granule Cell Layer; H: Hilus; SGZ: Subgranular Zone.*

← Figure 3.5 Lentivirus-mediated EGFP delivery to the SR. *Lentivirus-mediated delivery by stereotactic injections to the SR of the hippocampus does not result in substantial EGFP expression in the GCL, despite low* ***(A)*** *or high* ***(B)*** *EGFP expression, 1 week after injection. Right panels represent the boxed area in the left panels of the figure. Arrows (A) and arrowheads (B) indicate EGFP+ cells in the GCL. Scale bars: left panels 100 µm; right panels 20 µm. Each image shown is representative of 5 animals independently injected. SR: Stratum Radiatum; CA1: Cornu Ammonis 1; ML: Molecular layer; DG: Dentate Gyrus.*

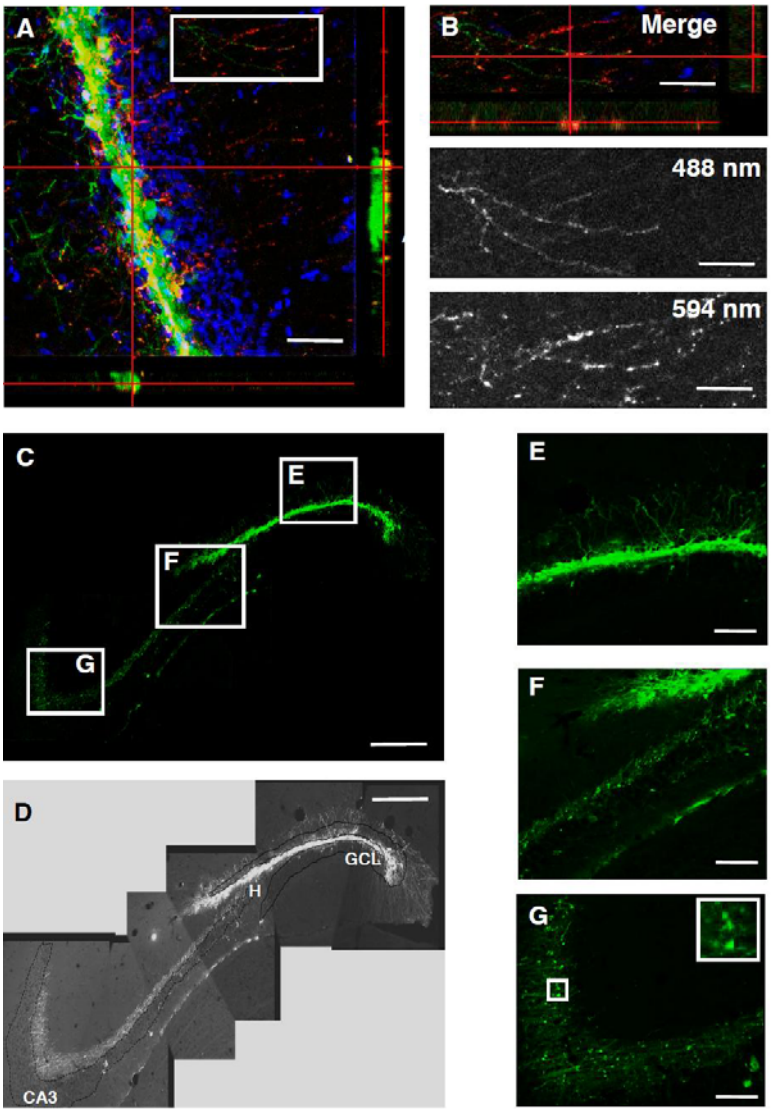

Figure 3.7 Transduction pattern of the CMV-EGFP lentivirus in DG and CA3 by EGFP immunohistochemistry. A*) Confocal microscope image and orthogonal projections onto the x–z (bottom) and y–z (right) planes showing co-localization (yellow) of the native EGFP fluorescence (green) and EGFP immunohistochemistry signal (red) in GC somata, one week after injection.* ***B)*** *Higher magnification of the boxed area depicted in A showing partial co-localization in dendrites of GC (top). The split panels corresponding to the EGFP native fluorescence signal (488 nm, center) and the EGFP immunohistochemistry signal (594 nm, bottom) are shown.* ***C*** *and* ***D)*** *Composite of 5 confocal z-projected stacks combining EGFP's native fluorescence and immunohistochemistry signal 5 weeks after injection, showing EGFP positive cells with their somata in the GCL* ***(E)*** *and projecting axons into the hilus* ***(F)*** *and the Stratum Lucidum of the CA3 field* ***(G)****, were the synaptic boutons of these axons are evident (F and inset in G). Each image shown is representative of 5 animals independently injected. Scale bars: A: 40 µm; B: 25 µm; C: 200 µm; E; F and G: 10 µm. GCL: Granule Cell Layer, H: Hilus. CA3: Cornu Ammonis 3.*

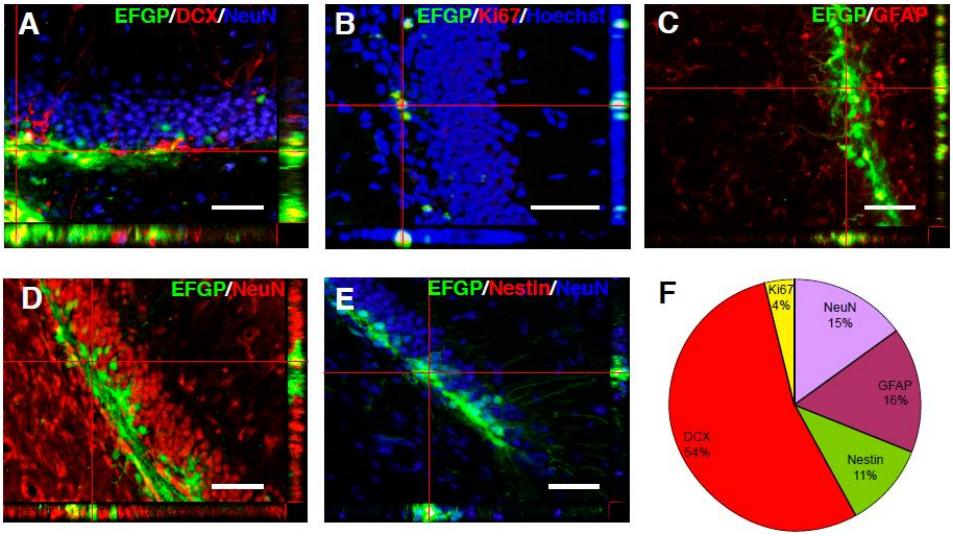

Figure 3.8 Identification and quantification of different cell types targeted by injection of CMV-EGFP in the DG. *See legend on the next page.*

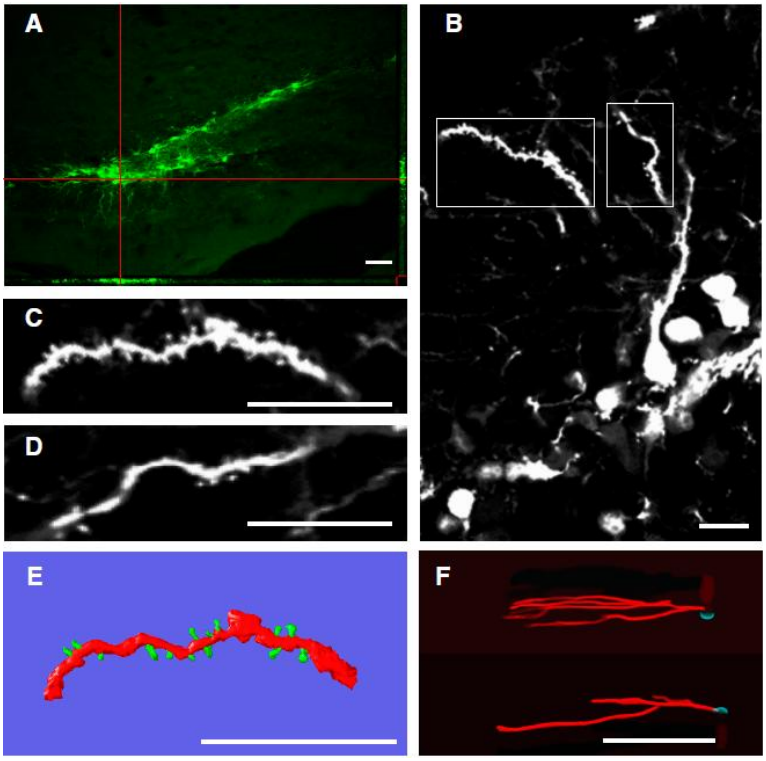

Figure 3.9 Morphological analyses and three-dimensional reconstructions of EGFP+/NeuN+ cells in the GCL. *See legend on the next page*

← ***Figure 3.8 Identification and quantification of different cell types targeted by injection of CMV-EGFP in the DG.*** *Examples of EGFP co-localization with different markers of neuronal differentiation within the GCL. The orthogonal projections onto the x–z (bottom) and y–z (right) planes of cells indicated by hairlines are shown to confirm double labelling throughout the extent of EGFP+ cells co-expressing DCX and NeuN **(A)**; Ki67 **(B)**; GFAP **(C)**; NeuN **(D)** or Nestin and NeuN **(E)**. Each image shown is representative of 5 animals independently injected. Scale bars: 20 µm. **(F)** Percentual distribution of EGFP+ cells expressing differentiation markers within the GCL, 1 week after the stereotactic injection normalized to the total number of EGFP+ cells.*

← ***Figure 3.9 Morphological analyses and three-dimensional reconstructions of EGFP+/NeuN+ cells in the GCL.*** *Representative examples of EGFP+ neurons located in the GCL 1 week after the stereotactic injection of CMV-EGFP. **A)** Confocal image showing EGFP+ cells in the DG. The orthogonal projections onto the x–z (bottom) and y–z (right) planes are shown to confirm EGFP expression throughout the extent of the cells indicated with hairlines. **B)** Z-axis projection of EGFP+ neurons from the area depicted in A, showing their morphological features. **C)** and **D)** higher magnification of the areas boxed in B. **E)** three-dimensional reconstruction of the dendritic segment depicted in C, shown as example of those used for dendritic protrusion analyses. **F)** Three-dimensional reconstructions of two example EGFP+ GCL neurons, showing their dentritic arborization and length. Cell somata are shown in cyan and dendrites in red. Similar neurons were used for quantitative analyses. Scale bars: A: 50 µm; B, C, D, E: 10 µm; F: 100 µm. (Full-resolution animated 3D-reconstructions are available at http://bio-imaging.liacs.nl/gallery).*

into the GCL, labelling Neuron-Specific Nuclear Protein (NeuN)+ cells present in all three sub-layers of the supra-pyramidal blade, the hilus and reaching the infra-pyramidal blade of the DG (Figure 3.6C). These results demonstrated that our stereotactic injections to the SR can positively transduce neurons of the GCL, including those located on the GCL3 and therefore that no physical (anatomical) obstacles may preclude lentivirus transduction of granule cells.

Although these findings support the conclusion that our lentiviral system is not able to induce transgene expression in GCL3 neurons, we tested once more this hypothesis in organotypic hippocampal slice cultures. Using this model, hippocampal cells are directly exposed to the virus-containing solution, avoiding the need of stereotactic injection [67]. During the first postnatal weeks neurons of embryonic origin are already present in the immature GCL, while progenitor cells that will eventually complete the neuronal layer are still present in the hilus [424]. Four days after transduction with CMV-EGFP lentivirus the vast majority of the EGFP+ cells were negative for NeuN (Figure 3.6D), therefore substantiating our conclusions. This is in agreement with previously reported observations, where we demonstrated that the CMV-EGFP lentivirus transduces nestin/GFAP+ neuronal progenitor cells in early postnatal hippocampal slices [67]. Moreover, others have shown that although transgene expression increases slowly with time after transduction of hippocampal slices with VSV-G pseudotyped lentivirus, it may remain restricted to CA1 and CA3 pyramidal cells [425].

Since we have used EGFP native fluorescence to directly detect transgene expression, one possible technical limitation in observing positive cells could have been a presumable low sensitivity of native EGFP fluorescence detection. Indeed, in the reference protocol for transgene delivery to granule cells the use of EGFP immunohistochemistry and subsequent indirect fluorescence detection is recommended [367]. Therefore, we followed this approach to account for possible low detectability of EGFP-expressing cells. As shown in Figure 3.7A,

immunohistochemistry increased the detection of EGFP, as expected. Importantly, this increase was most evident in the dendritic arborizations and axonal extensions of the labelled cells. To account for this observation we used an Alexa 594-labelled secondary antibody to discriminate in between EGFP native fluorescence detected with an excitation wavelength of 488 nm and the immunohistochemistry signal detected with an excitation wavelength of 594 nm (Figure 3.7A). This approach showed a partial co-localization of the two signals, with highest co-localization detected in cell somata, and only partial co-localization in dendrites and axons (Figure 3.7A, boxed area and 3.7B). This phenomenon has been previously described and explained by partial antibody penetration under experimental conditions similar to ours [426]. Therefore, in successive experiments we utilized a combination of native fluorescence and immunohistochemistry signal by using an Alexa 488-labelled secondary antibody. Following this approach, 5 weeks after injection the morphology of EGFP expressing cells transduced by the CMV-EGFP lentivirus was exposed with great detail. As recently described with other retroviruses [102;426], we were able to observe the axonal projections of EGFP labelled granule cells into the hilus and the stratum lucidum of the CA3 (Figure 3.7C-F). However, the distribution of EGFP+ cell somata within the GCL remained very similar to that observed using native fluorescence only, with almost no EGFP+ cells observed in the GCL3 (Figure 3.7G). Altogether these experiments further substantiate our conclusion that cells within the GCL3 are less permissive to lentivirus-delivered transgene expression.

Identity of EGFP+ cells in the GCL after CMV-EGFP injection.

To characterize the cell type(s) transduced by the CMV-EGFP lentivirus more accurately, we performed a series of immunohistochemical co-stainings for neuronal progenitor (nestin), glial (GFAP), immature neuron (DCX), proliferating (Ki67) and mature neuron (NeuN) cell markers [62] (Figure 3.8). EGFP+ cells present in the GCL were analyzed for co-expression of these markers one week after lentivirus injection (Figure 3.8A-E). Quantitative analyses of these samples demonstrated that the majority of the EGFP+ cells were DCX+, with phenotypes ranging from putative dividing neuronal progenitors to early post-mitotic immature neurons (Figure 3.8). Nestin+, GFAP+ and NeuN+ cells accounted each for approximately one third of the EGFP+ cells, while Ki67 was co-expressed in a small proportion of the cells (Figure 3.8F). NeuN+ cells were further analyzed for neuronal features such as the presence of dendritic spines (Figure 3.9). We found that 11±4 % of the EGFP+ neurons present in the GCL had simple dendritic arbors with dendritic spines (Figure 3.9B-D), phenotypically resembling immature, most probably adult generated neurons [94;368]. Quantitative analysis of spine density from EGFP+ neurons showed that these cells have relative low protrusion densities (Figure 3.9E; 7±2 protrusion/10 μm, n=5 neurons, 420 protrusions counted) and present morphological features compatible with immature neurons [427]. Three-dimensional reconstructions of EGFP+ cells revealed that these cells had narrow, low-complexity dendritic arbors, normally with one primary dendrite and relatively short secondary dendrites projecting into the ML (Figure 3.9F; mean maximal distance from soma 203± 20 μm, n=25 neurons), characteristics all compatible with being immature neurons [94;95].

DISCUSSION

In the present study we have used lentiviral vectors expressing EGFP from three different promoters in the mouse hippocampus and have identified sub-field specific differences in transgene expression in various cell types of the GCL of the DG. Furthermore, we have characterized the cell types transduced by these lentiviral vectors, concluding that they target primarily NPC and immature neurons present in the SGZ and more immature layers of the GCL. Our observations suggest the existence of intrinsic differences in the permissiveness to lentivirus transduction among populations of granule cells of the GCL. In particular, we show for the first time that mature neurons of the outer granule cell layer do not express lentivirus-delivered transgenes, despite successful expression in other hippocampal cell types. Therefore, only adult-generated neurons may be target for lentivirus-mediated transgene delivery within the GCL.

The DG of the mammalian hippocampus is progressively constructed through a complex developmental program. Embryology studies have demonstrated that the GCL can be divided into an outer shell and an inner core, originated from separate embryonic progenitor pools. These progenitors generate first the outer shell followed by the development of the inner core by later-born granule cells [428]. Therefore, the outer shell of the GCL is partially assembled during embryogenesis and the majority of dentate granule cells, located in the inner shell are generated after birth [429-431]. These and other observations have generated the hypothesis that, in contrast to the neocortex, the DG is built up following a life-long outside-in arrangement, where new cells are incorporated in the GCL following a downward gradient of positional cues [368].

In rodents, proliferative cells become largely confined to the SGZ at the base of the GCL after postnatal day 30 [430]. Therefore, during the juvenile and adult periods the SGZ is the source of newly produced granule cells [62].

Several groups have shown heterogeneous functional properties of granule cells in the adult hippocampus. In particular, new neurons generated by adult neurogenesis display increased synaptic plasticity and increased excitability suggesting that maturation of the neuronal phenotype includes changes in membrane excitability and morphology, as well as the establishment of appropriate connectivity [94;107;432]. Interestingly, it has been proposed that functional and morphological differences among granule cells are a function of their location within the GCL rather than of their relative age [76;433].

Herein we report that the three different lentivirus systems tested in this study, transduced mainly cells located in the SGZ and inner layers of the GCL. Cells expressing the reporter transgene EGFP one week after viral injection were mainly immature neurons expressing DCX. These observations resemble the EGFP expression profile achieved using MMLV-derived vectors that transduce only proliferating cells [94]. Therefore, the initial cell population hit by the lentivirus was most probably a subpopulation of NPC that evolved into the neuronal lineage as judged by the predominance of DCX+ cells one week after transduction, similar to reports using MMLV-vectors [94;95]. Moreover, retro- and lenti-viral vectors have been shown to target similar, although not completely overlapping, populations in the hippocampus [368]. Therefore, the use of adeno-

associated virus-derived vectors may be more adequate to target mature neurons of embryonic origin in the adult dentate gyrus [434]. An indubitable characterization of the cell type originally transduced by the lentiviral vector may request the use of cell type specific promoters restricted to NPCs [435]. However, in the adult dentate gyrus, DCX is only expressed in cells contributing to adult neurogenesis and therefore can be used as a bona fide marker of newborn adult-generated neurons [100;436].

Our observations are in agreement with the described ability of lentiviral vectors to transduce adult NPC in vivo [437]. The presence of subpopulations of EGFP+ cells expressing the NPC marker nestin and Ki67, a cell proliferation marker expressed during the active phases of the cell cycle [438] emphasize our conclusions.

Moreover, the reduced numbers of EGFP+/NeuN+ cells found, their morphology and their location in the inner layers of the GCL, indicate that these EGFP+/NeuN+ cells have most probably originated from a population of immature cells originally hit by the virus.

Crucial to sustain these conclusions are our experiments in which we delivered the lentiviral vector to the SR, situated between the CA1 and the outer shell of the GCL. If the pattern of EGFP expression restricted to the inner layers of the GCL would have been a mere mechanical effect of the steric hindrance generated by the tightly packed structure of the GCL [439], the lentiviral vector should have been able to transduce cells in the outer layers of the GCL, when delivered to the SR. Conversely, we observed strong EGFP expression in cells within the ML and CA1, demonstrating adequate diffusion of the lentivirus across different cellular structures. Moreover, EGFP+ cells were homogenously distributed within the CA1 layer, with profuse EGFP expression in the soma, axons and dendrites of cells phenotypically resembling mature pyramidal neurons. Our experiments using a peptide-cy5 conjugate, depicted in Figure 6, showed that this construct delivered into the SR, could effectively transduce the neurons located in the outer layers of the supra-pyramidal blade of the GCL and beyond into the hilus and the infra-pyramidal blade. These experiments demonstrated that stereotactic injection to the SR permits effective delivery to the GCL.

Our data from the CA1 cells demonstrated as well that the CMV promoter is indeed able to promote transgene expression in mature post-mitotic neurons, as previously described [420]. These observations made us to conclude that, although the use of different (cell-type specific) promoters is useful to promote different patterns of transgene expression in the GCL, cells present in the outer shell of the GCL only scarcely express transgenes delivered by lentiviral vectors. Interestingly, the Synapsin I promoter rendered an EGFP expression profile more similar to that of the CMV promoter than to that of the CaMKII promoter, in accordance to its expression in earlier neuronal developmental stages [383]. Therefore, although further experiments to investigate transgene expression mediated by different promoters at later times post-injection seems important to address the relevance of differential promoter use, it escapes the objective of the present study.

One potential drawback of the use of the CMV promoter may be its potential activation in astrocytes short time after injury, described in the cerebral cortex and caudate-putamen [440].

Nevertheless, this activation could be dependent on virus titers and other factors such as the particular CMV sequence used and the time after the injection [441]. For the interpretation of the data presented herein it is worth to take into account that sections surrounding the injection site were routinely discarded.

Specific transgene silencing after lentiviral vector-mediated delivery has been described before [442]. Although we cannot exclude from this set of experiments the possibility that transgene expression driven by the three promoters used in this study were selectively silenced in mature neurons present in the outer layers of the GCL, the fact that the CMV promoter was able to promote expression in cells of the CA1 makes this possibility unlikely.

Overall, our observations are in agreement with previous reports showing that lentiviral vectors can successfully transduce mitotic and post-mitotic cells [420;437;443]. However, the exact nature of the cell types and hippocampal sub-fields targeted by lentiviral vectors remains controversial. Previous reports did not find sub-field specific differences in GFP expression. This could be due to technical differences such as the use of different GFP variants and constructs, analysis of the samples at different time points after stereotactic injection or differences in the CMV promoter sequence used to control transgene expression [363;369]. Nevertheless, the disparity in EGFP expression reported herein between cells located in the inner or outer layers of the GCL seems to be a function of intrinsic differences between cells generated by embryonic or adult neurogenesis. In this context, disparities in transgene expression in granule cells, depending on their relative location within the GCL and their progression into the neuronal differentiation program, emphasize the heterogeneity between newly adult-generated neurons and pre-existing ones, probably originated during embryonic and/or early postnatal development.

Although further experiments will be required to clarify the exact nature of this heterogeneity among granule cells of the DG, regarding their permissiveness to lentivirus-delivered transgene expression, one possible explanation could be the differential expression of receptor proteins that recognize pseudotyping proteins by subpopulations of granule cells. However, VSV-G pseudotyped viruses have been shown to effectively transduce cells within the GCL of the DG [363;369]. This suggests that, although pseudotyping proteins can influence transduction efficiency and tropism to hippocampal cell types [384;419], the receptors for VSV-G glycoprotein are present in granule cells of the DG. Moreover, transgene expression from VSV-G pseudotyped lentivirus is pantropic in the rat brain, labelling a variety of glial and neuronal cell types depending on the promoter used to control transgene expression [443].

