Glucocorticoid receptor knockdown and adult hippocampal neurogenesis

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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].

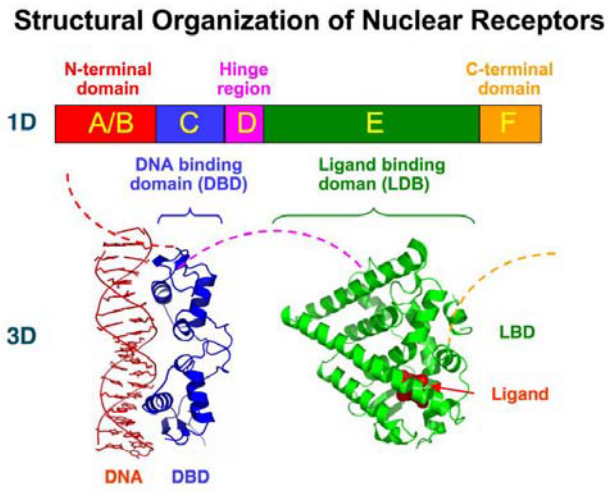

Figure 1.1 Structural organization of nuclear receptors like the GR. Top *– Schematic 1D amino acid sequence of a nuclear receptor.* ***Bottom*** *– 3D structures of the DBD (bound to DNA) and LBD (bound to hormone) regions of the nuclear receptor.*

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].

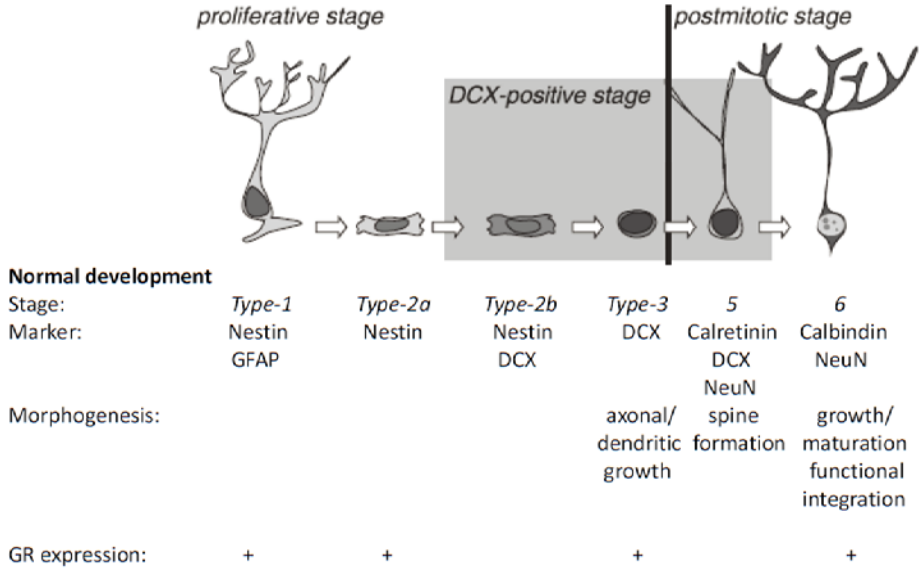

Figure 1.2 Proposed development of newborn neurons in the dentate gyrus. *Six stages of neuronal development in the adult hippocampus can be readily identified on the basis of morphology, proliferative ability, and expression of markers such as nestin, GFAP, DCX, calretinin, calbindin and NeuN. Development 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).

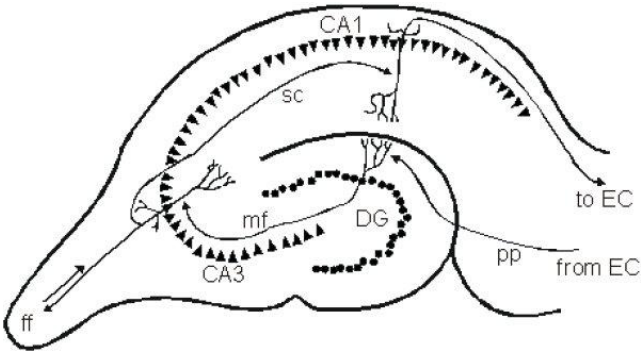

Figure 1.3 Hippocampal neuroanatomy. *Orientation of the dentate gyrus (black dots) and cornu ammonis (black triangles) and their connections with the trisynaptic circuit. Abbreviations: CA1-3 = cornu ammonis 1-3; DG = dentate gyrus; EC = entorhinal cortex; pp = perforant pathway; mf = mossy fibers; 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.

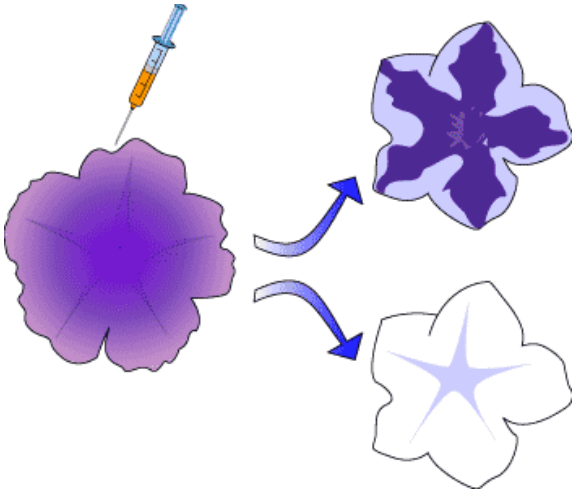

Figure 1.5 Discovery of RNAi phenomenon in purple petunias. *A variegated petunia, upon injection of the gene responsible for the purple colouring in petunias, the flowers became variegated or white rather than deeper purple as was expected.*

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].