Interestingly, even though cell mitosis is not a requisite for integration, transduction efficiency of lentiviral vectors is dependent on cell-cycle progression of target cells, with cells actively growing or arrested in phases other than G_0 being more efficiently transduced *in vivo* [420;444-446]. As demonstrated here, lentivirus transduced EGFP+ cells are in their vast majority positive for progenitor (nestin), astrocyte (GFAP), proliferation (Ki67) and immature neuron (DCX) cell markers. Furthermore, Schmetsdorf et al [447] have demonstrated that cells from distinct hippocampal fields, including CA1, CA3 and DG, express completely different repertoires of cell cycle-related proteins. Therefore, although a more thorough elucidation of the factors regulating

lentivirus transduction of post-mitotic granule cells is beyond the scope of this article, our observations demonstrating lentivirus-mediated transgene expression in NPC and immature neurons suggest that cell-cycle progression is an important determinant in lentivirus transduction efficiency of hippocampal granule cells *in vivo*.

Conclusion

Herein, we report on sub-field specific differences in permissiveness to lentivirus-delivered transgene expression in the mouse hippocampus. Most interestingly, we observed transgene expression preferentially in NPC and immature neurons present in the SGZ and inner layers of the GCL, where adult neurogenesis takes place and different subpopulations of granule cells exist. Based on our results, we conclude that this disparity in transgene expression observed between cells located in the inner or outer layers of the GCL seems to be a function of intrinsic differences between cells generated by embryonic or adult neurogenesis and therefore favour the hypothesis that cell-cycle progression of target cells is an important determinant of lentivirus transduction efficiency. These differences could be exploited in utilizing lentivirus for transgene delivery to NPC and immature neurons of the mouse hippocampus *in vivo*.

Acknowledgements

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GLUCOCORTICOID RECEPTOR REGULATES FUNCTIONAL INTEGRATION OF NEWBORN NEURONS IN THE HIPPOCAMPUS

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ABSTRACT

Stress is a major factor affecting adult hippocampal neurogenesis. However, the role of one of the major mediators of the stress response in neuronal progenitor cells (NPCs), the glucocorticoid receptor (GR), is unknown. Here, we show that specific GR knockdown in NPCs accelerated neuronal differentiation and migration. Strikingly, GR knockdown led to mis-positioning of adult newborn neurons, to altered dendritic arborization, to higher numbers of mature mushroom and thin spines and to larger mossy fiber boutons. In line with increased numbers of synaptic contacts, adult newborn neurons with GR knockdown exhibit increased mEPSC frequencies. Together, our data show a key role for GR expression levels in the appropriate formation of hippocampal neo-circuits.

INTRODUCTION

In the adult brain, neuronal progenitor cells (NPCs) exist in the sub-granular cell layer of the dentate gyrus, a subfield of the hippocampus that is involved in learning and memory formation. NPCs proliferate, and a subpopulation of these migrate and differentiate into granule neurons that are integrated into functional hippocampal networks (reviewed in [62]). Together, this process takes approximately 4 weeks and is known as adult hippocampal neurogenesis. Previous pharmacological and physical [280;448] and recent genetic manipulations [98;406;449] aiming at blockade of neurogenesis have provided convincing evidence for a role of neurogenesis in hippocampus-dependent memory formation.

Among the most profound and best-studied regulators of neurogenesis are glucocorticoids (GC), which are one of the main mediators of the stress response. In the brain, GCs have profound effects on hippocampal networks that underlie behavioural adaption to stress. Prolonged periods of elevated GC levels, induced by e.g. chronic stress, have been associated with alterations in neuronal plasticity [371] and decreased levels of neurogenesis [139;244;450].

GCs exert their effect by binding to two types of receptors i.e. glucocorticoid receptors (GR) and the mineralocorticoid receptor (MR) [14]. Despite the wealth of information on the effect of stress and circulating GC levels on neurogenesis, very little is known on the role of GR and MR in individual NPCs. GRs are known to be expressed in NPCs but the MR is not [56;57]. Two recent studies have suggested that tight GR regulation in NPCs is necessary for proper neurogenesis. Firstly, NPCs contain specialized retrograde transport mechanisms for GR translocation to the nucleus [67]. Secondly, neuron-specific miR-124, a non-coding small RNA molecule regulating proper neuronal differentiation of NPCs *in vitro* and *in vivo* [60;61], down-regulates GR protein levels [59]. Yet, how GR protein levels directly regulate adult-born NPC function is unknown.

To study the *in vivo* role of the GR in NPCs of the sub-granular layer of the DG requires an experimental approach that is not based on traditional pharmacological or genetic manipulations of the GR. Therefore, we used previously characterized lentivirus-based vectors that preferentially transduce a population of DCX+ neuronal progenitor cells and immature dentate granule neurons in the DG (further referred to as NPCs) [109;451] (see also CHAPTER 3). *In vitro*, lentivirus-based vectors containing shRNAs directed against the GR (pm-shGR) were found to gain more than 70% GR protein knockdown (Van Hooijdonk et al., unpublished data (CHAPTER 2)). Using this technique for shRNA transgenesis to knockdown GR protein expression, we found that GR knockdown accelerates neuronal differentiation of NPCs. Moreover, GR knockdown induced aberrant positioning of adult newborn granule neurons in the outer layers of the DG, a higher number of cells with complex dendritic trees and a higher abundance of thin / mushroom-shaped mature spines at the cost of more immature stubby-shaped ones. Strikingly, this aberrant cellular phenotype was associated with enhanced basal neuronal excitability as measured by mEPSC frequencies.

MATERIALS AND METHODS

Animals

Male BALB/c mice (6 weeks, Janvier Bioservices, Genest st Isle, France) were individually housed for one week in filtertop cages before stereotactic surgery. The mice had free access to food and water and were kept under a 12 hour dark/light cycle (lights on at 8.00 hrs) in a temperature (20°C) and humidity controlled room. Experiments were performed between 8.00 and 13.00 hrs. All experiments were approved by the committee on Animal Health and Care from the Leiden University, The Netherlands and the Netherlands ministry of VROM and were performed in strict compliance with the European Union recommendations for the care and use of laboratory animals.

Short hairpin (shRNA) constructs

Perfect match (pm) and two nucleotide mismatch (mm) control short hairpin RNA (shRNA) expression vector and mismatch (mm) control directed against the consensus sequence of mouse, rat and human GR were designed according to the described criteria [373;374]. The sequence for shRNA against mouse GR (NM_008173) was GATCCCCGAAAGCATTGCAAACCTCATTCAAGAGATGAGGTTTGCAATGCTTTCTTTTGGAAA for the pm, and GATCCCCGACAGCATTGCACACCTCATTCAAGAGATGAGGTGTGCAATGCTGTCTTTTGCAAA for the mm control (mismatch positions underlined). The sense and antisense oligonucleotides were annealed and cloned in *BglII* and *HindIII* sites of p-super vector (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Insertion of the oligonucleotides was confirmed by sequencing. The knockdown of the GR by pm-shGR was confirmed by Western Blot in rat PC12 cells and functionally tested in N1E-115 mouse neuroblastoma cells by using a Dual Luciferase (Promega Corp. Madison, WI)- based GC response element reporter gene assay as previously described (see CHAPTER 2).

Lentiviral vectors

P-super vector derived pm-shGR and corresponding mm-shGR constructs were subcloned into a lentiviral vector downstream of the H1 promotor. The lentiviral vector also contained EGFP downstream of the CMV promoter. These replication incompetent and self-inactivating Advanced Generation Lentiviral vectors were produced and titrated as previously described [67] (see CHAPTER 3). Titers of both viruses were comparable and ranged between $1x10^8$ and $1x10^9$ transducing U/ml. Virus suspensions were stored at -80°C until use and were briefly centrifuged and kept on ice immediately before injection.

Stereotactic surgery

Stereotactic injections were performed in the morning, following previously described methods [451] (see CHAPTER 3). For all experiments, (LV-) pm-shGR or -mm-shGR constructs (titers ranged between $1x10^8$ and $1x10^9$ transducing U/ml) were injected bilaterally into the hilus of the DG (AP:

-2.00 mm, ML: +/-1.50 mm, DV: -1.90 mm, relative to Bregma). After surgery, animals were placed under a heating lamp until awakening and checked upon daily. For immunocytochemical experiments a group of 40 mice (N=20 for pm-shGR and N=20 for mm-shGR) was stereotactically injected and sacrificed one week post injection (N=10 for pm-shGR and N=10 for mm-shGR) or five weeks post injection (N=10 for pm-shGR and N=10 for mm-shGR). The results at the five week time point for neuronal makers and GFP positioning were confirmed in the mice that underwent context and cue fear conditioning- (see CHAPTER 5). For electrophysiological experiments, 20 mice were stereotactically injected and sacrificed five weeks later (N= 10 pm-shGR and N= 10 mm-shGR). In all experiments, mice were only included for analysis based upon post-mortum histological evidence of an appropriately targeted micro-injection as visualized by GFP expression in SGZ. Animals with low (<100 GFP+ cells per section), absent or mis-positioned GFP expression were excluded from the experiment and further assessment.

Tissue preparation

For the immunohistochemical experiments, one to five weeks post injection (PI), mice were sacrificed and brains were fixated by transcardial perfusion and processed as described previously [451]. Serial, coronal sections of 20 um thickness, were obtained using a cryostat (Leica CM 1900). All brain sections containing the hippocampus were collected either free-floating in eppendorf tubes containing anti-freeze, or thaw-mounted on SuperFrost object glasses. Superfrost slides were stored at -80°C till further use, whereas free floating sections were stored at -20°C.

For the electrophysiology experiment, 5 week PI, mice at rest were sacrificed by decapitation (one mouse per day, between 9.00 and 9.30 am, when circulating levels of plasma corticosterone are low) Acute hippocampal slices (350 μM thick) for the electrophysiology recordings were made with a vibratome (model VT1000S; Leica, Germany) as described before [452] [30]. Hippocampal slices were stored at room temperature until recordings (for at least 1 h).

Immunohistochemistry

Immunofluorescent double and triple labelling staining procedures were performed following a standard procedure with thaw-mounted sections, and free floating as described [381;451] (see CHAPTER 3). Primary antibodies were from: Santa Cruz Biotechnology, Inc; Heidelberg, Germany (Doublecortin (C-18), used 1:200, Ki67 (M-19), used 1:100; GFAP, mouse monoclonal, used 1:1000); Chemicon-Millipore International BV, Amsterdam, The Netherlands (NeuN (A60), mouse monoclonal, used 1:200; GFP, mouse monoclonal, used 1:200), BD Biosciences, Breda, The Netherlands (Nestin, mouse monoclonal (556309), used 1:200), Abcam (Glucocorticoid receptor, (BuGR2), mouse monoclonal, used 1:500) or Molecular Probes/Invitrogen, Breda, The Netherlands (GFP, chicken polyclonal, used 1:500).

Electrophysiology

In acute hippocampal slices, GFP+ granule cells of both pm- and mm- shGR treatment groups and GFP- cells of both treatment groups were assessed for their physiological properties. Using patch

clamp techniques, spontaneous miniature excitatory postsynaptic currents (mEPSCs) were recorded as a measure of the resting membrane properties of newborn cells. Recordings of mEPSCs were performed as previously described [30]. All values shown in the results section are the average ± SEM of the data. Statistical analysis between control and a single treatment on a given group of cells was carried out using a Student's *t*-test (paired when applicable). If more than two conditions were investigated on a given set of cells, we applied a general linear model for repeated measures (within-subjects contrast). Comparison of several conditions between sets of cells was performed with ANOVA, followed by *post hoc* multiple comparisons of the mean (Tukey). In all cases, $P < 0.05$ indicated significance.

Histological analyses and confocal microscopy

Quantification of EGFP+ cells and quantitative analysis of different classes of neuronal cells in the hippocampus of treated animals were performed using the optical fractionator sampling method, as described by Encinas and Enikolopov [81]. Briefly, every tenth hippocampal section was collected starting at the DG following the fractionator scheme, to ensure that each slice is 200 nm apart from the next slice within each collected set of approximately 11 slices [67;451]. For quantification of EGFP+ cells, three sets of slices from at least three independently injected animals from each experimental group were used. Sections surrounding the injection site were routinely discarded. For quantitative analysis of neuronal cell-types other three sets of slices from at least three independently injected animals from each experimental group were used. Confocal images were acquired using a Nikon C1si Spectral confocal microscope, as described [67]. Expression of markers and cell-localization analyses were done counting more than 50 EGFP+ cells per animal. Co-localization was assessed through the entire z-axis of each cell, using an optical slice of 0.3–0.6 µm. Morphology was analyzed from three-dimensional reconstructions of series of sequential confocal images taken at 0.3–0.6 µm intervals in EGFP+ cells.

Image analysis

For EGFP+ cell-localization analyses within the DG or CA1 sub-fields, maximum intensity z-axis projections of series of sequential confocal images were constructed using ImageJ, as described [67]. Using these projections, EGFP+ cells were automatically identified and counted using Cell Profiler (http://www.cellprofiler.org) [414]. This procedure was validated by comparison to manual counting performed by an experienced operator using the optical fractionator method sampling scheme and unbiased stereology estimation of cell numbers as described by West and co-workers [415]. The "pipeline" used to automate cell counting was composed of the following Cell Profiler's modules, in the specified order: LoadSingleImage, ColorToGray, CorrectIllumination_Calculate, CorrectIllumination_Apply, IdentifyPrimAutomatic. By using this pipeline we routinely found a strong correlation between the manual unbiased stereology method and the automated procedure (r=0.985, Pearson's correlation test performed with GraphPad Prism 4, GraphPad Software, Inc., La Jolla, CA). EGFP+ cells were individually pseudo-coloured to facilitated visualization and cell-localization maps were generated using Cell Profiler. Subsequently, based on

a previously described manual method to study granule cell location within the GCL [416] the GCL was subdivided in four 2-cell-body-wide sub-layers using ImageJ (http://rsb.info.nih.gov/ij/) to generate a superimposed grid, guided by Hoechst 33342 staining of cell nucleus. These sub-layers were denominated: sub-granular zone (SGZ) and granule cell layer (GCL) 1 to 3, as described by others [94;278;368]. Then, the pseudo-coloured cell-localization maps generated with Cell Profiler were used to manually assign and count individual EGFP+ cells to the 4 sub-layers of the GCL of the DG. In all cases, EGFP+ cells present in the apex of the DG were excluded from the analyses. A similar procedure was used in experiments comprising EGFP+ cells in CA1.

GR knockdown measurements

Confocal images were analyzed by an operator blind to the treatment, using *ImageJ* 1.40 software for *Windows* (an open source public domain developed by NIH, USA). Clear-shaped and non-overlapping $EGFP^+$ cells in the granule cell layer of the DG of either perfect match- or mismatch shRNA groups were selected by using *"free hand selection"* mode, guided by Hoechst staining. Sections surrounding injection sites were discarded. The intensity of EGFP+ and mouse-anti-GR-Alexa 594 immuno-labelling in these cells was measured by using Region of Interest (ROI) manager program. The degree of GR knockdown at protein level was estimated by normalizing expression levels in EGFP+ cells within the GCL and sub-granular zone (SGZ) to expression levels in equal numbers of GR+ CA1 neurons within the same hippocampus slice, which were not targeted by the lentivirus delivered to the hilar region.

Quantification of different cell-type markers

EGFP+ cells were automatically identified and counted using Cell Profiler from z-projected confocal images. From the same images, cells positive for each individual co-stained marker were also automatically identified and counted with Cell Profiler using the corresponding confocal channel. Cells positive for each marker analyzed were expressed as percentage of total EGFP+ cells per layer. All image analyses procedures were performed in hippocampal sections from at least three independently injected animals as described above. In all cases, image analyses were performed by an operator blind to the treatment.

Quantification of GFP+ cell distribution among different DG sub-layers

For each DG sub-layer, the number of GFP+ cells were counted –blind to treatment- using Image J *Cell counter* and *ROI Manager* program, respectively. Hoechst/GFP+ cells were counted separately for each DG sub-layer according to the following steps. At first, the DG in the photo was rotated to the horizontal or vertical position. After the rotation, the DG was divided by grids. Grids' size was decided by blades' width (only including dense area), which was fitted by 6 grids (2 grids for one layer: the GCL 1, 2&3). The blades' width is not the same everywhere. Thus an average size of grids was chosen for each picture. The neurons outside the GCL1 but next to it (less than 2 grids distance) were counted as SGZ neurons. Then GFP+ cells were counted over all DG sub-layers.

Later same procedure was followed for quantification of Hoechst/GFP/DCX+ and Hoechst/GFP/NeuN+ neurons.

Dendrite tracing and three-dimensional reconstructions.

Three-dimensional reconstructions of dendritic arbors was performed as described previously [451], using TDR3D software package (http://bioimaging.liacs.nl/tdr3dbase.html), and using a simulated fluorescence process-based algorithm [418]. Series of confocal images of EGFP+ neurons were taken at 0.3–0.6 µm intervals from at least three independently injected animals. All cells used for morphological analyses were positive for the neuronal marker NeuN.

Sholl analysis

Three-dimensional reconstruction of the entire dendritic processes of EGFP+ neurons was made from Z-series stacks of confocal images as described above. All EGFP+ dentate granule cells with largely intact dendritic trees were analyzed for total dendritic length and branch number. Measurements did not include corrections for inclinations of dendritic process and therefore should be considered to represent projected lengths [368]. Sholl analysis for dendritic complexity was carried out using the Sholl Analysis Plugin for ImageJ (http://www-biology.ucsd.edu/labs/ghosh/software/ShollAnalysis_class). Data shown were from a 22 individual EGFP+ dentate granule cells from at least 4 animals per experimental group.

Dendritic spine three-dimensional reconstructions and shape classifications.

Three-dimensional reconstruction of dendritic segments was done from Z-stacks of confocal images series of 100-200 confocal planes taken at 0.1 µm intervals using a 63x oil immersion objective. The confocal stacks were then deconvolved with Huygens Deconvolution Software (Scientific Volume Imaging b.v, Hilversum, the Netherlands). Spine density reconstructions and analysis were performed by automated three dimensional detection and shape classification based on a Rayburst sampling algorithm [453] using NeuronStudio software (http://www.mssm.edu/cnic/tools-ns.html).

Confocal analysis of mossy fiber boutons.

Mossy fiber boutons in the CA3 area were analyzed as described by others [102]. Z-stacks of confocal images series of 100-200 confocal planes were acquired at 0.75 µm intervals with 40X oil lens (numerical aperture, 1.3; Nikon) and a digital zoom of 6. Confocal stacks were then deconvolved Huygens Deconvolution Software.

Statistical analysis

All comparisons of GR knockdown animals with control animals were statistically tested using unpaired Student's t-test. When more than two groups were compared we used a one-way ANOVA test with Tukey's post test if $P<0.05$, as described by van Hooijdonk et al [451]. All values

shown in the results section are the average ± SEM of the data. In all cases, $P < 0.05$ indicated significance.

RESULTS

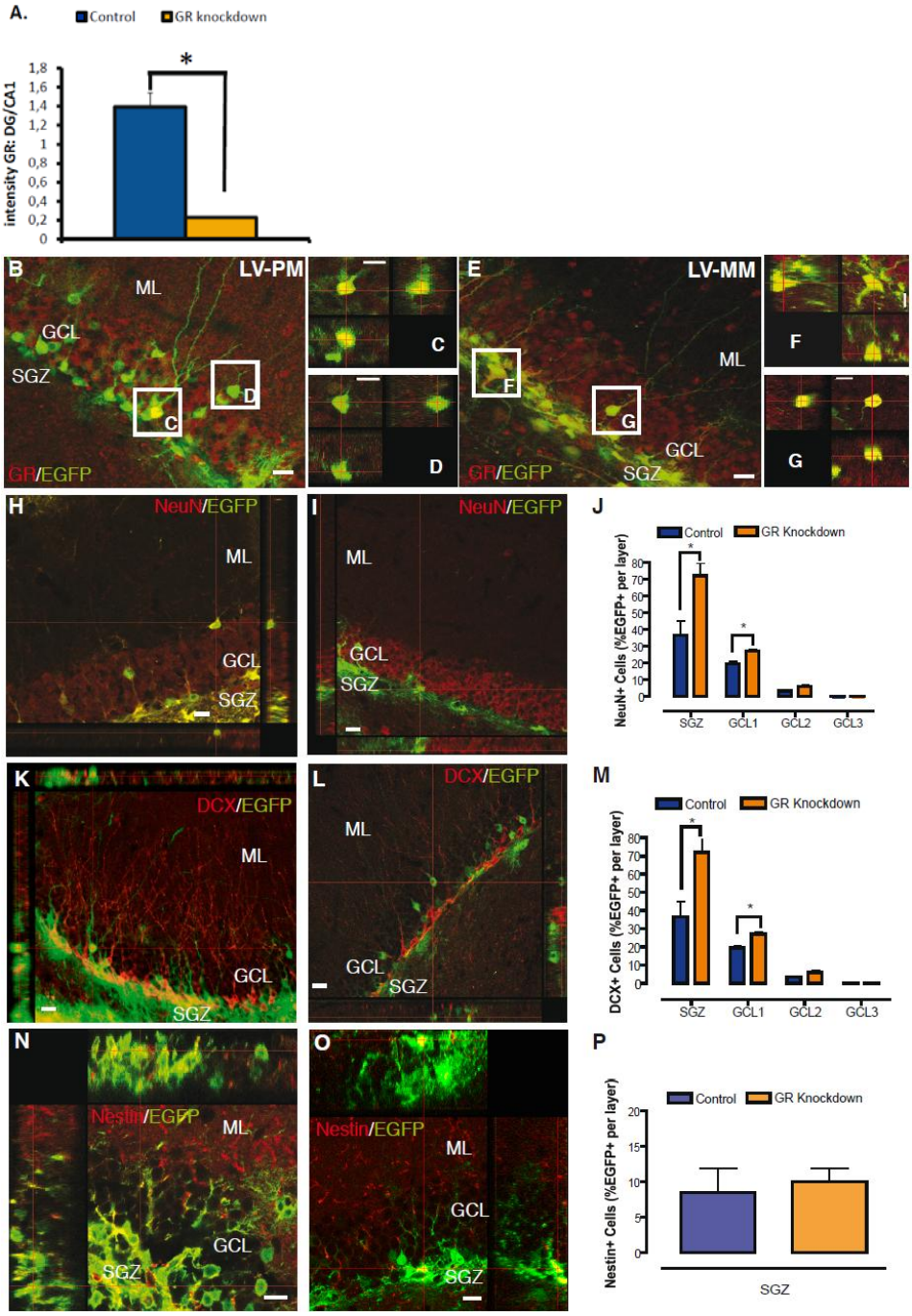
GR knockdown *in vivo*

To knockdown the GR in NPC and their neurogenic progeny *in vivo*, lentivirus suspensions were delivered to the DG of the adult mouse hippocampus by stereotactic injections to the hilar region. Immunohistochemistry to detect the GR confirmed effective knockdown in LV-pm-shGR (further referred to as GR knockdown) EGFP+ cells, while LV-mm-shGR (further referred to as control) was ineffective on GR expression (Figure 4.1 B-G). After semi-quantification, we observed a significant GR knockdown of 85% in LV-pm-shGR EGFP+ cells, as compared to LV-mm-shGR EGFP+ analyzed in the same way (Figure 4.1 A). This partial knockdown, is comparable to that observed *in vitro* (>70%, see CHAPTER 2), and is consistent with previous *in vivo* observations [360].

GR knockdown leads to accelerated neuronal differentiation of NPCs

As different levels of GCs affect the fate of NPCs during embryogenesis [454] and in the adult brain [123], we first investigated the effect of GR knockdown on neuronal differentiation one week after stereotactic delivery of our lentiviral constructs. We found a significantly increased proportion of EGFP+ cells positive for the mature neuron marker NeuN within the internal layers of the GCL, compared to LV-mm-shGR injected controls (Figure 4.1 H-J). This was accompanied by a concomitant increase in the proportion of EGFP+ cells positive for the immature neuron marker DCX in the same layers (Figure 4.1 K-M). Interestingly, the proportion of EGFP+ cells positive for the neuronal progenitor marker Nestin -exclusively located in the SGZ in both experimental groups- was unaffected by GR knockdown (Figure 4.1 N-P). These results strongly suggest that GR knockdown accelerates neuronal differentiation in newborn cells, without affecting survival of neuronal progenitors in the DG, as judged by the numbers of Nestin+ cells.

→ ***Figure 4.1 Effective GR knockdown in vivo regulates neuronal differentiation.*** *(A to H) Validation of GR knockdown by lentiviral constructs in vivo. Semi-quantification of GR knockdown in EGFP positive cells of the dentate gyrus* ***(A)****. GR expression levels detected by immunohistochemistry in EGFP+ cells within the GCL and SGZ were normalized to expression levels in equal numbers of individual GR+ cells in the CA1 area within the same hippocampal section, negative for EGFP (125 cells per experimental group, * p< 0.0001, unpaired Student's t-test). The effective (LV-PM,* ***B****) or ineffective (LV-MM,* ***E****) lentiviral constructs were delivered by stereotactic injection into the hilus and GR knockdown was analyzed in cells expressing the enhanced green fluorescent protein (EGFP, green) by GR immunohistochemistry (red) 1 week PI (post-injection). Orthogonal projections corresponding to boxed areas in B show a sample cell positive for GR expression* ***(C)*** *and a sample cell negative for GR expression* ***(D)****. Cells with highest GR depletion were always found in the proximity or within the Molecular Layer (ML). Orthogonal projections corresponding to boxed areas in E show two sample cells positive for GR expression (****F, G****) irrespective of their relative positioning within the granule cell layer (GCL) or the sub-granular zone (SGZ). Sample orthogonal projections from confocal z-stack images corresponding to animals injected (1 week PI) with LV-PM* ***(H, K, N)*** *or LV-MM* ***(I, L, O)****, showing EGFP and* ***(H, I)*** *NeuN,* ***(K, L)*** *DCX and* ***(N, O)*** *Nestin co-immunostainings. Distribution plots of EGFP and* ***(J)*** *NeuN,* ***(M)*** *DCX and* ***(P)*** *Nestin double-positive cells within four subdivisions of the GCL (SGZ, GCL1-3; Experimental Procedures). Values represent mean ± SD (n = 5 animals per group); * p< 0.05, unpaired Student's t test. Nestin positive cells were only observed in the SGZ. Scale bars: 10 μm.*


A.
Control
GR knockdown
intensity GR: DG/CA1
B
LV-PM
ML
GCL
SGZ
C
D
GR/EGFP
E
LV-MM
F
G
H
NeuN/EGFP
I
J
Control
GR Knockdown
NeuN+ Cells (%EGFP+ per layer)
SGZ
GCL1
GCL2
GCL3
K
DCX/EGFP
L
M
DCX+ Cells (%EGFP+ per layer)
N
Nestin/EGFP
O
P
Nestin+ Cells (%EGFP+ per layer)

GR knockdown in NPCs leads to an altered dendritic tree and dendritic spine profile

During neuronal differentiation, new granule cells migrate from the sub-granular zone outwards, to the edge of the cell layer [368;455]. This is accompanied by changes in the dendritic arborization of neurons in the DG, with cells in the outer layer showing more dendrites branching from the soma and more complex dendritic arborization than cells in the inner layer [433]. Consistent with these observations we found that GR knockdown induced differential morphological features in EGFP+/ NeuN+ neurons (Figure 4.2 A-B). To characterize these differences, we reconstructed dendritic arborization of individual EGFP+ neurons in the DG. Compared to control animals, EGFP+ neurons in GR knockdown animals consistently showed more complex dendritic arbors (Figure 4.2 C-D).

Consistent with previous observations [433], Sholl analyses demonstrated the existence of two different populations of EGFP+ neurons. We named these populations A and B cells. 'A' cells have simpler dendritic arbors than B cells (Figure 4.2 E-F). Moreover, near the cell body, A cells present a unique primary dendrite with a smooth, a-spiny proximal dendritic domain and a spiny distal dendritic domain, while in B cells both the proximal and distal dendritic domains present abundant spines (Figure 4.2 C-D). Interestingly, the number of B cells was significantly increased in GR knockdown animals, with all B cells located in the most external third of the GCL (Figure 4.2 G). These observations are consistent with a premature progression into neuronal differentiation of newborn neurons in the adult hippocampus after GR knockdown.

The morphological alterations after GR knockdown are likely regulated by GR-responsive genes [219]. In previous work, we have identified a number of genes like BDNF, LIM kinase 1(LIMK-1) and Calcineurin A, that also regulate spine dynamics as GR -responsive [22;456-458]. Therefore, we reasoned that GR could be involved in regulating dendritic spine morphology in newborn neurons of the adult hippocampus. Multiple studies have demonstrated that newborn neurons in the adult hippocampus integrate into pre-existing circuits, receiving fully functional excitatory inputs and forming morphologically mature synapses, which is reflected in the shape of the dendritic spines [95;427;459]. Since mature granule neurons receive the majority of their synaptic input through dendritic spines, their numbers and morphology is indicative of their connectivity within hippocampal circuits [95].

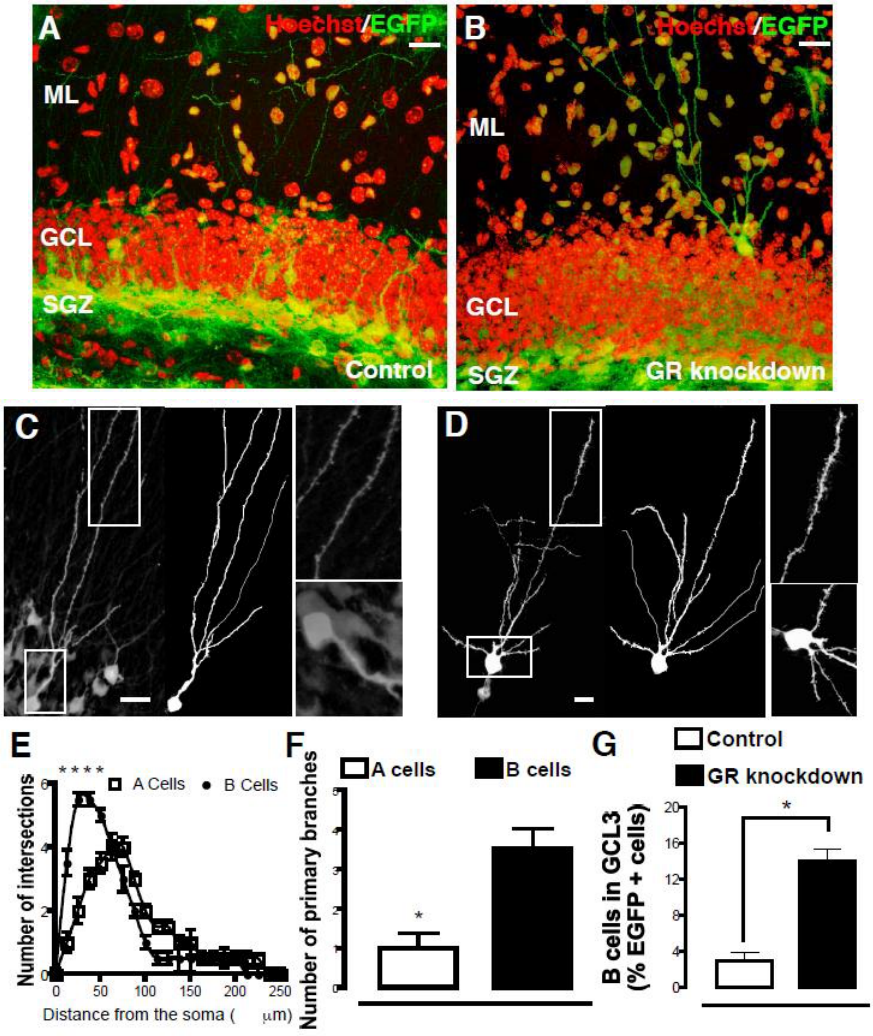


Figure 4.2 GR regulates dendritic morphology of newborn neurons. *Sample confocal images showing denditic morphology of EGFP+ cells and their location within the GCL from animals injected with LV-MM* ***(A)*** *or LV-PM* ***(B).*** *3D confocal reconstruction of somas and dendrites of EGFP+ newborn neurons, obtained 1 week PI (****C****, Control;* ***D****, GR knockdown). Left panels show original images, center panels corresponding 2D projected dendritic trajectories and right panels show details of the distal (top) or proximal (bottom) to soma dendritic domains corresponding to boxed areas in the original image. Panels showing proximal domains were rotated 90 degrees clockwise. Only EGFP+ neurons with obvious dendritic spines were considered for analysis. Scale bar: 10 μm.* ***(E)*** *Sholl analysis of dendritic complexity of EGFP+ neurons 1 week PI. Values represent mean ± SD (n=5 animals per group), * p< 0.05, one-way ANOVA.* ***(F)*** *Dendritic properties of EGFP+ newborn neurons 1 week PI. Values represent mean ± SD (same cells as in E, n=5 animals per group), * p< 0.05, unpaired Student's t-test.* ***(G)*** *Presence of "B" cells (results section) in GCL3 (Experimental Procedures) as percentage of total EGFP+ cells in GCL in control vs. GR knockdown animals. * p< 0.05, unpaired Student's t-test.*

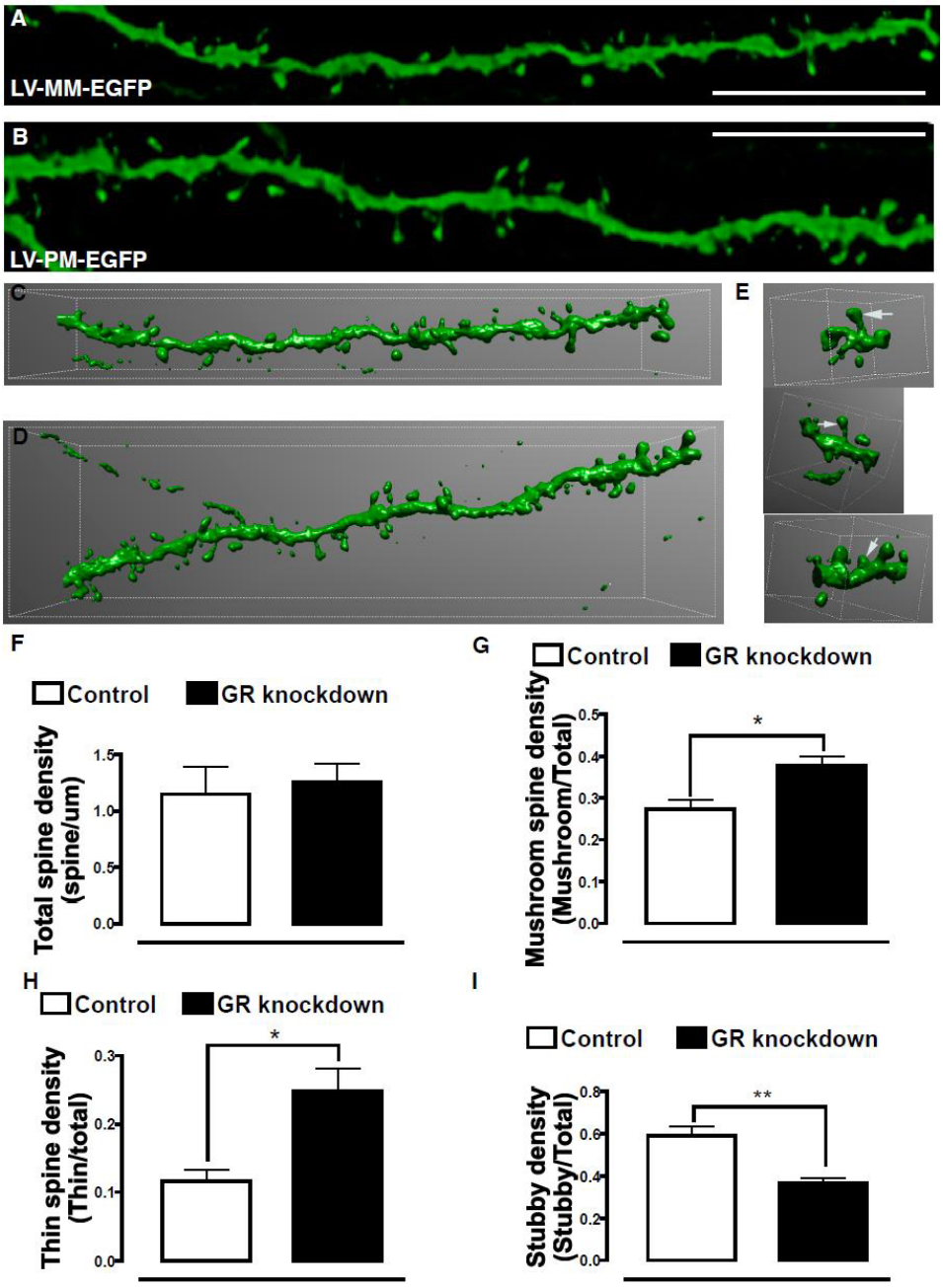


Figure 4.3 GR regulates dendritic spine maturation in newborn neurons. *Sample deconvolved confocal images of spiny dendrites of newborn neurons in* ***(A)*** *Control or* ***(B)*** *GR knockdown animals and their corresponding 3D reconstructions (**C**, Control; **D**, GR knockdown). Scale bar: 10 μm.* ***(E)*** *examples of 3D reconstructions of individual mushroom (top), thin (middle) or stubby (bottom) dendritic spines, indicated by arrows. Spine density measurements corresponding to* ***(F)*** *total,* ***(G)*** *mushroom,* ***(H)*** *thin and* ***(I)*** *stubby shaped spines in Control vs. GR knockdown animals. Values represent mean ± SD (n = 5 animals per group), * p< 0.05, unpaired Student's t-test.*

Recently, we have shown that one week after stereotactic delivery of our lentiviral vector, only a small percentage of EGFP+ cells present in the GCL are positive for the mature neuron marker NeuN and have simple dendritic arbors with dendritic spines, whereas the vast majority of EGFP+ cells are positive for the immature neuron marker DCX, with phenotypes ranging from putative dividing neuronal progenitors to early post-mitotic immature neurons [451]. Therefore, we analyzed the density and shape of dendritic spines from EGFP+/NeuN+ neurons in control and GR knockdown animals with visible spines in the proximal molecular layer (Figure 4.3 A-E). We found no significant difference in total spine density between EGFP+/NeuN+ cells in control and GR-knockdown animals (Figure 4.3 F). The average spine densities that we found are consistent with spine densities previously observed in young newborn hippocampal neurons [95].

Dendritic spines can be categorized into at least three different types based on their morphology: mushroom, thin and stubby [460;461]. From these, stubby (headless) spines are associated with developing neurons while thin and mushroom spines, with increased head size, are more abundant in mature neurons [460;462]. Strikingly, we found a significant increase in the percentage of mushroom and thin spines in EGFP+/NeuN+ cells from GR-knockdown compared to control animals (Figure 4.3 G-H). This increase in mushroom and thin spines was compensated by a significant decrease in stubby spines in GR-knockdown neurons (Figure 4.3 I). Overall, stubby spines were the predominant group of spines (59.2±1.5%) in control animals, while in GR-knockdown animals mushroom spines slightly predominated (37.8±0.7%). Interestingly, although thin spines were the minority group, they constituted the group with the largest fold-change in GR-knockdown neurons (11.7±0.5% and 24.8±1.1 %, 2.11 fold, control vs. GR-knockdown, respectively). These results are again consistent with an accelerated maturation of newborn neurons after GR knockdown in NPCs. Moreover, as the size of spine head may correlate with the efficacy of the corresponding synapse [95], our results strongly suggest a role for GR protein levels in controlling synaptic efficacy in newborn granule neurons of the adult hippocampus.

GR knockdown results in altered mossy fiber boutons

The connectivity of neurons is determined by both afferent input through dendrites and efferent output through axons. As early as 10 days after new granule cells are born in the adult hippocampus they project their axon into the hilus and CA3 areas [95;105;439]. The maturation of dendritic spines of newborn cells has been suggested to be matched with similar development of axonal output [102]. Our observation that GR knockdown animals exhibit increased dendritic spine maturation suggests therefore that axonal synaptic output of newborn cells is also increased. These synaptic contacts, often visible as large mossy fiber synaptic boutons, constitute functional synapses of newborn granule cells with hilar interneurons and CA3 pyramidal neurons. The cross-sectional area of mossy fiber boutons reflects the maturity of the corresponding synapse [102]. Earlier it was reported that a relative increase in GR activation in the hippocampus, e.g. in mineralocorticoid receptor knockout animals [463] or after chronic stress [464], correlates with decreased mossy fiber projections, suggesting a role for corticosteroid receptors in the regulation

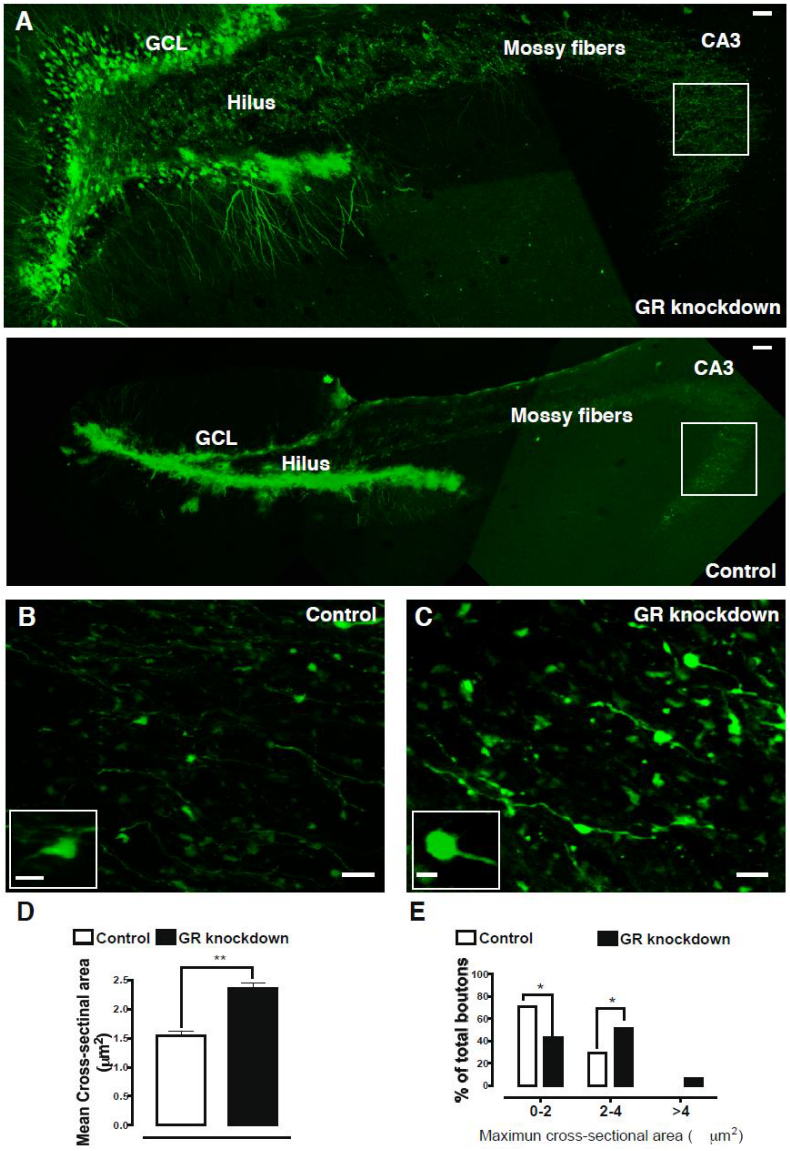


Figure 4.4 GR regulates mossy fiber boutons maturation. (A) *Sample confocal images showing an overview of the hippocampus with a subpopulation of lentivirus-transduced cells in green (EGFP) in GR knockdown (top) or Control (bottom) animals, 1 week PI. Images are compositions of 5-6 individual images obtained at 40X and processed using the automatic Photomerge function of Adobe Photoshop CS2 (Macintosh version). Examples of mossy fiber axons labelled with EGFP in the CA3 corresponding to* ***(B)*** *Control or* ***(C)*** *GR knockdown. Insets show high magnification examples of giant boutons in the CA3. Scale bars: 10 μm (A-C) and 2.5 μm (insets).* ***(D)*** *Cross-sectional area of EGFP+ boutons at the largest section, calculated from 3D reconstructions of original images exemplified in B and C in Control vs. GR knockdown animals.* ***(E)*** *Frequency distribution plot of the size of mossy fiber boutons in Control vs. GR knockdown animals. Giant mossy fiber boutons in CA3 were grouped according to their size, and the percentage of boutons in each size group was calculated. In all cases values represent mean ± SD (n = 5 animals per group); *p< 0.05, unpaired Student's t-test.*

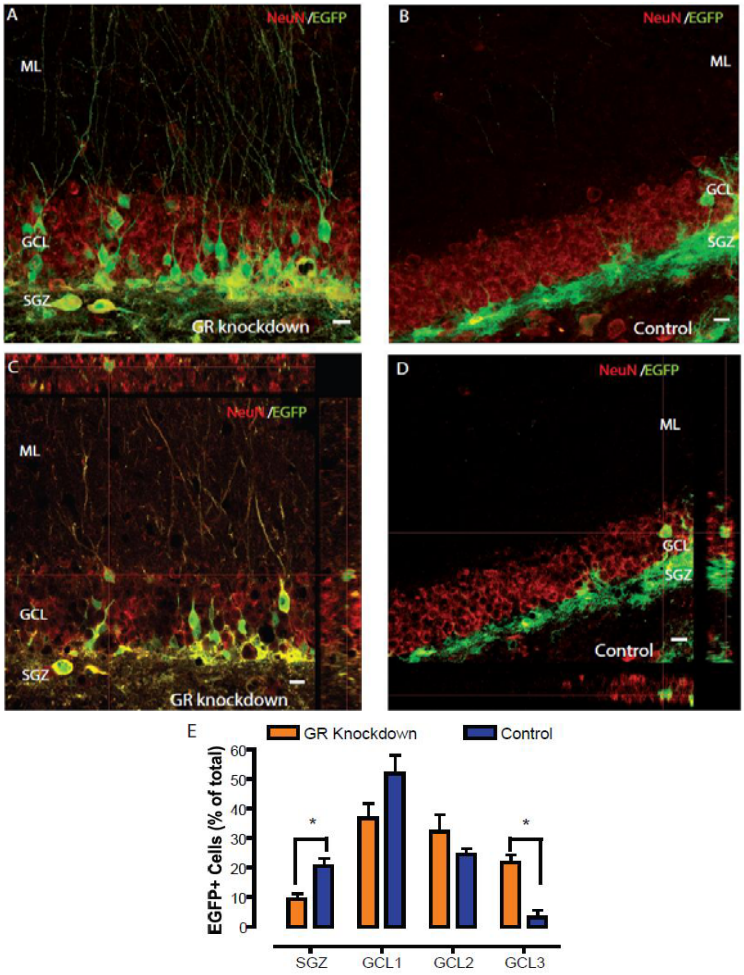


Figure 4.5 GR regulates positioning of newborn neurons within the GCL. *See for legend next page.*

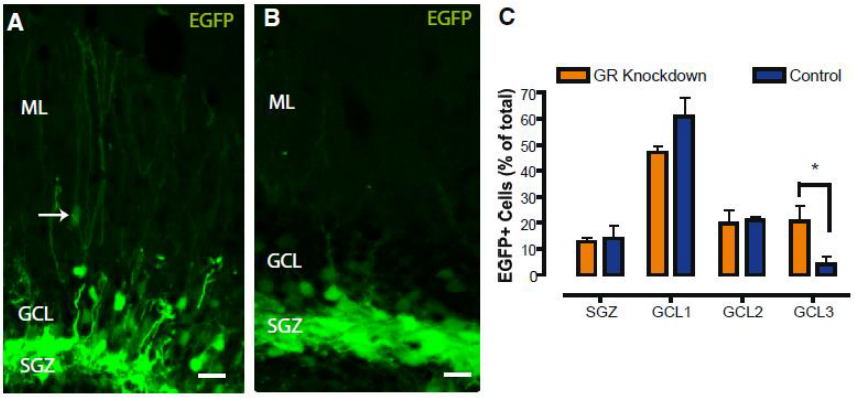


Figure 4.6 GR regulates resting membrane electrophysiological properties of newborn neurons (part 1).

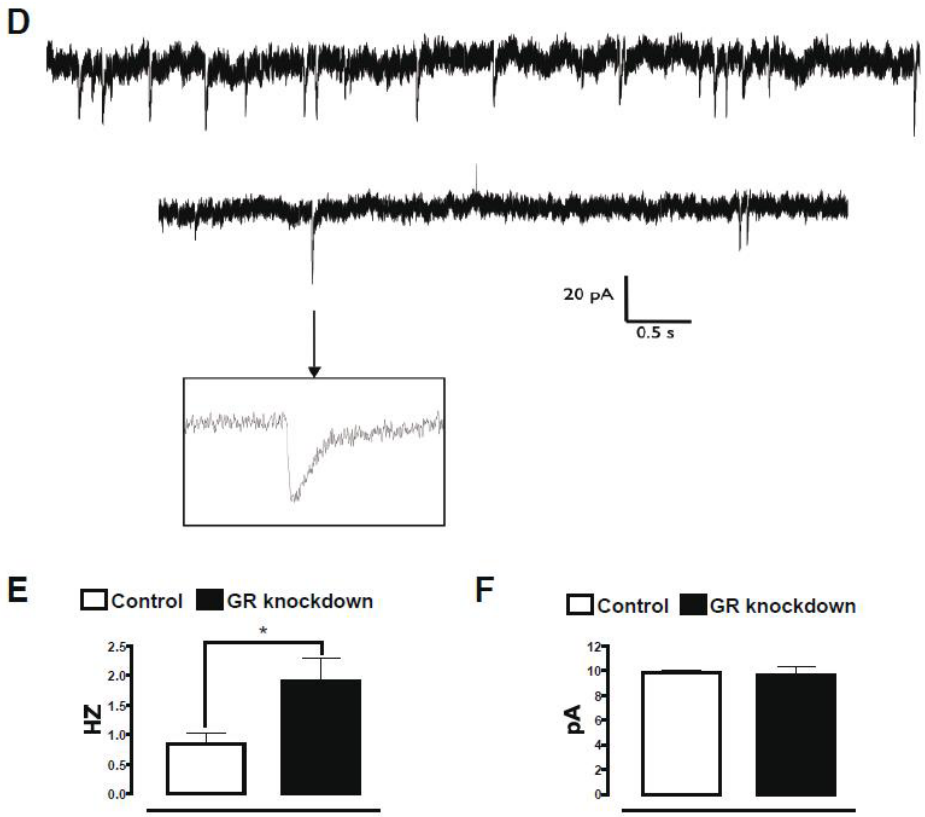


Figure 4.6 GR regulates resting membrane electrophysiological properties of newborn neurons (part 2). *Sample confocal images of EGFP+ (green) immunostaining from (**A**) GR knockdown or (**B**) Control animals 5 weeks PI, showing the persistent mis-location of EGFP+ cells after GR knockdown. The arrow in (**A**) shows a sample EGFP+ cell ectopically located in the ML. (**C**) Bar graphs showing the relative positioning of EGFP+ cells within four subdivisions of the GCL (SGZ, GCL1-3; methods section) normalized to total EGFP+ cell numbers 5 weeks PI in Control vs. GR knockdown animals. Values represent mean ± SD (n = 5 animals per group); * p< 0.05, unpaired Student's t-test. (**D**) Examples of paired mEPSC recordings in GR knockdown (top) or Control (bottom) animals treated with LV-PM (top) or LV-MM (bottom), 5 weeks PI. Inset shows a sample mEPSC. Scale bars represent 0.5s, 20 pA. mEPSC mean frequency (**E**) and amplitude (**F**) in animals in Control vs. GR knockdown animals. Values represent mean ± SD: n= 10 animals per group; * p< 0.05, unpaired Student's t-test.*

← ***Figure 4.5 GR regulates positioning of newborn neurons within the GCL.*** *Sample confocal images of EGFP (green) and NeuN (red) immunostainings in (**A**) GR knockdown or (**B**) Control animals, showing the differential positioning of EGFP+ neurons within the pre-existing granule neurons of the GCL, 1 week PI. Corresponding orthogonal projections (**C**, GR knockdown; **D**, Control) showing examples of EGFP/NeuN double-positive neurons. (**E**) Bar graphs showing the relative positioning of EGFP+ cells within four subdivisions of the GCL (SGZ, GCL1-3; Experimental Procedures) normalized to total EGFP+ cell numbers, 1 week PI. Values represent mean ± SD (n = 5 animals per group); * p< 0.05, unpaired Student's t-test.*

of mossy fiber connectivity. Therefore, we studied the layout of mossy fibers in GR-knockdown animals by analyzing axons and axon terminals in the CA3 area using confocal microscopy.

We found that the mossy fiber boutons in CA3 were substantially larger in one week GR-knockdown animals than those in the control group animals (Figure 4.4 A-C). The mossy fiber boutons in GR-knockdown animals had a significantly larger mean cross-sectional area than those in control animals (Figure 4.4 D, p=0.007; 2.4±0.1μm^2 vs. 1.5±0.1 μm^2 GR-knockdown, n=109, and Control, n=102, respectively. p=0.007). Moreover, a frequency distribution analysis of mossy fiber bouton size showed that in control animals the majority (70.7 %) of the boutons had a cross-sectional area smaller than 2 μm^2, while in GR-knockdown animals the majority (57.2 %) of the boutons had a cross-sectional area larger than 2 μm^2. In contrast, 6.3% of the boutons in GR knockdown animals had a cross-sectional area larger than 4 μm^2, which was not observed in control animals (Figure 4.4 E). These results suggest that, together with those presented in previous sections, GR-knockdown in adult-born granule cells induces a differential connectivity pattern to hippocampal circuits that may result in increased excitability.

GR knockdown leads to altered positioning of adult newborn cells

In our analysis of dendritic arborisation and spine morphology of newborn cells, we noted a marked dispersion of EGFP+ cells within the pre-existing granule cells of the granule cell layer (GCL) 1 week after treatment with LV-pm-shGR, which was nearly absent with LV-mm-shGR. Moreover, EGFP+ cells that retained high GR expression were located in the internal layers of the GCL, while EGFP+ cells with more profound GR knockdown were consistently located in more external layers of the GCL (Figure 4.5 A-D). These results suggest a role for the GR in the accurate positioning of newborn cells within the pre-existing GCL. In line with this are recent findings that excess glucocorticoids retard neuronal migration of NPCs during the cortical development [465]. Therefore, we proceeded by comparing in detail the relative contribution of EGFP+ cells to the pre-existing GCL in groups of animals treated with LV-pm-shGR (GR knockdown) or LV-mm-shGR (control) and their progression into neuronal differentiation (Figure 4.5 E). We found that the proportion of EGFP+ cells located to specific GCL subdivisions, termed SGZ and GCL1-3 from the hilus to the Molecular Layer (Methods section), was significantly different in GR knockdown compared to control animals. The majority of the cells in control animals were located in the inner layers, which is in line with normal migration patterns of adult-born new neurons [76;94]. Strikingly, in GR knockdown animals, the majority of the cells had progressed towards the outer layers, with a significant higher number of EGFP+ cells in GCL3 for GR knockdown animals (Figure 4.5 E). This seemed to be accompanied by a significant reduction of the number of EGFP+ cells in the SGZ. Thus, GR knockdown leads to an altered positioning of adult newborn neurons in the different layers of the DG.

GR knockdown leads to sustained mis-positioning of NPCs and affects basal membrane excitability

To check if 5 weeks of GR knockdown also resulted in mis-positioning of newborn cells we have analysed the location of EGFP+ cells in the different DG layers (Figure 4.6 A-B). For the position of EGFP+ cells in the different layers, time was not significant for both treatment groups. In fact, significantly ($p< 0,05$) higher numbers of EGFP+ cells were found in GLC3 in GR knockdown animals compared to control animals, indicating long-lasting effects of GR knockdown on the formation of hippocampal neo-networks (Figure 4.6 C).

To further test the contribution of newborn cells of GR knockdown animals in hippocampal circuitry we analysed the potential synaptic strength by recording spontaneous miniature excitatory postsynaptic currents (mEPSCs) as a measure of the spontaneous excitatory transmission (Figure 4.6 D), with whole cell voltage clamp (holding potential V_H=-70 mV). EGFP+ cells within the granule cell layer were identified as neurons by the shape and location of their soma and dendritic tree. Consistent with the increased proportion of mature spines, EGFP+ cells in GR-knockdown animals presented a significant ($p=0.03$) increase in the frequency of mEPSCs, as compared to EGFP+ cells in control animals (Figure 4.6 E). The mean frequency of mEPSCs in control EGFP+ cells was highly comparable to that of neighbouring non-EGFP cells. No differences in mEPSC amplitude were detected between GR-knockdown and control EGFP+ cells (Figure 4.6 F) or neighbouring EGFP- cells. Collectively, these data corroborate that GR-knockdown in newborn hippocampal neurons induces drastic increases in basal membrane excitability, thus suggesting their participation in hippocampal circuitry.

DISCUSSION

Here we show that GR protein levels in NPCs are a key determinant for functional integration of adult born granule cells in hippocampal neo-networks. Firstly, GR knockdown leads to an accelerated neuronal differentiation of newborn cells. Secondly, downregulation of GR leads to a significant change in the relative positioning of newborn cells in the external layers of the GCL, or even in the molecular layer. Thirdly, lowering GR protein levels results in a clear shift in spine morphology, with more mature thin and mushroom spines and less immature stubby spines. Fourthly, $EGFP^+$ neurons exhibited increased neuronal activity after GR knockdown, a finding with possible consequences for hippocampal circuitry.

Several studies showed that aberrant GC signalling impairs neurogenesis in the sub-granular zone of the adult dentate gyrus [109;123]. However, the role of the main mediator of glucocorticoids action in NPCs, i.e. the glucocorticoid receptor, remains elusive. This role may be direct or indirect since GRs are expressed in virtually every cell type in the DG, which confounds the interpretation of studies aiming to unravel the contribution of the GR in NPCs using classical transgenesis models. Therefore, we here used lentiviral vectors to specifically knockdown GR in the neurogenic niche of the dentate gyrus [451]. Our data clearly indicate that aberrant GC signalling may target directly the fate of NPCs and that GR, expressed in NPCs, has a key role in proper integration of newborn neurons into existing hippocampal neuronal circuits.

The process of adult hippocampal neurogenesis comprises of several stages: proliferation, survival, migration, neuronal differentiation and functional integration into the hippocampal trisynaptic circuitry. Thus far, stress, and aberrant GC signalling have been mainly associated with antineurogenic effects at the initial stages (reviewed in CHAPTER 1.3.2); cell proliferation [82;137;158;466] and cell survival [92;122]. These stages are mainly associated with quantitative aspects of neurogenesis.

In this study, we have found several lines of evidence that GR is involved in later stages of neurogenesis. GR knockdown in NPCs resulted in accelerated differentiation. In addition, our study revealed morphological alterations in dendritic arborisation, dendritic spines and boutons. Perhaps most importantly, our study showed that GR in NPCs controls correct positioning of NPCs as well a physiological evidence for an aberrant functional integration of NPCs into hippocampal networks. Our results indicate therefore GR knockdown in NPCs might have possible implications for the functioning of the hippocampus. In a study by Herbert and Wong (2006), a first indication for an involvement of GR in later stages of neurogenesis was observed. Systemic injections with high concentrations of GCs, thus activating GR, "discouraged" the acquisition of neuronal fate in a time-dependent fashion [123]. GCs given during the post-mitotic interval were shown to reduce the differentiation of newly formed cells into mature neurons as determined 1 and 4 weeks after treatment. Together, these data imply that GR's are not only involved in regulating the number of newborn neurons (referred to as "quantitative" aspects), but also in controlling several aspects of neuronal development, such as spine and bouton formation of newborn neurons (referred to as "qualitative" aspects).

How to explain these qualitative aspects of neurogenesis upon GR knockdown? One possible explanation is underlying the molecular function of GR as a transcription factor. This implies a more or less direct GR-mediated effect on differentiation, migration and integration. However, the signalling pathway downstream of the GR is not yet resolved. A first indication as to the nature of these signals comes from a study showing that the phenotype of newborn cells after knockdown of Disrupted-In-Schizophrenia 1 (DISC1; [368], a gene of which genetic variants have been implicated in the pathogenesis of schizophrenia [467], is strikingly reminiscent of what we observed after GR knockdown. Similarly as in our study, DISC1 knockdown in NPCs leads to enhanced excitability, accelerated neuronal maturation and synapse formation and to aberrant integration of newborn cells into hippocampal neo-networks. This similarity in phenotype after GR and DISC1 knockdown suggests that both proteins may be involved in the same signalling cascade directing newborn granule cells to their destination. One such potential converging pathway might be GSK3beta, a kinase involved in the control of cell proliferation and direct target of the anti-depressant lithium, as both both DISC1 and GR have been shown to control GSK3beta activity in neuronal progenitor cells [56;468].

A second indication can be inferred from the observation that GR knockdown leads to a significant increase in the frequencies of spontaneous mEPSCs as well as in the numbers of mature-type mushroom and thin spines, indicative of increased glutamatergic neurotransmission [469]. This may be the result of overall acceleration of neuronal differentiation by increased neuronal activity

caused by GR knockdown. Neuronal activity is a major determinant for the maturation rate of newborn cells. Alternatively or in combination with other factors, the GR may control more directly synapse formation and glutamatergic neurotransmission. Indeed, we have reported that the expression of several important components affecting spine formation, such as BDNF [456], LimK-1 and calcineurin A [22], is controlled by GRs at the genomic level. More recently, synaptic GR-dependent glutamate receptor clustering has been reported by us and others [470;471]. In addition, GRs are known to alter glutamate signalling [89;132-136]. Also, a number of excitatory stimuli are known to be influenced by GR-mediated transcriptional regulation. Excitatory stimuli of NPCs are thought to release BDNF and also activate glutamatergic signalling via NMDA receptors, calcium entry, and activation of transcription factors like CREB and AP-1 [64;472-476]. Activated GR is well-known to dampen these excitatory stimuli, for example by downregulation of BDNF [456;477] or by repression of AP-1 and cAMP signalling [478-480]. However, the signalling pathways downstream of GR are a matter requesting further investigations.

Either way, our data indicate a crucial role for appropriate GR expression levels in NPCs for progression of neuronal differentiation and functional integration into existing networks. The question therefore arises to what extent endogenous factors affect GR expression. Recently we showed that GR protein levels are down-regulated by microRNA-124 [59], a non-coding RNA that is endogenously highly expressed specifically in neuronal cells. Interestingly, recent *in vivo* experiments identified microRNA-124 as a master switch that turns on a neuronal differentiation program in neuronal progenitor cells [61] suggesting that reduced GR protein levels are necessary to keep neuronal differentiation within physiological range, a notion that is in line with our data. Equally interesting, several risk factors for the pathogenesis of psychiatric disorders have been shown to result in reduced GR protein levels. To illustrate, decreased maternal care in early life in rats, a rodent model for depression, reduces GR protein levels in the hippocampus [273]; chronic stress, a major risk factor for several psychiatric disorders, is associated with reduced GR protein and mRNA levels in the hippocampus [51;302;481] and aging impairs negative feedback action of glucocorticoids on the HPA-axis that is associated by reduced hippocampal GR protein levels [482]. Extrapolating, our data indicate that reduced GR protein levels under these circumstances can impair hippocampal function by re-organizing hippocampal neo-networks.

In conclusion, we have demonstrated a crucial role for GR expression levels in migration, differentiation and integration of newborn granule cells into hippocampal networks. As the GR is a target for stress-induced elevation in glucocorticoids and for a broad range of pharmacological steroid-based agents, our data suggest that these factors may affect correct integration of newborn cells with possible consequences for neuroplasticity and hippocampal function.

5

GR KNOCKDOWN IN NEWBORN DENTATE GRANULE NEURONS RESULTS IN IMPAIRED FEAR MEMORY

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*Both authors contributed equally

This paper, in combination with the paper of CHAPTER 4 was submitted to Neuron

ABSTRACT

In a previous study, we found evidence for a regulating role of glucocorticoid receptors (GRs) in the development of newborn dentate granule neurons. To what extent GRs in these cells contribute to hippocampus-dependent memory processes, has yet to be investigated.

In this study, we examined the role of GRs in a population of neuronal progenitor cells (NPCs) and immature dentate granule neurons for the formation of contextual fear memory. Lentiviral vectors expressing perfect match-shRNA for the GR were applied by microinjections into the dentate gyrus of male BALB/c mice to specifically knockdown glucocorticoid receptor proteins in the newborn granule neurons. Mismatch-shRNA injections served as control. Four weeks later, when immature dentate granule neurons were matured, mice were trained and tested in a Pavlovian fear conditioning task. This task was designed to allow measurement of fear memory (expressed as freezing) for both context and cue within the same procedure.

Our data demonstrate dependency of GR signalling in newborn dentate granule cells for facilitation of consolidation of fear memories. Knockdown of GR destabilized memory consolidation to the conditioned context, resulting in a less strong expression of fear behaviour; i.e less “passive” freezing and more “active” scanning coping style. In line with our previous study (see CHAPTER 4), these data suggest a key role for GR in the formation of hippocampal neo-networks that coordinate hippocampal memory formation.

INTRODUCTION

The dentate gyrus (DG) of the hippocampus is implicated in memory processes involving discrimination between similar contexts [483] and the encoding of spatial information [73;184-186;484]. The granule cell layer of the dentate gyrus is a heterogenous structure formed by granule neurons of different ages, morphologies and electrophysiological properties [76;402]. The sub-granular zone of the DG also contains neuronal progenitor cells (NPCs). During a process called adult neurogenesis, NPCs proliferate and a subpopulation of these differentiate, migrate to the granular cell layer and mature into functionally active granule neurons that are incorporated in the hippocampal trisynaptic circuit. This process takes about four weeks. Previous pharmacological and physical [280;366;448] and recent genetic manipulations [98;406;449] have provided convincing evidence that these adult born granule neurons also play a role in hippocampus-dependent memory formation. At least four weeks after modulation or elimination of NPCs for example, hippocampal function was affected as shown by weakening of contextual fear conditioning [296;366;449;485-487].

Both hippocampus-dependent cognitive functions as well as neurogenesis are regulated by glucocorticoids (GCs) [5]. These stress hormones affect memory consolidation [149;209], and neurogenesis [122;123]. GCs exert their action via high affinity mineralocorticoid and low affinity glucocorticoid receptors (MRs and GRs respectively). MR and GR are known to be abundantly expressed in the granule neurons of the DG [54]. In NPCs, GRs but not MRs are expressed [56;57]. However, the GR role in NPCs is largely unknown which might be due to technical limitations to specifically manipulate its expression in this cell population.

We hypothesize that GCs via GR may affect hippocampal-dependent cognitive performance by regulating the development of NPCs. To examine this in detail, in a previous study we have applied lentiviral vectors using stereotactic injections targeted at the sub-granular zone of the DG. We showed successful transduction of a specific population of DCX+ NPCs and immature dentate granule neurons (further referred to as NPCs) [451] (see CHAPTER 3). We used this technique to knockdown GR protein expression by RNA-interference technology (GR knockdown; Van Hooijdonk et al., unpublished data (CHAPTER 2) and indeed found evidence for an orchestrating role of GR in the formation of hippocampal neo-networks (Van Hooijdonk and Fitzsimons et al., *submitted* (CHAPTER 4)). GR knockdown was shown to have major impact on NPC differentiation, positioning, morphology and physiology. In fact, specific GR knockdown in NPCs accelerated neuronal differentiation and migration. Strikingly, GR knockdown led to mis-positioning of adult newborn neurons, to altered dendritic arborization, to higher numbers of mature mushroom and thin spines and to larger mossy fiber boutons. In line with increased numbers of synaptic contacts, adult newborn neurons with GR knockdown exhibit increased mEPSC frequencies. This suggests GR knockdown in NPCs might affect neuronal signalling and thereby result in a destabilized cognitive performance. To what extent GRs in NPCs contribute to hippocampus-dependent cognitive processes is topic of further investigation. Here, we assessed the effects of GR knockdown in NPCs on context and cue fear conditioning. For our experiments, we have selected the Balb/c mouse strain. Balb/c mice are known for their increased stress susceptibility, high

emotional expression and superior cognitive performance in hippocampal-dependent tasks such as context and cue fear conditioning [308;484;488]. We found that GR knockdown resulted in a destabilized memory consolidation to the conditioned context. This resulted in a less strong expression of fear behaviour; i.e less "passive" freezing and more "active" scanning coping style. Our data demonstrate dependency of GR signalling in newborn dentate granule cells for facilitation of consolidation of fear memories. In line with our previous study (see CHAPTER 4), these data suggest a key role for GR in the formation of hippocampal neo-networks that coordinate hippocampal memory formation.

MATERIALS AND METHODS

Animals

We chose for BALB/c mice as this mouse strain expresses strong context- and cue-related fear memories in fear conditioning [484;489]. Male BALB/c mice (6 weeks, Janvier Bioservices, Genest st Isle, France) were individually housed for one week in filtertop cages before stereotactic surgery. The mice had free access to food and water and were kept under a 12 hour dark/light cycle (lights on at 8.00 hrs) in a temperature (20°C) and humidity controlled room. Experiments were performed between 8.00 and 13.00 hrs. All experiments were approved by the committee on Animal Health and Care from the Leiden University, The Netherlands and the Netherlands ministry of VROM and were performed in strict compliance with the European Union recommendations for the care and use of laboratory animals.

Small interference (siRNA) constructs

Perfect match (pm) short hairpin RNA (shRNA) expression vector and mismatch (mm) control directed against the consensus sequence of mouse, rat and human GR were designed. The sequence for shRNA against mouse GR (NM_008173) was GATCCCCGAAAGCATTGCAAACCTCATTCAAGAGATGAGGTTTGCAATGCTTTCTTTTGGAAA for the pm, and

GATCCCCGACAGCATTGCACACCTCATTCAAGAGATGAGGTGTGCAATGCTGTCTTTTGCAAA for the mm control. The sense and antisense oligonucleotides of 64 bp long were annealed and cloned in *BglII* and *HindIII* sites of p-super vector (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Insertion of the oligonucleotides was confirmed by sequencing. The knockdown of the GR by GR knockdown was tested by Western Blot and quantitative PCR in rat PC12 cells (Van Hooijdonk et al., unpublished (CHAPTER 2); Van Hooijdonk and Fitzsimons et al., *submitted* (CHAPTER 4)) and functionally tested in N1E-115 mouse neuroblastoma cells by using a Dual Luciferase (Promega Corp. Madison, WI)- based GC response element reporter gene assay as previously described [67].

Lentiviral vectors

P-super vector GR knockdown and corresponding control constructs were subcloned into a lentiviral vector downstream of the H1 promotor. The lentiviral vector also contained EGFP downstream of the CMV promoter. These replication incompetent and self-inactivating Advanced Generation lentiviral vectors were produced and titrated as previously described [451] (CHAPTER 3). Titers of both viruses were comparable and ranged between $1x10^8$ and $1x10^9$ transducing U/ml. Virus suspensions were stored at -80°C until use and were briefly centrifuged and kept on ice immediately before injection.

Stereotactic surgery

Stereotactic injections were performed in the morning, following previously described methods (CHAPTER 3) [367;451]. LV-pm-shGR knockdown (n=25 GR knockdown mice) and LV-mm-shGR (n=25 control mice) constructs were injected bilaterally into the hilar region of the DG (AP: -2.00 mm, ML: +/-1.50 mm, DV: -1.90 mm, relative to Bregma). After surgery, animals were placed under a heating lamp until awakening and checked upon daily. Four weeks after injection, mice were subjected in the context and cue fear conditioning procedure to assess their learning and memory capacities for a fearful event. This fear conditioning experiment was repeated with another cohort of injected mice (N=20 GR knockdown mice and N=20 control mice) with similar results. In addition, the brains of these mice were used for the morphological analysis of NPCs described in CHAPTER 4.

Fear conditioning apparatus

Fear conditioning was conducted in a conditioning chamber (25 cm x 25 cm) enclosed by 4 black Plexiglas walls (35 cm high), one embedded with a speaker (25 cm high) connected to a tone generator (70 dB). A 3 cm plastic rim covered the top of the walls. The floor consisted of 37 stainless steel bars 5 mm in diameter spaced 0.5 cm apart, connected to a shock generator (0.4 mA). Underneath the stainless steel bars, tissues were placed to collect faeces and urine. A white light source (260 Lux) was placed 20 cm above the conditioning chamber together with a camera for later behavioural analysis from video tape.

A radio on the other side of the experimental room produced 20 dB of background noise. The light intensity of the experimental room was 90 Lux. After each animal the chamber was cleaned with tap water and the tissues in the container replaced by clean ones.

Fear conditioning procedure

Our fear conditioning paradigm allows to differentiate between context and cue related behavioural responses in the same setting [484;490]. During conditioning (day 1), three minutes of baseline recording was followed by 6 light/tone (CS) + shock (US) pairings with a one minute interval. Pairings were as follows; light and tone were given simultaneously for 20 seconds; an electric foot shock (0.4 mA) was administrated during the last two seconds. Two minutes after the last pairing, the mice were returned to their home cage. At 48 (day 3) and 72 hrs (day 4) after the

initial conditioning, the same procedure was repeated without shocks to test memory and extinction of fear behaviours during alternating context and additional cue episodes. The procedure lasted 12 minutes per mouse on each testing day. At the end of the testing session of Day 4, animals were sacrificed, and upon a fixation procedure, sections of the hippocampus were assessed for EGFP expression in the dentate gyrus around the injection site. Animals with low (<100 EGFP+ cells per section), absent or mis-positioned EGFP expression were excluded from the experiment and further behavioural assessment.

Corticosterone radio immune assay

At 4 time points before, during and after the fear conditioning procedure blood was collected via tail incision or after decapitation. Under resting conditions, two days before the fear conditioning experiment, blood samples were taken by tail incision during the morning (7.00 hrs, circadian nadir) and evening (18.00 hrs, circadian peak), i.e. one hour after light on and before light off [491]. A third, peak stress blood sample was collected 30 minutes after the start of conditioning at Day 1. A fourth, habituated stress blood sample was collected 60 minutes after the last memory testing session on Day 4. Corticosterone concentrations were measured in blood plasma using a commercially available radio immune assay kit (ICN Biomedicals, Inc), as described before [308].

Behavioural assessment

Fear can elicit multiple behavioural responses, for example encompassing behavioural inhibition such as freezing in response to threatening contexts. We registered freezing as expression of fear behaviour during alternating context and cue episodes. Freezing is defined as complete immobility of the body and head, being devoid of interaction with the environment. For a more precise evaluation of fear behaviour we additionally analysed scanning. Scanning is defined as immobility of the body, while the head is moving horizontally from side to side. Although being immobile, the animal is still actively interacting with its environment. Scanning and freezing are interdependent behaviours that express a different quality of fear. With automatic scoring they are often measured together as “total immobility” [194]. To ease comparison of our work with others, we also calculated the total immobility score. All behaviours were scored from video tape using a semi automatic scoring program (The Observer 4.1, Noldus, Wageningen, The Netherlands). Videotaped behaviour was analysed by an experimenter blind to the treatment.

Statistical analysis

Endocrine and behavioural analysis of selected GR knockdown and control mice was performed after testing, based upon two criteria: 1) post-mortem histological evidence of an appropriately targeted micro-injection as visualized by EGFP expression in SGZ, and 2) indication of an appropriate, average acquisition of fear conditioning at Day 1. Data are shown as mean ± SEM and $p \leq 0.05$ was accepted as level of significance for all statistical testing. Endocrine analysis consisted of a General Linear Model (GLM)-Repeated Measures for comparing blood plasma corticosterone concentrations between the different treatment groups over the four time points. In addition,

Students T-test statistics were used to compare corticosterone concentrations of GR knockdown and control groups at each time point. Fear behaviour is expressed as percentage of e.g., freezing per Day for context and cue on episodes. A comparison in average freezing behaviour was determined first by a GLM-Repeated Measures analysis for all treatment groups: 1) uni- and bilateral injected mice, (2) sham and mm-shGR control groups, and 3) mm-shGR and GR knockdown injected mice. Difference in average freezing behaviour and progression over episodes/ Days between GR knockdown and control mice was subsequently determined by GLM-Repeated Measures statistics. Difference in context only and cue on induced freezing behaviour between GR knockdown and control mice were determined with a two-way ANOVA. Furthermore, a two-way ANOVA was performed to determine on which episodes GR knockdown and control mice differ in freezing behaviour. Significant treatment difference between GR knockdown and control mice in fear (freezing, scanning and total immobility) and other behaviour was determined with a GLM multivariate analysis followed by an ANOVA to determine which specific behaviours differed. A GLM-Repeated Measures was subsequently done to determine differences in total immobility or scanning behaviour and progression over Days between GR knockdown and control groups for context as well as cue on episodes. A Paired Samples T-test was done to compare freezing to context and cue for both GR knockdown and control groups for Day 3 and Day 4. A Students T-test for independent variables was used to compare the percentage of freezing or scanning for context and cue on episodes between groups per Day. Factor analysis was subsequently performed to determine a treatment specific behavioural structure. The factor analysis uses cross-mouse comparisons to distinguish the relation between behavioural parameters. It includes as much data as possible in each factor to minimize residual variance from the original dataset. The Principal Component Analysis (PCA) was performed with a Varimax rotation on variables with communalities over 0.65, that is, of which at least 65 % of the variance is explained by the Factors extracted. The number of extracted Factors was not pre-defined; as described before [489], Factors with an Eigenvalue over 1 were accepted. Factor scores were subjected to a one-way ANOVA to determine treatment differences.

RESULTS

Assessment of treatment groups for EGFP expression, HPA activity and freezing.

Mice with appropriate EGFP expression at the injection site and freezing behaviour during conditioning on day 1 were selected for the two experimental groups: GR knockdown and control. Twenty-one out of N= 25 GR knockdown mice showed EGFP expression in the SGZ of the DG in at least one brain hemisphere (N= 17 mice bilateral EGFP expression and N= 4 unilateral EGFP expression). Eleven out of N= 25 control mice were selected for further analysis (of which N= 7 bilateral EGFP expression and N= 4 unilateral EGFP expression). One mouse from the control group was not selected because of a complete lack of freezing behaviour.

We further assessed whether unilateral or bilateral EGFP expression differentially affected corticosterone secretion and freezing behaviour. We found no differences between the groups (corticosterone: GR knockdown F(89,91) 3.093, p=0.082); control F(38,40) 2.305, p= 0.137; freezing: for GR knockdown F(1,39) 0.88, p= 0.354); for control F(1,15) 0.66, p= 0.428). Therefore, we pooled the data of mice with unilateral or bilateral EGFP expression in the GR knockdown (n= 21) or control groups (n= 10). This is also in line with earlier reports on the connectivity between hippocampi, indicating there is a strong connectivity by contralateral commissural projections of mossy cells in the hilus, and lesion studies in mice and humans (Amaral et al., 1990, 2007; Hoz et al., 2005; Groticke et al., 2008; Batchelor et al., 2008) [70;72;492-494].

Some of the mice (n= 14) did not show EGFP labelling of the cells indicating that no lentivirus was injected, i.e, "sham-injected" control geroup. One mouse did not freeze during acquisition and was discarded from further analysis. Comparison of the sham and EGFP expressing control groups revealed comparable corticosterone secretion (F(83,85) 1.53, p= 0,220) and freezing over the three testing days (F(1, 65) 3.15, p= 0.081), excluding an effect of the lentiviral microinjection.

Because the mm-shRNA construct differs only two nucleotides from the pm-shRNA construct, we consider the control group as the most appropriate control group. Therefore, we continued our endocrinological and behavioural analysis by comparing control and GR knockdown groups.

GR knockdown in dentate granule neurons does not affect plasma corticosterone

GR activation in the hippocampus after stress exposure is known to limit further activation of the stress system via facilitation of behavioural adaptation [9;11;495]. Knockdown of hippocampal GR might therefore affect HPA activity, and thereby indirectly influencing cognitive performance. To control for this, we measured plasma corticosterone concentrations at four time points. Basal plasma corticosterone concentrations were determined the morning and evening two days prior to the start of fear conditioning. Compared to these basal morning and evening corticosterone levels, there was an expected robust corticosterone response 30 min following acquisition on Day 1 (Figure 5.1). In line with previous studies from our group [489;491], this indicates that plasma corticosterone levels had increased enough to activate the GR during the fear conditioning paradigm. The corticosterone levels of the fourth sample were low and similar to basal. At all measured time points, corticosterone concentrations were comparable between mm- and GR knockdown groups. GR-knockdown in NPCs therefore did neither affect the level nor the rhythmicity of corticosterone secretion. This suggests that newborn dentate granule cells are not involved in hippocampal inhibition of the HPA axis. In addition, this indicates that the behavioural effects are due to the selective downregulation of GR expression and not indirectly, due to differences in ligand availability.

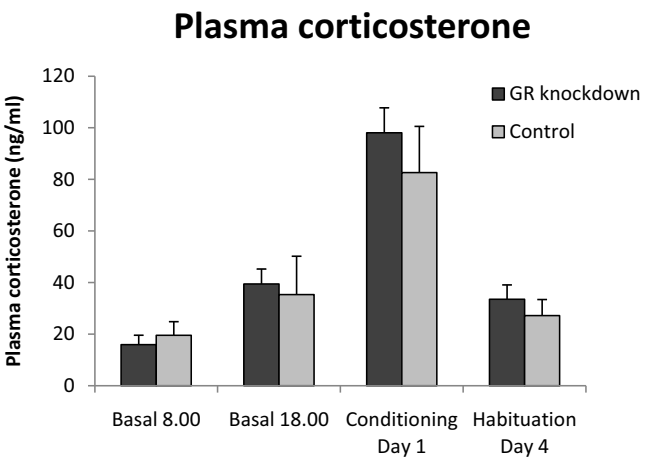

Figure 5.1. Plasma corticosterone concentrations in GR knockdown and control at four time points (mean ± SEM). *Basal morning and evening blood samples were taken two days before the start of fear conditioning. A third blood sample was taken 30 min after the start of conditioning on Day 1 and the fourth sample 60 minutes after the start of memory testing on Day 4.*

Freezing during context and cue episodes per testing day

Context and cue fear conditioning is a form of Pavlovian conditioning elicited by pairing a neutral stimulus (CS: light and tone = cue) with an aversive unconditioned stimulus (US: e-shock) in a distinctive context. Acquisition of the CS-US association is known to require the amygdala [496]. Acquisition of a context-US association is regarded as a hippocampus-dependent task, involving a spatial/ contextual component that involves the dentate gyrus and amygdala [497;498]. Proper acquisition elicits fear behaviours for the expected aversive stimulus, such as freezing, which is regarded as an adaptive species-specific defence behaviour [499]. This is emotionally arousing as well as stressful, triggering GC secretion as well as subsequent GR activation and thus making it suitable for investigating GR function. After 48 (day 3) and 72 hours (day 4), mice were tested for their fear memory related to alternating cue-on and context episodes. Freezing is the predominant expression of fear.

Perfect match-shGR and control treated mice differed significantly in freezing behaviour over the three testing days (Figure 5.2; main effects of treatment: $F(1,85)$ 20.483 $p<0.0001$; day: $F(2,85)$ 9.908 $p<0.0001$; interaction treatment-day: $F(2,85)$ 3.391 $p=0.038$). Both, freezing during context and cue differed significantly between treatments over the Days (cue / context: main effect treatment: $F(1,91)$ 17.519, $p<0.0001$ / $F(1,85)$ 19.588 $p<0.0001$; Day $F(2,85)$ 9.439, $p<0.0001$ / $F(2,85)$ 11.517, $p<0.0001$; interaction treatment-Day: $F(2,85)$ 3.975, $p=0.022$ / $F(2,85)$ 3.205, $p<0.046$).

During acquisition on Day 1, freezing behaviour was comparable between GR knockdown and control groups for cue and context episodes. During memory testing on Day 3, GR knockdown treated mice showed about 50 % less context-related freezing ($F(1,30)$ 7.322, $p=0.011$) while on

Day 4 both, context and cue-related freezing were decreased by 50 % (context: F(1,30) 15.456, p< 0,001; cue: F(1,30) 18.026, p<0.0001) (figure 5.2) compared to control mice. Mismatch-shGR mice expressed similar high cue-related freezing on Days 3 and 4. Perfect match-shGR treated mice discriminated between freezing to context (less) and cue (more) on Days 3 and 4 (both p<0.01). Mismatch-shGR treated mice showed significantly less freezing to context than cue on Day 4 (p<0.0001).

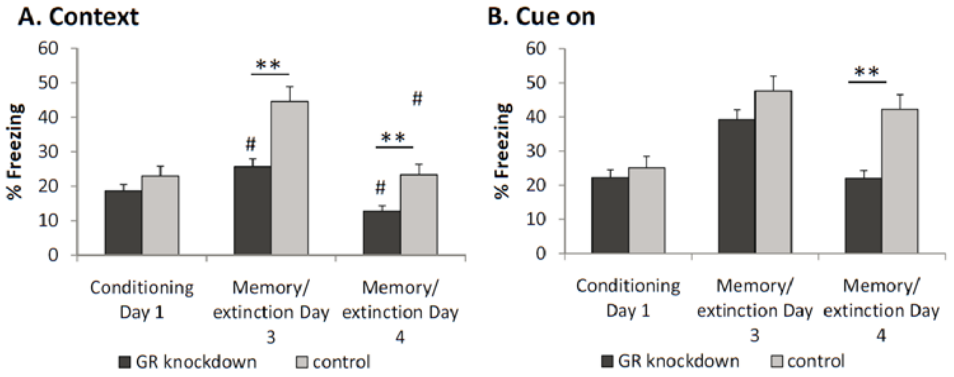

Figure 5.2. Percentage of freezing during context (A) and cue (B) episodes per testing day (mean ± SEM). *(A) freezing to context. B) freezing to cue on. Dark bars: GR knockdown, light bars: control. Between groups ** p< 0.011 GR knockdown compared to control. Within groups # p< 0.01 context freezing compared to cue freezing.*

Progression of freezing behaviour over alternating context and cue episodes

During acquisition of fear on day 1, mm- and pm- shGR injected mice significantly increased freezing over context and cue episodes (context: F(5,145) 47.291, p< 0.0001; cue: F(5,145) 54.779, p<0.0001; Figure 5.3). This pattern was similar in both groups, indicating a proper learning curve independent of treatment. During memory testing on day 3, context-related freezing decreased over time, comparably in both groups (F(5,140) 6.779, p<0.0001), while cue-related freezing did not change over time. On testing day 4, context-related freezing changed over time (F(5,140) 12.416 p<0.0001), with a different time course in both groups: freezing is low with little change over time in the GR knockdown groups, while there is a significant decrease of context freezing in the control group (interaction F(5,140) 2.604, p=0.028). Cue-related freezing decreases in both groups (F(5,140) 2.846 p=0.018).

On day 3, significant differences in freezing behaviour were observed for several context and the first cue episodes (all p < 0.05; figure 5.3). Perfect match-shGR treated mice showed less freezing than control mice during the initial 3 minutes in the box before the first cue (F(1,111) 10.35, p< 0.002). This shows that GR knockdown treated mice freeze less when first exposed to the context previously associated with a shock, and also freeze less when the cue is presented for the first time.

During testing for memory on day 4, freezing behaviour during all cue and context episodes was significantly less expressed in GR knockdown than control mice (ANOVA for all episodes: $p < 0.05$; GLM for context: $F(5,155)$ 2.79, $p= 0.019$; GLM for cue: $F(5,155)$ 4.97, $p<0.0001$).

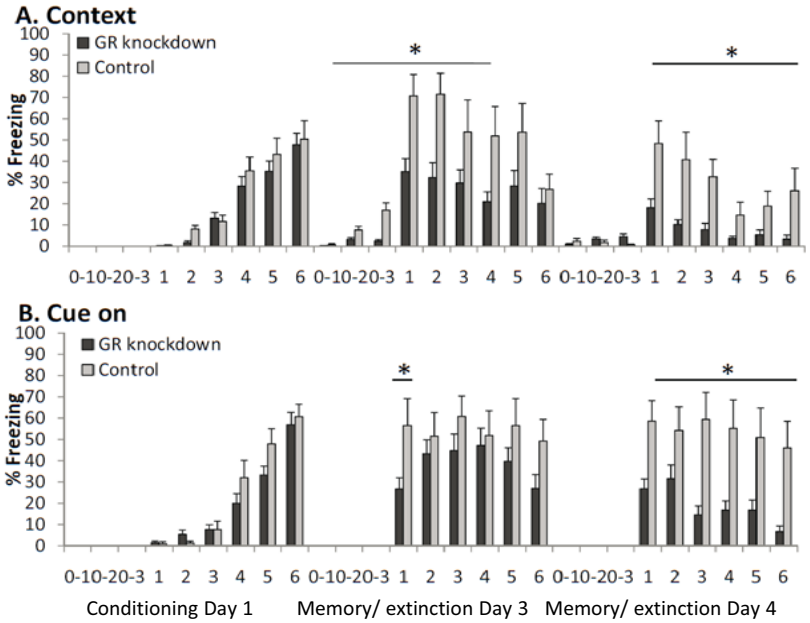

Figure 5.3. Percentage of freezing to context (A) and cue on (B) over the alternating episodes per testing day (mean ± SEM)*. From episode 0-3 onwards paired context and cue on episodes are separated in this graph for better distinction of the freezing patterns between the two groups. Dark bars: GR knockdown; light bars: Control. The lines indicate the context or cue on episodes with significant differences between treatment groups. Freezing to the context was significant at Day 3 for episodes 0-1 to 0-3 and 1 to 4 ($p<0.05$). For Day 4 context freezing was significant for episodes 1-6 ($p<0.05$). Freezing to cue on was significant at Day 3 only for the first episode ($p<0.05$), while on Day 4 episodes 1 to 6 were significant between GR knockdown and control groups ($p<0.05$).*

Scanning: a more active fear behaviour

In addition to freezing, we also assessed another fear-related behaviour: scanning. Freezing and scanning are often represented together as "total immobility". Throughout the 3 days of the context and cue fear conditioning paradigm, the percentage of time in which mice displayed "total immobility" was comparable to GR knockdown and control mice. Comparable to the differences found for freezing, scanning differed significantly between GR knockdown and control mice and changed over days (main effect of treatment: $F(4,81)$ 2.835, $p=0.03$; day: $F(8,164)$ 9.071 $p<0.0001$). During conditioning, scanning in context and cue episodes was comparably high between the groups. Perfect match-shGR mice keep the same level of scanning behaviour shown during conditioning also on the memory testing days, while scanning decreases in the control mice. Thus, on testing Days 3 and 4, scanning behaviour was increased in GR knockdown mice

during context and cue episodes (context: F(1,84) 5.613 p=0.02; cue: F(1,84) 8.484 p=0.005) compared to control mice (figure 5.4).

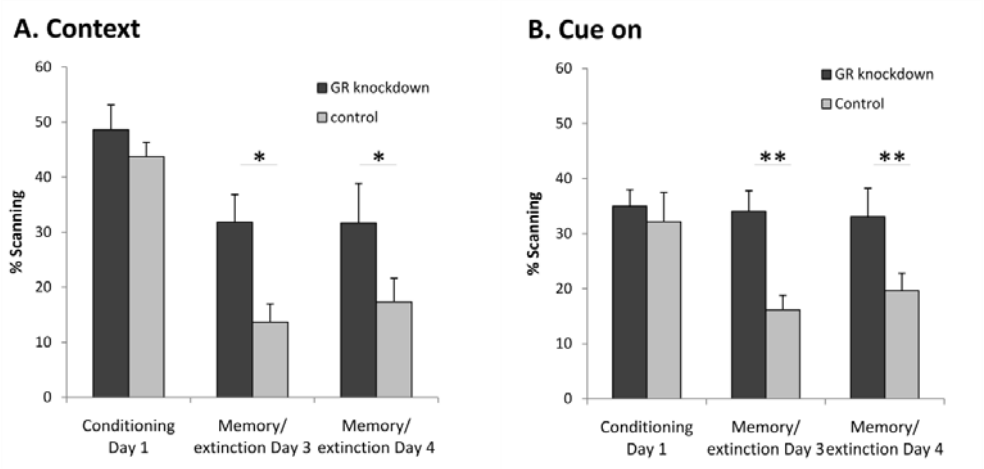

Figure 5.4. Percentage of scanning during context (A) and cue (B) episodes per testing day (mean ± SEM). *Dark bars: GR knockdown, light bars: control. Between groups: * p<0.05 and ** p<0.005 compared to control. Within groups: not significant.*

Factor analysis

Factor analysis resulted in the extraction of two factors explaining 83 % of total variance (table 5.1). Factor 1 was classified as passive fear, factor 2 was classified as fear activity. Further ANOVA showed a significant treatment effect (F(1,1349) 12.466, p<0.0001) on factor 1, indicating less passive fear behaviour in GR knockdown treated mice.

	Factor 1: Passive fear	Factor 2: Fear activity
Total immobility	0.890	
Freezing	0.828	
No. Scan		0.978
No. Freeze		0.943

Table 5.1. Principal component analysis over all data, with Varimax rotation and Kaiser normalisation. *Behavioural parameters are represented as factor loading per factor. Factor loadings with equal value are positively correlated, while loadings with opposing values are negatively correlated. Loadings < 0.7 are not included in this table.*

DISCUSSION

In this study we have shown that four weeks of RNAi-mediated GR knockdown (GR knockdown) in NPCs resulted in a destabilized memory consolidation during context and cue fear conditioning. Specifically, GR knockdown resulted in less context- dependent freezing during memory testing on Day 3, and lower context- and cue- induced freezing on Day 4, despite a comparable acquisition during Day 1. Furthermore, analysis of all fear behaviour showed a shift from a passive coping strategy (freezing) to a more active coping strategy (scanning) in GR knockdown injected mice. This was verified by factor analysis. Supported by the lack of effects on circulating plasma corticosterone, these data confirm the involvement of hippocampal GR expression levels in NPCs in cognitive performance.

Several studies have shown the involvement of glucocorticoids and GR activation in fear conditioning [149;209]. However, the role of GR in specific hippocampal cell populations, i.e. neuronal progenitor cells, remains elusive. Thus far, it has been difficult unravelling GR function in the different cell types, because of a lack of discriminating techniques. Therefore, we here used lentiviral vectors to specifically knockdown GR in the neurogenic niche of the dentate gyrus [451]. Our data add to the growing evidence that NPCs are important substrates underlying hippocampal cognitive performance. In addition, we show for the first time that GR expression in NPCs is involved in memory consolidation for a fearful event.

GR knockdown in NPCs is not involved in appraisal or acquisition

Hippocampal-dependent learning and memory can be separated into distinct phases; on the one hand appraisal (evaluation of the situation) and acquisition (learning), on the other hand consolidation (memory formation) and retrieval (memory recollection) [183]. Extinction occurs when a conditioned response to a stimulus decreases when a reinforcer is omitted [500]. A lack of memory as “end product” can be caused by a disruption in each of these phases. For the GR, its role in memory during contextual fear conditioning has been observed in several studies using systemical loss of GR expression, inhibited GR activation by adrenalectomy or GR [199;200;209;501-504]. In our study we aimed for a more specific investigation of the function of the GR in NPCs in relation to a specific phase of memory. To this end, we used a 4-day paradigm for context and cue fear conditioning. Although it is difficult to discriminate between phases, this paradigm gives more insight in the memory phases affected by GR knockdown in NPCs.

Interestingly, the acquisition of fear behaviour in GR knockdown mice was comparable to control mice during conditioning on day 1. During the initial minutes in the fear conditioning apparatus and the following episodes of alternating context and cue pairings followed by a footshock, we did not observe any difference between GR knockdown and control mice. Freezing increased during each subsequent episode in a similar fashion for both GR knockdown and control mice. These learning curves suggest the mice learned equally well and therefore may imply that appraisal and acquisition are not affected by adult born neurons and more specifically not by GR knockdown in

NPCs. This supports the finding of others, that altering GR activation by GR (ant-) agonist treatment before acquisition does not affect fear behaviour to a shock [489;505].

Also, upon a non-GR related genetic manipulation of NPCs, no effects of acquisition could be found [487]. In this study, the expression of the pro-differentiative gene PC3 (Tis21/BTG2) was induced specifically in nestin-positive cells, which is in line with the GR knockdown data described in Chapter 4. In line with the data described in this chapter, the authors also found that an accelerated differentiation of NPCs did not affect acquisition. This suggests that acquisition was not affected by GR knockdown in NPCs, and consequently, that different hippocampal circuits and signalling pathways are involved in acquisition of context and cue fear conditioning.

GR knockdown in NPCs leads to destabilized memory consolidation for the context

In contrast to a role for GR knockdown in NPCs in appraisal and acquisition, a role in memory consolidation is more likely. Memory consolidation is the process by which a fragile short term memory trace is transferred into stable long term memory. Stress-mediated activation of GRs, has been strongly associated with a facilitative effect on memory consolidation [149;209]. Specifically, GCs have been shown earlier to enhance memory consolidation of emotionally arousing experiences [506], a situation we tested in context and cue fear conditioning.

Fourty-eight hours after conditioning, during first minutes of memory testing, GR knockdown treated mice showed less freezing response when placed in the fear conditioning chamber (context) and the first cue. This strongly indicates that for context, mice seem to have remembered less about the fearful foot shock on Day 1. After the first cue exposure, a number of subsequent context episodes still showed decreased freezing of GR knockdown mice as well. Freezing in response to the cue however, was comparably high to control mice and also significantly higher from context freezing in GR knockdown mice. This indicates mice are able to differentiate between context and cue episodes.

Processing context and cue information is known to take place in different brain areas. The cue related fear response is related to the amygdala [498]. This explains well the lack of effect on cue freezing upon GR knockdown in the dentate granule neurons. In contrast, in the dorsal hippocampus, the DG together with the CA3 serves to encode spatial and contextual information [187;245], so less GR function in the DG might affect encoding of context information during memory testing. Another explanation for less contextual freezing during (initial) memory testing might be a loss of the facilitative effects of GR activation on memory consolidation; i.e. a destabilized consolidation [507]. In line with this, a hippocampal GR modulatory effect on contextual fear memory has been shown [489;508;509], although not specific for the DG.

GR-mediated facilitation of memory consolidation of emotionally arousing experiences such as fear conditioning is also critically dependent upon GC induction of sympathic activity in the amygdala. In our study, GC levels were high during conditioning, thus also able to activate GRs throughout the brain, including the amygdala. GR knockdown however was restricted to NPCs in

the DG. It would therefore be interesting to further investigate the link between NPCs and amygdala activation in context and cue fear conditioning.

Indeed, the involvement of NPCs in processing contextual information has been shown previously. Similar to our study, Farioli-Vecchioli et al for example showed that accelerated differentiation of NPCs by conditionally expressing the PC3 transgene specifically affected contextual memory for fear conditioning (but not acquisition) [487]. Also, focal X-irradiation, and genetic ablation of GFAP+ NPCs impaired contextual fear conditioning but not cued conditioning [366;510]. These studies support our evidence that GR in NPCs is involved in contextual memory consolidation.

GR knockdown in NPCs facilitates extinction of fear memory and leads to a shift from passive fear coping to active fear coping

Overall freezing seems to decrease during memory testing days 3 and 4. Interestingly, total fear memory (total immobility= freezing + scanning) does not differ between groups on both day 3 and 4. This suggests that mice indeed remember the negative event, although expressing a different quality of fear: scanning. In fact, the reduction of freezing behaviour seems to be compensated by a relative increase in scanning. On both memory testing days scanning is relatively increased in GR knockdown compared to control mice during both context and cue episodes.

This can be due to (i) lower fear memory or (ii) better memory for the new "safe" situation or (iii) better retrieval. It is difficult to distinguish between these alternative explanations in our paradigm. However, we can conclude that the process(es) that underlie less freezing behaviour on day 4 probably take place between the two memory testing days and thus involves memory consolidation. Therefore this is also in line with the evidence for a role of GR knockdown in destabilization of memory consolidation: Possibly, less consolidation of the fearful event lowers (not extinguishes) fear perception for the adverse context during later memory testing and induces a more active coping mechanism to the fearful environment.

Perfect match-shGR treated mice do not differ in freezing behaviour at the end of memory testing day 3, while they do show less overall freezing from the beginning of the memory testing day 4. Furthermore, progression of freezing behaviour on day 4 is not similar compared to controls, although a significant lowering of freezing behaviour is present in both GR knockdown and control treatment groups. This additionally shows that short term extinction is similar between GR knockdown and control mice, and that only absolute freezing levels differ.

Thus, GR knockdown in NPCs seems to affect memory consolidation. Several studies have shown impaired GR function disrupts consolidation [509;511-513], thus excluding the argument that in mice with GR knockdown consolidation of memory for the new "safe" situation is improved. In this line of reasoning, the hypothesis regarding decreased fear memory is most likely due to impaired consolidation in GR knockdown treated mice.

Still, a careful consideration of our GR knockdown animal model is necessary. Firstly, this study is the first to show GR knockdown in NPCs, therefore far more specific than any other pharmacological or genetical animal models thus far. Nevertheless, in our study we have manipulated the GR irreversibly four weeks before memory testing. Therefore our shGR mice

were already different from the start of the fear conditioning procedure, even though at the time of acquisition equally high GC levels were present. Keeping in mind that GCs and GR activation need a narrow time window to affect any of the individual memory phases [183;514], an supplementary experiment would be necessary. In addition to the GR knockdown and control mice in which during conditioning high GC levels were observed, a group of ADX mice should be investigated. ADX mice are depleted of GC secretion and therefore are expected to have similar memory impairments to GR knockdown mice. However, GC treatment just after acquisition should rescue this phenotype; mimicking the control group. Although not specific for NPCs, such an experiment would present final evidence that GR is critical for memory consolidation.

Destabilization of memory consolidation in Balb/c mice is adaptive

BALB/c mice represent an emotional and stress sensitive mouse strain and good spatial learners [489]. Similar to wild type BALB/c mice our control mice lack discrimination of context and cue. This indicates a generalised and even potentiated fear response [484]. In contrast, during memory testing, GR knockdown results in a pattern of fear behaviour that indicates discrimination between the context and cue. As discussed above, this indicates GR knockdown in NPCs affects context information processing in the DG and shows a relatively more pronounced fear response to the cue. Recognizing the cue as a threat, and respond with freezing in anticipation of the shock, can be considered as an adaptive response. The decrease in freezing to the context was compensated by an increased scanning which indicates a shift to a more active coping strategy that might allow possibilities to escape the expected aversive event (Brinks et al., 2008). Therefore, a lowered fear response secondary to GR knockdown in BALB/c mice seems an adaptive response rescuing emotionally overwhelmed BALB/c mice from a generalised fear response.

The long term blocking of GR by RNAi and the acute behavioural results we have seen for memory consolidation of a fearful event, suggests that in newborn dentate granule neurons GR is especially important to deal with stressful challenges of the hippocampus. This is also in line with GRs function as a sensor of salient and/ or threatening stimuli [3].

How do NPCs contribute to memory consolidation for context and cue fear conditioning?

Opposed to a direct role of GR in memory consolidation, an alternative possibility is a rather indirect role of GR. In a previous study, we have shown GR knockdown in NPCs affected proper differentiation and positioning, morphology, physiology and synaptic plasticity of adult born dentate granule neurons (see CHAPTER 4). This evidence strongly suggests that GR knockdown in NPCs affects functional incorporation of newborn dentate granule neurons into the hippocampal neo-circuitry and thereby affecting hippocampal function.

Adult born dentate granule neurons have indeed been shown to contribute to the hippocampal circuitry as well as pre-existing dentate granule cells [95;98;103-108]. In fact, they display plastic properties making them exceptionally suited for contribution to hippocampus-dependent

cognitive performance. Kee et al demonstrated that newborn granule cells are preferentially activated in hippocampus-dependent learning tasks.

In numerous studies of eliminating or genetically modulating NPCs, the involvement of adult born dentate granule neurons in hippocampus-dependent memory formation and contextual fear conditioning has been stressed [296;366;449;468;485-487;515;516]. However, not all studies uniformly report an involvement of NPCs in context fear conditioning [98;406]. These conflicting data can be explained by differences in experimental design and NPC targeting techniques [110].

As there is at present no indication that these NPC manipulations affect GRs, it is likely that both GR and NPCs in combination are the substrate underlying our behavioural findings. This is underlined by the observations that successful memory consolidation are dependent on *de novo* protein synthesis, long term changes in synaptic plasticity [517] and the fact that the GR is a transcription factor that is involved in dentate gyrus neurogenesis and synaptic plasticity (CHAPTER 4).

Conclusions

All together, our data demonstrate dependency of GR signalling in newborn dentate granule cells for facilitation of consolidation of fear memories. Knockdown of GR destabilized memory consolidation to the conditioned context, resulting in a less strong expression of fear behaviour; i.e less “passive” freezing and more “active” scanning coping style. These results, in combination with the evidence for a role of GR in the maturation of NPCs (see CHAPTER 4), are in line with our hypothesis that GR knockdown in NPCs affects neuronal function and may thereby modulate cognitive performance. The precise mechanisms underlying this exciting phenomenon, is a matter for additional experimentation.

6

GENERAL DISCUSSION

OVERVIEW

6.0 INTRODUCTION TO GENERAL DISCUSSION

Impaired GR signalling has been associated with hippocampal dysfunction and stress-related diseases. The underlying mechanisms are still unknown and therefore necessitate the development of animal models in which these processes can be carefully manipulated and investigated. Previously, GR has been investigated in various pharmacological and genetic models. While often mutually consistent, the available models sometimes show a conflicting or contradictory phenotype. As the GR is involved in a pleiotropy of functions in different cell types of the hippocampus [3], it is important to scale down and to carefully dissect GR function at discrete hippocampal subfields.

The objective of my thesis was therefore to investigate in detail the role of the GR in a specific subfield of the hippocampus: the dentate gyrus. For this purpose, we developed a novel mouse model to specifically knockdown GR expression using RNA-interference. To our surprise we observed that GR knockdown occurred selectively in a population of DCX+ neuronal progenitor cells the SGZ of the DG. Hence, this unexpected finding allowed to focus the objective on the role of GR in the neurogenic niche of the hippocampus. In the second part of my PhD project I have used this model to test the hypothesis that GRs in these newborn cells in the DG contribute to aspects of cognitive performance. For this purpose, the (sub-) cellular morphology and physical properties of GR knockdown cells were investigated. Subsequently, the effects of GR knockdown were characterized in a hippocampal-dependent task: context and cue fear conditioning.

We have described the design and selection of potent GR-shRNA constructs in CHAPTER 2 and a new strategy for specifically targeting neural progenitors in the SGZ by lentivirus-mediated delivery of shRNAs in CHAPTER 3. Using this strategy, we achieved GR knockdown of approximately 85% specifically in a population of newborn granule neurons. Also, we discovered a new role for GR in the development and functional integration of newborn granule neurons (CHAPTER 4). Finally, pharmacological experimentation suggested evidence for the involvement of GR in contextual fear memory and my data strongly suggest that this action of glucocorticoids, at least partly, is executed by GR expressed in NPCs. Our RNAi mouse model further revealed that basal and stress-induced plasma corticosterone concentrations were not different from the values observed in control mice, suggesting that GR in NPCs is not involved in hippocampal inhibition of the HPA axis (CHAPTER 5).

In this chapter I will discuss these experimental results in a broader context. I will compare the functional results of the RNAi mouse model with other GR animal models. I also will propose a mechanism for the involvement of granule cell GR in the new findings of GR in neurogenesis and hippocampal function. Furthermore I will discuss the possible consequences and implications of the new insights gained in the present study.

6.1 LOCAL APPLICATION OF LV-RNAI-MEDIATED GR KNOCKDOWN

6.1.1 A comparison of our shGR mouse model with other transgenic animal models for GR

In this thesis, I have described a novel mouse model in which GR expression was specifically reduced by 85% using RNAi. Short hairpin RNAs were delivered into the hippocampus using stereotactic injections of lentiviral vectors. In fact, to our surprise we found lentiviral vectors to transduce a specific population of DCX+ NPCs and their progeny of newborn dentate granule neurons (further referred to as NPCs). This LV-shGR mouse model enabled us to investigate the hypothesis that GRs in NPCs in the DG underlie hippocampal features of cognitive processes.

GR function has been investigated already for decades in various animal models using pharmacological and genetic approaches (see CHAPTER 1.6). For investigating our hypothesis, our newly developed LV-shGR mouse model has several advantages over these conventional approaches. A major advantage for example is that our LV-shGR mouse model seems specific for DCX+ NPCs, while more conventional strategies target GR more widely in numerous cell populations. The GR is expressed in almost all brain cell types and is associated with an enormous diversity in functions [3]. Therefore, conventional models could mask the cell-specific effects and thereby confound the interpretation of GR function in individual cell populations [113]. Equally important, even subtle differences affecting GC signalling through GR may affect the phenotype. As described in CHAPTER 1, GC signalling in the hippocampus depends not only on factors such as GR expression and GC concentrations, but also on receptor-specific characteristics, timing, and importantly, on cellular context. This context is critical in the dentate gyrus, since it is a very heterogeneous environment consisting of different cell types and cell populations from different origin and age [62;70]. Therefore, compared to more conventional approaches such as classical transgenesis, more cell-specific GR manipulations are required to give new insight in cell type-specific GR functions.

A second advantage of our LV-shGR mouse model is the lack of possible developmental disruptions. Several transgenic GR mice are associated with developmental disruptions and/or compensation mechanisms. For example, the GR appears to be critical for embryonic development and absence leads to lethality or severe hyper-adrenalism and wasting [319]. As mentioned before, GR is expressed in virtually every brain cell, executing different functions. Cell-specific GR manipulation by viral delivery in the adult brain circumvents possible developmental problems by preventing compensation and confounding effects. As I showed specific targeting of lentivirusses to NPCs (CHAPTER 3), our LV-shRNA mouse model seems ideal to investigate the role of GR in newborn dentate granule neurons in adult mice.

A third advantage is that RNAi-mediated GR manipulation allows for a partial knockdown and not a complete knock-out of GR protein expression. On average, we found a 85% knockdown of GR protein expression in NPCs. This is critical, since the extent to which the GR expression is manipulated has consequences for the phenotype. GR can be over-expressed or knocked out fully in transgenic animals, but can also have a partial knockdown of function or expression as for example mediated by antagonists or RNAi. Since GR is an essential transcription factor, full

ablation is developmentally lethal. However, reductions of GR mRNA and protein expression and function have been shown in physiological conditions such as chronic stress, early life stress, aging and elevated GC concentrations [51;273;481;518-520]. Also in line with our findings, previously reductions in GR expression have been associated with cognitive and neurogenic and physiological effects *in vivo* [312;313;318;321]. GR protein levels are also endogenously determined by RNAi. Recently our group showed that GR protein levels are down-regulated by microRNA-124 [59], a non-coding RNA that is endogenously highly expressed specifically in neuronal cells, such as NPCs [61]. The approach of a (RNAi-mediated) reduction of GR expression is therefore considered more resembling naturally occuring, physiological circumstances.

Despite these advantages of our shGR mouse model, there are more differences between different GR animal models that might be more a drawback or disadvantage. Each approach comes with its own pros and cons (see CHAPTER 1, Table 1.1). A first potential disadvantage of our shGR mouse model is associated with the delivery of shRNA constructs; the stereotactic injections into the brain. Every intrusion, may involve a potential hazard, since neuronal damage or inflammations secondary to neuronal damage may interfere with the phenotype. Also, since the small and restricted location of NPCs in the sub-granular zone of the dentate gyrus, injections have to be very precise. Minor variations can lead to a mis-positioned injection. This is reflected in the relatively high numbers of experimental animals we have used for each experiment. Also pharmacological experiments may be influenced by variations in technique or individual differences between experimental animals. This is not the case with genetic animal models (although breeding costs a lot of animals, effort and money).

A second potential disadvantage of our approach is the use of exogenous materials in the brain; lentiviral vectors and shRNA constructs. As extensively discussed in CHAPTER 2, this may lead to non-specific effects or off-target effects. These non-specific effects may be difficult to circumvent. However, it is possible to adapt the experimental design with appropriate controls. We therefore used mismatch-shRNA constructs that were only different from perfect match shRNA in two nucleotide point mutations.

For our experiments aimed at investigating the role of GR with respect to adult hippocampal neurogenesis and cognition, our LV-shGR mouse model has proved to be an excellent approach. Nevertheless, it is important to keep in mind that investigating the different aspects of GC/GR signalling may require different approaches. For example, genetic approaches alter gene expression at the level of the DNA. Posttranscriptional RNAi alters gene expression at the level of mRNA. Both methods have in common that both mRNA and protein expression is altered. In contrast, pharmacological methods alter GR function -but not expression- at the level of the protein. The availability of all different models is also necessary to confirm experimental data and obtain robust evidence for GR function. In this respect, the best proof is when a certain result is established using different methods. This also accounts for our LV-shGR model, as the phenotype of RNAi-mediated reduction of GR was at the behavioural level in support of other studies using antagonists and adrenalectomy with hormone substitutions (see CHAPTER 5) [200;209;489;504]. Our

model therefore has the important advantage of high target specificity combined with flexibility and easiness of use.

6.1.2 Lentiviral vectors target neuronal progenitor cells and immature dentate granule neurons

In CHAPTER 3, I described how lentiviral vectors target a specific population of adult born dentate granule neurons referred to as NPCs. This evidence was based on the analysis of cell-lineage specific markers. In CHAPTER 4 we studied the effects of GR knockdown during the development of these cells. Several lines of evidence in this study indicated GR knockdown results in an accelerated neuronal differentiation. This hypothesis was based on the analysis of the expression of cell-lineage specific markers and the examination of morphological parameters such as dendritic arborisation, dendritic spines and boutons. One remarkable observation was that one week PI GR knockdown in NPCs resulted in a significant increase of the percentage of a more mature and stable type of spines compared to control. Typically, spine formation in adult born neurons takes place during the third and fourth week of neuronal development [64;102;427]. How is this finding to be explained in the light of the cell population targeted by the lentiviral vector?

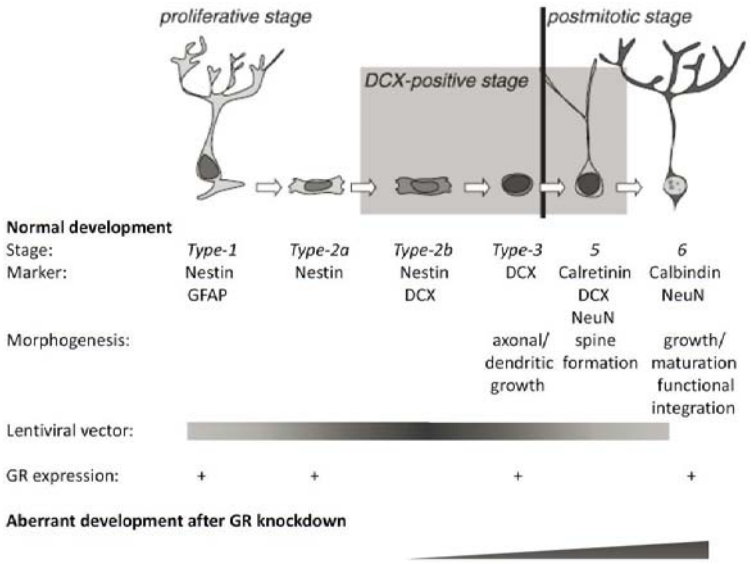

Figure 6.1 Proposed development of newborn neurons in the dentate gyrus (see also CHAPTER 1). *Six stages of neuronal development in the adult hippocampus can be readily identified on the basis of morphology, proliferative ability, and expression of markers such as nestin, GFAP, DCX, calretinin, calbindin and NeuN. The gray line indicates the developmental stages that are suggested to be transduced by the lentivirus. Figure modulated from references [57;62-64].*

One possibility is that lentiviral vectors do not target NPCs around the stage of cell division, but in a later stage. The development of a newborn neuron takes about four weeks during which the cell passes several stages. Each of these stages has its own characteristics (see figure 6.1). According to Kempermann et al (2004) neuronal development is hypothesized to originate from a putative

stem cell (called a type-1 cell) [62]. This cell has radial glia and has astrocytic properties, such as GFAP expression. Neuronal development then progresses over three stages of putatively transiently amplifying progenitor cells (Type-2a, type-2b and type-3 cells), which appear to be increasingly determined to the neuronal lineage to an early post-mitotic stage. Type-1, Type-2a and type-2b cells for example express Nestin. Type-2b and type-3 cells express DCX during the second and third week after cell division. DCX is therefore regarded as a marker for immature neurons [100;135]. During the third and fourth week spine formation appears [64;102;427]. From the post-mitotic stage onwards, cells express the mature neuron marker NeuN in the fourth week.

In our study (CHAPTER 3), the majority of the cells targeted by the lentivirus (> 50%) was DCX+ already one week PI. Cells positive for NeuN constituted 15%. These cells were characterized by their morphology and dendritic spine profile as probably late stage immature neurons. Cells positive for a very early neuronal marker Nestin constituted 11%. Four percent of the LV-targeted cells were positive for the neuronal proliferative marker Ki67. The remaining 16% of the cells were positive for GFAP, a marker for putative stem cells and glial cells. In addition, the cell population targeted by the lentivirus was typically located in the sub-granular zone and inner layers of the granule cells layer. As discussed in CHAPTER 3, these data suggest that the lentivirus targets newborn neurons of several developmental stages; i.e. early neuronal progenitors (KI67+ and Nestin+), as well as later stage DCX+ immature neurons. This finding is confirmed using three different lentiviral systems and is in line with studies by others showing that in the CNS, lentiviruses may target both dividing as well as type 2b, type-3 and/or (some) type-5 cells *in vivo* [353;355;369;370;437].

This hypothesis may explain why in CHAPTER 4 dendritic spines were observed to be present on NPCs in such a short time as 1 week PI. We suggest that the NPCs in which the spines were present, the lentivirus transduced these respective cells in stage type-3 or stage 5. Of course, more research is necessary to clarify this issue. However, the differences between spine morphology and other morphological characteristics between GR knockdown and control are likely not explained by the lentivirus but by treatment. Therefore we hypothesised in CHAPTER 4 that GR knockdown results in accelerated differentiation of newborn neurons.

The term “neuronal progenitor” has been used in literature to loosely describe all dividing cells with some capacity to differentiation into neurons [64]. For the purpose of describing the population of newborn cells targeted by the lentiviral vector we have used the term NPC to cover the cells targeted by the lentivirus from the KI67+ and Nestin+ early stages to immature neurons expressing DCX and the early NeuN expressing stages. Other studies of neurogenesis *in vivo* have often used retroviral vectors, Murine Maloney Leukemia Viruses (MMLV) for example, that target a similar but smaller population of NPCs. These retroviruses transduce proliferating cells only [94]. The low numbers of cells transduced at a certain time point in an animal may be a drawback for certain studies. Often, high numbers of cells are necessary for behavioural studies and comparison between several treatments (in my case GR knockdown versus control). By targeting cells of a more broad developmental stage, as lentiviruses do, a higher number of cells can be analysed.

CONCLUSION: The ability to study GR function in a specific cell population of newborn dentate granule neurons -NPCs- using LV-RNAi, makes our model unique compared to other existing animal models. Therefore the shGR mouse models may provide a valuable new approach to study gene function in restricted brain regions.

6.2 GR MAY CONTRIBUTE TO HIPPOCAMPAL PLASTICITY BY MODULATING NEUROGENESIS

In this thesis I have characterized a new mouse model in which NPCs have been targeted by LV-delivered shRNAs directed against the GR. I found significant effects of GR knockdown on neurogenesis, neuroplasticity and hippocampal-dependent memory. Here, I will further discuss how neurogenic alterations may contribute to cognitive performance. Subsequently, I will discuss how GRs may modulate hippocampal function by controlling the maturation and proper integration of newborn neurons into the hippocampal neo-network.

6.2.1 Are neurogenic alterations a substrate for cognitive processes?

Although described in separate chapters, the cognitive effects of GR knockdown in NPCs (chapter 5) were observed in the same animals of which the morphology of NPCs has been analysed (chapter 4). Therefore, the accelerated neuronal differentiation and aberrant positioning and connectivity of newborn dentate granule neurons are associated with the impaired memory consolidation for a fearful event. This anatomical co-localization of neurogenic alterations and impaired memory consolidation in the NPCs of the dentate gyrus seems to be interdependent. Whether altered neurogenesis is causally underlying the observed changes in memory consolidation is a tempting speculation. However, the causal relationship between neurogenesis and hippocampal function is as yet still not fully established. Since this possibility was first outlined by Barnea and Nottebohm (1994) [521], a number of intriguing correlations has been described. Recently, a number of publications provided evidence for a causal relationship:

Firstly, NPCs are important for hippocampus-dependent cognition, since ablation of NPCs by different methods has shown detrimental effects for hippocampus-dependent cognition [98;280;406;449;522]. In fact, the relevance of NPCs for memory consolidation in fear conditioning has been shown by others. At least four weeks after modulation or elimination of NPCs for example, hippocampal function was affected as shown by weakening of contextual fear conditioning [296;366;449;486;523;524]. Remarkably, these results were obtained with different methods. Neurogenesis could for example be disrupted with whole brain or focal (directed to hippocampal region) fractionated ionizing/ X irradiation [296;366;486;524]; genetically targeted ablation [365;449] of neurogenesis by over-expressing pro-aptoptic genes in NPCs; or by reducing neurogenesis with the toxin methylazoxymethanol acetate (MAM) [523]. Our data extend these observations by selectively manipulating one gene by RNAi in newborn granule cells in the sub-granular zones.

Secondly, NPCs exhibit unique physiological properties during certain critical periods of maturation, which make them suitable for neuroplasticity and signalling underlying hippocampal memory [104;524]. It has been established with a variety of different techniques that newborn neurons become within a month functionally integrated in the trisynaptic circuit where they construct long-lasting connections [92;94;95;103;105-107;439;525;526]. In fact, Kee et al demonstrated that newborn granule cells are preferentially activated in hippocampus-dependent learning tasks, suggesting they uniquely contribute to memory formation in the dentate gyrus [527]. Also, in line with our findings, this study shows that these granule cells are mature, contain more mushroom/thin spines, have increased excitatory electrical firing capacity and form altered functional synapses with their target cells in the CA3 region (see figure 6.2).

Thirdly, aberrant neurogenesis underlies hippocampal dysfunction. Recent evidence has suggested that correct coordination of hippocampal memory tasks is critically dependent on the correct timing of the initial stages of NPC maturation and on connection to pre-existing circuits [487]. In this study, the pro-differentiative transgene PC3 was conditionally expressed in Nestin+ NPCs. This resulted in an accelerated differentiation of NPCs combined with profound morphological changes. Three to four weeks later, these mice exhibited impairments in spatial and contextual memory in several hippocampus-dependent tasks. Similar observations have also been observed by others studying pathological conditions in the brain. It has been found that in mouse models for epilepsy [516] or schizophrenia [368;468], alterations in neurogenesis are also correlated with impairments in hippocampal memory formation. Seizure-induced malformation of dendritic outgrowth of newborn cells has for example been associated with impaired hippocampal memory formation [528].
DISC1 knockdown in the dentate gyrus also results in impaired performances of mice in several hippocampus-dependent tasks as well as comparable aberrant placement in hippocampal neo-circuits [368;468]. In our study we found that GR knockdown in NPCs resulted in accelerated neuronal differentiation and aberrant positioning and connectivity of newborn dentate granule neurons. These morphological and physiological alterations are associated with the impaired memory consolidation. In fact we delivered the LV-shRNAs into the dorsal section of the hippocampus, an area of the hippocampus that is particularly associated with cognitive functions [245;529].
Above discussed publications indeed suggest a causal link between neurogenesis and certain aspects of hippocampal function. Aberrant neurogenesis as a substrate seems therefore the most likely explanation of our behavioural findings.

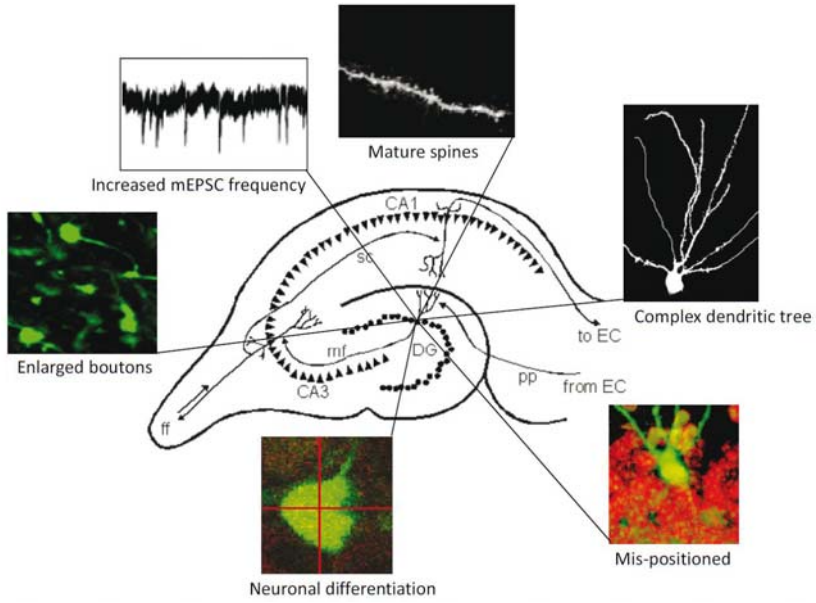

Fig 6.2 GR knockdown- mediated alterations in NPCs of the dentate gyrus (see also CHAPTER 1).
Summary of the main cellular findings in NPCs. Orientation of the dentate gyrus (black dots) and cornu ammonis (black triangles) and their connections with the trisynaptic circuit. Abbreviations: CA1-3 = cornu ammonis 1-3; DG = dentate gyrus; EC = entorhinal cortex; pp = perforant pathway; mf = mossy fibers; sc = Schaffer collaterals; ff = fimbria fornix. (Adapted from Kim et al., 2002) [71]

6.2.2 Are GRs modulating cognitive performance by regulating neurogenesis?

As outlined above, a causal relationship between adult hippocampal neurogenesis and hippocampal function seems evident. If this hypothesis holds true and new neurons in the adult hippocampus are indeed involved in the formation of new memory [448], the GR knockdown-induced accelerated neuronal differentiation, aberrant positioning and functional integration of newborn dentate granule neurons may account for the cognitive deficits observed in context and cue fear conditioning. This is in line with our hypothesis proposed in CHAPTER 1: that hippocampal GRs may affect hippocampal function by modulating neurogenesis. So, what role plays GR in this relationship?

The neurogenic actions of glucocorticoids mediated by GR are likely to be direct [123], since newly-formed cells express GR at birth and the expression of these receptors increases over time [57]. Also, NPCs in the sub-granular zone of the DG are closely associated with the vasculature, indicating that factors from the blood (such as GCs) may have a direct impact on NPCs [124;530]. An intriguing argument for this hypothesis comes from a study of the effects of diabetes on neuroplasticity and cognition [172]. Diabetes is known to influence the HPA-axis. In this study it was demonstrated in two independent animal models, that diabetes impairs hippocampus-dependent memory, perforant path synaptic plasticity and adult neurogenesis, AND that glucocorticoids contribute to these adverse effects. The diabetic animal models suffered from reduced insulin,

hyperglycemia, increased corticosterone, impairments in neurogenesis, synaptic plasticity and learning. Typically, these changes in neuroplasticity and hippocampal function could be reversed when physiological levels of corticosterone were maintained. In a similar study, aberrant effects on neurogenesis and hippocampal function of diabetes-induced hypercorticism could even be attenuated by treatment with the GR-antagonist mifepristone [148]. The authors of both studies suggested therefore convincingly that the cognitive impairment in diabetes may result from glucocorticoid-mediated deficits in neurogenesis and synaptic plasticity.

However, our findings on accelerated neuronal differentiation and aberrant functional integration after GR knockdown might be secondary effects; e.g. GR-mediated effects on cell proliferation or cell survival/ cell selection. It is known that spatial learning depends on both the addition and removal of newborn neurons in the hippocampus (Dupret et al., 2007) [98]. Neuronal networks seem to be sculpted by a tightly regulated selection and suppression of different populations of newborn neurons. GR may play a role in this selection process by affecting the numbers of newborns cells in certain developmental stages. GCs are implicated in cell proliferation [82;137;158;466], as well as apoptotic cell death [5] and cell survival of newborn neurons [92;122]. Immature DCX+ cells have for example been shown to undergo apoptosis when they were also GR+ [57]. In this line of reasoning, it could be that because of GR knockdown in the neuronal progenitors more cells survive which are not destined to become functionally mature. Speculatively, this could be an interesting explanation for the further aberrant path of altered migration, neuronal maturation and inappropriate integration of shGR targeted newborn neurons underlying impaired memory consolidation in our study. However, in our study the proportion of EGFP+ cells positive for the neuronal progenitor marker Nestin was unaffected by GR knockdown. A finding which suggests that GR knockdown accelerates neuronal differentiation in newborn cells, without affecting survival of neuronal progenitors in the DG.

Alternatively to cell survival/ death, GR may also play another role in cell selection during neuronal development. Newborn neurons need to be adequately connected into the hippocampal circuitry to function [531]. In our study we found several morphological alterations that suggest an altered functional integration of newborn neurons upon GR knockdown. GR knockdown resulted in mis-positioned cells, a more complex dendritic arborization of NPCs, an increased number of spines of a mature phenotype, an altered synaptic bouton profile, and an increased frequency of mEPSCs (see Figure 6.2). Combined with the memory impairments observed, these results could explain a possible role of GR in appropriate pruning and modulation of morphological characteristics of newborn neurons. This is again very suggestive, although GCs already previously have been associated with altered dendritic morphology and synaptic transmission [5].

An alternative explanation for GR involvement in neurogenesis and hippocampal function –or perhaps in combination- may be that GR function was reduced during memory formation due to GR knockdown in NPCs, regardless of the position or maturation stage of the newborn cells. Indeed, loss of GR function by adrenalectomy or pharmacological inhibition of GR activity has been shown to impair memory for contextual fear conditioning in rats [200;209;504]. However, in these studies the function of GR in NPCs was not studied in detail like in our study.

The above discussed theories and their underlying mechanisms need to be investigated further. However, a role of GR in hippocampal function by modulating the development of NPCs is plausible in respect to the hippocampal function in behavioural adaptation. The unique physiological properties of newborn neurons make them particularly suited to respond to and integrate stimuli during memory formation [92;110]. The enhanced plasticity of newborn neurons has been suggested to allow preferential association of representations that are closely related in time [404]. As there is a continuous cycle of neurogenesis in the adult hippocampus, this implies distinct age-cohorts of which the different populations of neurons might also be distinct in their modulated history during their development. This phenomenon might underlie the appreciation of different experiences or memories labelled in context and time, which precisely reflects the cognitive function of the hippocampus [282]. Strikingly, newborn neurons of the hippocampus only constitute a very small part of the whole neuron population. It may therefore well be that the adult granule neurons are part in existing circuits underlying learned behaviours and that the newborn neurons function more in challenging conditions when new strategies need to be learned and applied which need a higher level of cellular plasticity. At this level, stress, and GCs through GR likely play a role as a functional modulator in the sensitivity and adaptation of NPCs for future situations [466].

CONCLUSION: There is a clear relationship between GC signalling and neurogenesis on one hand, and GC signalling and hippocampal function on the other hand. Although the involvement of newborn neurons in hippocampal functioning is still not fully established, our evidence suggests that adult born dentate granule neurons contribute to contextual memory for a fearful event. GR knockdown in this cell population results in altered quantitative properties of neurogenesis and simultaneously impaired memory for a task associated with DG function. Our findings therefore strongly suggest that GR-mediated neurogenic alterations are indeed a substrate for hippocampal-dependent cognitive processes. Speculatively, GR signalling in NPCs contributing to hippocampal-dependent memory may promote behavioural adaptation.

6.3 IN VIVO KNOCKDOWN OF GR IN NPCS: IMPLICATIONS FOR STRESS-RELATED-BRAIN DISORDERS

If, as discussed above, GR is indeed critical for maturation, migration and functional integration of newborn dentate granule neurons to adaptive hippocampal functioning; aberrant GR signalling in NPCs may contribute to hippocampal pathology and disease. Similar to our results, studies of others have shown aberrant neurogenesis in relation to hippocampal dysfunction and neuropathologies. Epileptic seizures for example, have been shown to induce dispersion of at least some of the newborn neurons to ectopic locations. The granule cell layer of the dentate gyrus in patients with temporal lobe epilepsy (TLE) is often abnormal due to dispersion and the

presence of ectopic granule like cells in the hilus and inner molecular layer [92;532;533]. Hilar-ectopic granule-like cells are also observed in several animal models of TLE and may persist for months [516;533;534]. Similar to our findings, these aberrantly integrated cells display and accelerated functional maturation resulting in persistent hyperexcitability, and exhibit a much higher percentage of persistent basal dendrites than is normally found [526;535;536]. Therefore, it is suggested that hilar-ectopic granule cells integrate abnormally and might contribute to seizure generation and propagation [533].

As reviewed above, schizophrenia-associated gene DISC1 knocked down by RNAi results in a phenotype in which newborn dentate granule neurons were ectopically located. In addition, DISC1 knockdown was also shown to lead to soma hypertrophy, accelerated dendritic outgrowth with appearance of ectopic dendrites, enhanced intrinsic excitability, and accelerated synapse formation of new neurons [368]. The results from Duan et al suggest that DISC1 orchestrates the tempo of functional neuronal integration in the adult brain and demonstrates essential roles of a susceptibility gene for major mental illness in neuronal development including adult neurogenesis.

In respect to the GR, aberrant GC signalling before has been linked with damage to hippocampal integrity and cognitive function, as well as reductions in cell proliferation and newborn cell survival [1]. In fact, there is strong evidence that the alterations in GR expression and activity ("GR resistance") [52;537;538] are implicated in the pathogenesis and course of stress-related-neuropsychiatric disorders, such as depression [4;113;219;230;232;238-240;304;539-541]. Although the underlying molecular mechanisms are still far from clear (discussed in CHAPTER 4), "natural" reductions of GR in the hippocampus have been observed. To illustrate, decreased maternal care in early life of rats, a rodent model for depression, reduces GR protein levels in the hippocampus [273]; chronic stress, a major risk factor for several psychiatric disorders, is associated with reduced GR protein and mRNA levels in the hippocampus [51;481;520] and aging impairs negative feedback action of GCs on the HPA-axis that is associated by reduced hippocampal GR protein levels [482].

At the level of newborn dentate granule cells, our shGR animal model could represent an endophenotype of GR-resistance. This is an interesting possibility since the DG is known to be exquisitely sensitive to circulating GCs and therefore may likely be one of the primary regions where GR-resistance could occur.

HPA-targeted therapy could be beneficial to resolve the stress-associated hippocampal dysfunction. Antidepressants, although not specifically targeting the stress system [542], have been found to resolve HPA hyperactivity [543] and improve neurogenesis and cognition (reviewed in [109]). Similar findings have been reported for anti-glucocorticoids [150;151]. It would be interesting to investigate this. In particular, since recent evidence points out that just increasing neurogenesis is not sufficient [293;294]. New therapies should suppress aberrant integration of newborn neurons or enhance the correct integration. Our shGR animal model may be instrumental to study the effects of such (antidepressant) drugs (whether or not specifically targeting GR). In addition, our shGR mouse model seems suitable to study the effects of several risk factors of hippocampal pathology

and stress-related-diseases, such as chronic stress. Therefore, our RNAi mouse model may be useful to investigate and explain the neurological alterations resulting from GR reductions and associated hippocampal dysfunctions and pathology.

CONCLUSION: GR knockdown in NPCs resulted in a phenotype of aberrant neurogenesis that is possibly associated with hippocampal dysfunction and neuropathologies. Down-regulation of GR expression has been observed under several natural (pathological) circumstances. Our shGR animal model may therefore be useful to study a specific endophenotype: the effects of GR – mediated alterations in the development of newborn neurons underlying hippocampal (dys-) function. Using this animal model, the sensitivity for stress-related brain disorders involving the hippocampus can be investigated.

6.4 PERSPECTIVES

The studies described in this thesis have yielded a wealth of information on GR function in modulating neurogenesis and fear memory. The results discussed fit well together in the concept of how GCs modulate the brain by regulating cellular processes. Further characterization of our shGR animal model will give more insight in the underlying molecular and cellular mechanisms.

To further understand the alterations of newborn neuronal differentiation and aberrant incorporation in the dentate gyrus, it would for example be informative to study the cellular consequences of GR manipulation on a shorter time interval. In our experiments we have investigated the effects of GR knockdown 1 and 5 weeks PI. As these results were comparable, the aberrant process likely starts before one week PI. I suggest therefore in a future study to investigate a shorter time point such as 3 days PI. An alternative approach for such as study could be the use of a selective GR antagonist in combination with GFP-labelled NPCs by lentiviral delivery. Also, birth dating studies of NPCs with BrdU are important to obtain further insights in the characteristics of the cell population targeted by lentiviral vectors and at which time points of neuronal development GR plays a role.

Then, it may be useful to investigate the quantitative effects (e.g. proliferation and survival of NPCs) in our shGR mouse model as well. For example, proliferation rate en cell death/ cell survival numbers could give necessary information to compare our GR knockdown phenotype with that of other models.

In addition, it would be informative to further investigate the electrophysiological properties of the newborn cells with GR knockdown. The experiments described in CHAPTER 4, investigated signalling of GR knockdown neurons in resting state by measuring mEPSCs. However, how these adult born neurons would underlie cognition, could be better understood by investigating the LTP properties.

To further characterize our shGR animal model it is relevant to study its behavioural and physiological response in the context of both chronic and acute stress. In my thesis, I have

reported the effect of GR knockdown on NPCs under "basal" conditions. Although I tested cognition under acute stress conditions (context and cue fear conditioning), I did not address the effects of chronic stress. Chronic stress however, is associated with disease states. As GR (dys-) function is also associated with stress-related disease states, e.g. "GR resistance", our shGR animal model may provide a useful addition to the already known animal models for anxiety and depression. And as discussed above, it could be a valuable addition to study the biological mechanism of and/ or sensitivity for stress-related-brain disorders in respect to neurological and cognitive disturbances.

This is especially relevant since so many drugs used in clinic affect GC/GR signalling. Extensive use of such GR antagonistic or agonistic drugs may have prominent effects on neurogenesis and hippocampal function. Recently the antiglucocorticoid RU38486 has been shown for example to relief psychotic and depressive symptoms within one week of treatment [544-546] as well as boost neurogenesis in animals [150;151]. Vice versa, high systemic doses of prednisolone (GR agonist) may have adverse effects on the hippocampus, while suppressing autoimmune disease. Using our shGR animal model we can further study the neurological and molecular mechanisms underlying such effects. On the long run, more insight could therefore possibly lead to more cell specific therapeutics or even an important new drug target.

CONCLUSION: Further characterization of our shGR animal model is necessary in the pursuit of a better understanding of how GCs modulate the brain by regulating cellular processes.

6.5 CONCLUSIONS

The shGR mouse model characterized in this thesis is a valuable addition to the current animal models for the investigation of GR function in the brain. The approach using LV-shRNA to specifically knockdown GR in a specific cell population, the neural progenitor cells (NPC) in the dentate gyrus is unique and validated in this thesis. In respect to the phenotype of this model, my study has led to the following conclusions:

Knockdown of GR in NPC's accelerates neuronal differentiation and migration. GR knockdown alters positioning of newborn neurons in the dentate gyrus. The altered dendritic and synaptic organization corresponds to the enhanced excitability measured in de dentate circuit.

Knockdown of GR in NPC's destabilized memory consolidation in a fear conditioning paradigm.

The data suggest a key role for GR in the formation of hippocampal neo-circuitry that coordinates the function of the hippocampus in memory formation for an aversive experience. This conclusion leads me to argue in favour of the hypothesis that hippocampal GRs may affect hippocampal function by modulating neurogenesis.

7

SUMMARY/ SAMENVATTING

SUMMARY

Glucocorticoid stress hormones and their glucocorticoid receptors (GRs) have a profound influence on the brain by modulating processes such as neuronal plasticity and cognition. Aberrant glucocorticoid signalling negatively affects cognitive performance and the integrity of neuronal structures and therefore, has been implicated with several stress-related neuropsychiatric disorders. In addition, many known drugs affect glucocorticoid signalling. This makes an in dept understanding of the effects of glucocorticoid signalling in the brain very important.

Particularly sensitive to stress hormones is the dentate gyrus of the hippocampus, a brain structure implicated in memory processes and encoding of spatial information. The dentate gyrus is a very heterogeneous structure, consisting of different cell types, of different age, origin and physiological properties. Of particular interest are the neuronal progenitor cells (NPCs), known to express GR. These cells are located in the sub-granular zone and are the substrate for adult hippocampal neurogenesis. Neurogenesis is a form of neuroplasticity. It involves the continuous process of development of new functional neurons from NPCs. Studies in rodents suggest that these newborn neurons may contribute to the function of the dentate gyrus in spatial and contextual memory.

The finding that newborn granule cells contribute to memory formation and that NPCs express GR has culminated in the **hypothesis that hippocampal GRs may affect hippocampal function by modulating neurogenesis**. The underlying mechanism is not clear and needs detailed study. The main objective of my thesis was therefore to investigate the role of the GR in hippocampal NPCs in relation to neuroplasticity (described in CHAPTER 4) and learning and memory processes (described in CHAPTER 5). Thus far, technical limitations have prevented the study of the function of GR in individual cell types. Because of this, the second objective of my PhD project was to investigate the applicability of a new refined technique to study GR in NPCs *in vivo*: lentiviral-shRNA injections in the dentate gyrus (described in CHAPTER 2 and 3). This approach makes it possible to down-regulate GR expression specifically in NPCs. The mouse model in which this approach was developed is further referred to as the shGR mouse model.

In CHAPTER 2 the design and optimization of effective RNA-interfering constructs targeting the glucocorticoid receptor were described. To achieve potent knockdown of the GR, we have designed four different sequence-specific short interfering RNA constructs. These constructs were cloned into pSuper vector in a short hairpin (sh) format. Subsequently, pSuper-shRNA constructs were transfected into a neuronal cell line and assessed for their potency to down-regulate GR protein levels. Using Western Blot analysis we determined the efficacy of the different constructs compared to sham, empty vector and corresponding mismatch shRNA. We found four effective pm- shRNAs, one (pm-GR3) with high potency to yield more than 90% GR protein knockdown, whereas the 3 others were less potent (pm-GR2 ~ 60%, pm-GR1 ~ 46% and pm-GR4 ~ 25%

respectively). Pm-GR3 was subsequently cloned into a lentiviral vector and its potency was verified, gaining > 70% GR protein knockdown. Using shRNA constructs it was possible to specifically down-regulate GR expression both via plasmid- and lentiviral vectors in a neuronal cell line. Therefore, this lentiviral vector was used in further studies to deliver shRNA into the dentate gyrus.

In CHAPTER 3 the cell types are described that are effectively transduced *in vivo* by the lentiviral vector. In contrast to the CA1 subfield of the hippocampus, lentiviral injection in the granule cell layer of the dentate gyrus revealed sub-field specific differences in transgene expression. Furthermore, lentiviral vectors were found to target primarily NPCs and immature neurons present in the sub-granular zone and more immature layers of the granule cell layer. The targeted cell population was further referred to as NPCs. Also, lentiviral vectors in the granule cell layer do not target mature granule neurons. These observations suggest the existence of intrinsic differences in the permissiveness to lentiviral transduction among various hippocampal cell types. Therefore, it was concluded that amongst hippocampal granule cells, only adult-generated neurons are target for lentivirus-mediated transgene delivery. These properties make lentiviral vectors excellent systems for over-expression or knockdown of genes in neuronal progenitor cells, immature neurons and adult-generated neurons of the mouse hippocampus *in vivo*.

In CHAPTER 4 the new approach of lentiviral-shRNA injections in the dentate gyrus was successfully used to study the effects of GR knockdown on neurogenesis. It was observed that specific GR knockdown in NPCs accelerated neuronal differentiation. Also, GR knockdown in NPCs led to altered morphological properties of the newborn neurons, as reflected by altered dendritic arborization, higher numbers of mature mushroom and thin spines and larger mossy fiber boutons. Strikingly, GR knockdown led to mis-positioning of adult newborn neurons, suggesting aberrant migration into the granule cell layer. In line with increased numbers of synaptic contacts, adult newborn neurons with GR knockdown exhibited increased mEPSC frequencies. Therefore we suggested that GR protein levels play a key role in the development of NPCs and the appropriate formation of hippocampal neo-circuits.

In CHAPTER 5 we further characterized the phenotype of the shGR mouse model at the behavioural level. The role of GRs in NPCs was studied in respect to the formation of contextual fear memory. To this aim, four weeks after lentiviral injections when NPCs were matured and expected to be functionally incorporated into the hippocampal circuitry, GR knockdown and control mice were trained and tested in a Pavlovian fear conditioning task. This task was designed to allow measurement of fear memory (expressed as freezing) for both context and cue within the same procedure. Results demonstrated the dependency of GR signalling in NPCs for facilitation of consolidation of fear memories. Knockdown of GR destabilized memory consolidation to the conditioned context, resulting in a less strong expression of fear behaviour; i.e relatively less “passive” freezing and more “active” scanning coping style. In line with the results described in

CHAPTER 4, these data suggest a key role for GR in the formation of hippocampal neo-networks that coordinate hippocampal-dependent memory formation.

In CHAPTER 6 the results are discussed leading me to argue in favour of the hypothesis that hippocampal GRs may affect hippocampal function by modulating neurogenesis. The data also demonstrate the validity of the newly developed shGR mouse model for further study on glucocorticoids and neurogenesis in adaptation to stress.

SAMENVATTING

Stresshormonen (glucocorticoïden) oefenen grote invloed uit op de hersenen. Hormonen die in het bloed circuleren, kunnen de hersencellen binnendringen en daar hun effect uitoefenen. Dit doen zij voornamelijk door middel van binding aan specifieke glucocorticoïdreceptoren (GR). Op deze manier beïnvloeden glucocorticoïden verschillende processen waarbij de hippocampus betrokken is, zoals cellulaire neuroplasticiteit en leer- en geheugenprocessen, die als doel hebben de aanpassing aan stress te bevorderen. Echter, in sommige situaties kan er sprake zijn van een verstoorde glucocorticoïdwerking, bijvoorbeeld bij langdurige stress of ziekte. Een verstoorde glucocorticoïdwerking heeft niet alleen een negatieve invloed op deze processen, maar ook een beschadigend effect op de hersenen zelf. Dit fenomeen wordt zelfs in verband gebracht met de ontwikkeling en het verloop van stressgerelateerde ziekten zoals depressie. Op dit moment weten we nog te weinig over het mechanisme dat hieraan ten grondslag ligt. Het is daarom erg relevant om te weten wat precies de effecten van glucocorticoïden in de hersenen zijn en hoe deze effecten via de GR tot stand komen.

Bijzonder gevoelig voor stresshormonen zijn de neuronen in de gyrus dentatus van de hippocampus. Het neuronale circuit dat deze neuronen vormen is betrokken bij geheugenprocessen, vooral wat betreft de tijd, plaats en context van met name emotionele ervaringen. De gyrus dentatus is een zeer heterogene structuur. Het bestaat uit verschillende celtypen en cellen van verschillende leeftijd en herkomst. Deze groepen neuronen hebben ieder hun specifieke eigenschappen. Interessant voor deze studie zijn vooral neuronale progenitorcellen. Deze cellen beschikken over de GR en zijn daarom gevoelig voor glucocorticoïden. Bovendien, in tegenstelling tot de meeste hersencellen, is dit celtype in staat zich te delen. Hierbij ontstaan nieuwe functionele neuronen die onderdeel worden van het neuronale circuit in de gyrus dentatus. Dit proces, neurogenese genoemd, duurt ongeveer een maand en bestaat uit verschillende ontwikkelingsfasen: celdeling, -selectie, -migratie, -differentiatie, -rijping en de daadwerkelijke functionele integratie van het volwassen neuron in het circuit. Er wordt daarom aangenomen dat deze nieuwe neuronen bijdragen aan de functie van de gyrus dentatus in het geheugen voor de context.

Deze achtergrond heeft geleid tot de **hypothese** die in dit proefschrift onderzocht is: **het glucocorticoïdhormoon beïnvloedt via GR in neuronale progenitorcellen de functie van de gyrus dentatus door modulatie van neurogenese.** Omdat het precieze mechanisme nog onduidelijk is, was het onderzoek naar de rol die GR speelt in neurogenese (Hoofdstuk 4) en gedrag (Hoofdstuk 5) de voornaamste doelstelling van mijn proefschrift. Voorheen was het technisch niet mogelijk om de functie van een gen (zoals de GR) te onderzoeken in verschillende celtypen in de muis. Omdat de GR tot expressie komt in verschillende celtypen en waarschijnlijk verschillende functies in deze cellen heeft, is het voor deze studie essentieel dat de GR alleen in neuronale progenitorcellen onderzocht wordt. Om deze reden was de tweede doelstelling van mijn

proefschrift het ontwikkelen van een nieuwe techniek (RNA-interferentie) om de expressie van de GR in neuronale progenitorcellen van muizen te manipuleren. Om RNA-interferentiemoleculen in de neuronale progenitorcellen te brengen zijn lentivirussen gebruikt als vector (Hoofdstuk 2 en 3). De lentivirussen zijn met behulp van een stereotact in de gyrus dentatus van muizen geïnjecteerd. Dit muismodel is het shGR-muismodel genoemd.

In Hoofdstuk 2 is de ontwikkeling en de effectiviteit van de shRNA-constructen beschreven. Om een goede downregulatie (verlaging) van GR-expressie te verkrijgen, zijn in eerste instantie vier verschillende constructen ontworpen. Deze constructen zijn vervolgens in een plasmidvector ingebouwd en getest in een neuronale cellijn. Met Western Blot zijn de GR-concentraties bepaald bij het toepassen van de verschillende constructen en specifieke controles. Alle vier constructen gaven enige downregulatie van de GR. Construct “pm-GR3” had de hoogste effectiviteit met een verlaging van meer dan 90% van de GR-expressie. Dit construct werd dan ook geselecteerd en ingebouwd in de lentivirale vector. Opnieuw werd de effectiviteit getest in de neuronale cellijn. Het lentivirus met het pm-GR3-construct gaf meer dan 70% downregulatie van GR. Daarom werd deze combinatie gebruikt in het vervolgonderzoek om de GR te verlagen in de neuronen van de muis.

In Hoofdstuk 3 werd eerst gekeken naar de soort celtypes in de gyrus dentatus die het lentivirus infecteert. Een week na stereotactische injecties in de gyrus dentatus van de muis werden de cellen gelabeld door het lentivirus, bestudeerd. In tegenstelling tot neuronen van een andere hippocampale structuur, bleek er in de gyrus dentatus een verschil te zijn tussen verschillende cellagen. Sommige cellagen werden geïnfecteerd, andere niet. Nadere bestudering van deze cellagen en hun cellen gaf aan dat het lentivirus vooral neuronale progenitorcellen en onvolwassen neuronen infecteert. Deze celpopulatie, bestaande uit neuronale progenitorcellen en nieuwgeboren neuronen in verschillende ontwikkelingsstadia, werd verder aangeduid als NPC. Het lentivirus infecteert geen volwassen neuronen in de gyrus dentatus. De mogelijke verklaringen voor deze eigenschap van het lentivirus werden geëvalueerd. Er werd bovendien geconcludeerd dat dit lentivirus erg geschikt is als vector om RNA-interferentie over te brengen in NPC’s. Door deze eigenschap kunnen lentivirussen goed gebruikt worden in onderzoek naar de functie van genen in NPC’s.

In Hoofdstuk 4 werd deze lentivirus-RNA-interferentie-aanpak succesvol toegepast. Er werd onderzocht wat de effecten van GR-downregulatie in NPC’s zijn op neurogenese. Een week van GR-downregulatie in NPC’s resulteerde al in een versnelde neuronale differentiatie. Bovendien bleek GR-downregulatie een effect te hebben op de morfologie van de NPC’s. Dit kwam onder meer tot uiting in een veranderde structuur van de dendrieten-“boom”, een toegenomen aantal dendritische spines met een volwassen fenotype, en een vergrote doorsnede van zogenaamde mossy fiber boutons. Opvallend was dat ook de positie van de NPC’s met GR-downregulatie in de granulaire cellaag verschilde van de controlemuizen. Dit duidt op een foutieve migratie. De

effecten van GR-downregulatie waren in overeenkomst 1 en 5 weken na injectie. De morfologische resultaten wekken de indruk dat de synaptische contacten - de manier waarop neuronen contact leggen met andere neuronen in het neuronale circuit - toegenomen zijn. In overeenstemming met deze bevindingen, vonden we ook een toegenomen frequentie van mEPSC's wat wijst op een verhoogde excitabiliteit. Op basis van deze resultaten, samen met de veranderde migratie suggereerden we dat de GR-expressie mogelijk een cruciale rol speelt in de ontwikkeling van nieuwgeboren neuronen en de correcte integratie van deze neuronen in het neuronale circuit van de hippocampus. Ook werden de moleculaire mechanismen bediscussieerd die mogelijk ten grondslag liggen aan deze functie van GR.

In Hoofdstuk 5 is vervolgens angstgemotiveerd gedrag van de shGR-muis onderzocht. Hiertoe werd onderzocht hoe GR's in NPC's betrokken zijn bij het geheugen voor een stressvolle en angstige gebeurtenis. ShGR-muizen en controlemuizen werden vier weken na injectie met het lentivirus (waarbij werd verondersteld dat de door het lentivirus geïnfecteerde NPC's inmiddels volledig ontwikkeld waren) getest in een Pavloviaanse taak die "context and cue fear conditioning" heet. Tijdens context and cue fear conditioning is het mogelijk het angstgeheugen van de muizen te meten door herhaaldelijk de gedragsrespons op de omgeving (context) en op de geconditioneerde stimulus te meten. Angstgedrag bij muizen wordt gekenmerkt door immobiliteit en kan zowel door "freeze"- als door "scan"-gedrag tot expressie gebracht worden. Uit de mate van het angstgemotiveerd gedrag enkele dagen later kan een indruk worden verkregen van het verloop van de leer- en geheugenprocessen. Uit de resultaten bleek dat glucocorticoïdwerking via GR in NPC's belangrijk is voor de bevordering van de consolidatiefase van het geheugen. shGR-muizen leken een minder goed geheugen te hebben voor de angstige gebeurtenis.

In Hoofdstuk 6 werd de mogelijke betekenis van de resultaten uit de verschillende experimenten bediscussieerd. **De resultaten lijken de hypothese te ondersteunen dat de GR in neuronale progenitorcellen de functie van de gyrus dentatus beïnvloedt door modulatie van neurogenese. Bovendien ondersteunen de resultaten de validiteit van dit nieuw ontwikkelde shGR-diermodel voor verder onderzoek naar glucocorticoïden en neurogenese in aanpassing aan stress.**

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CURRICULUM VITAE/ PUBLICATIONS

CURRICULUM VITAE

Lenneke van Hooijdonk is geboren op 24 april 1980 te Dordrecht. Zij behaalde haar HAVO diploma in 1997 en haar VWO diploma in 1999, beide aan het Katholieke Scholengemeenschap te Etten-Leur. Vervolgens begon zij in 1999 met de studie Biologie aan de Universiteit Leiden. Het propeadeuse jaar behaalde zij cum laude. Tijdens de doctorale fase volgde ze de richting Organismale Zoölogie, aangevuld met de theoretische vakken van de richting Medische Biologie. Als onderdeel van deze opleiding doorliep zij twee stages: aan de afdeling Medische Farmacologie van het LACDR in de groep van Prof. Dr. E.R. de Kloet, en aan de afdeling Psicobiologia e Psicofarmacologia, IRCCS Santa Lucia/ Università degli studi di Roma, “La Sapienza”, onder leiding van Dr. M. Ammassari-Teule. Aan het einde van haar studie was zij enkele maanden student-assistent bij het zoölogische practicum van Dr. H. Berkhoudt. Het doctoraal diploma behaalde zij in 2004 cum laude. Aansluitend volgde het promotie onderzoek aan de afdeling Medische Farmacologie van het LACDR in de groep van Prof. Dr. E.R. De Kloet, onder zijn begeleiding en die van Dr. E. Vreugdenhil. De resultaten zijn beschreven in dit proefschrift. Sinds maart 2009 is zij werkzaam als Programmaleider Onderzoek bij Hersenstichting Nederland.

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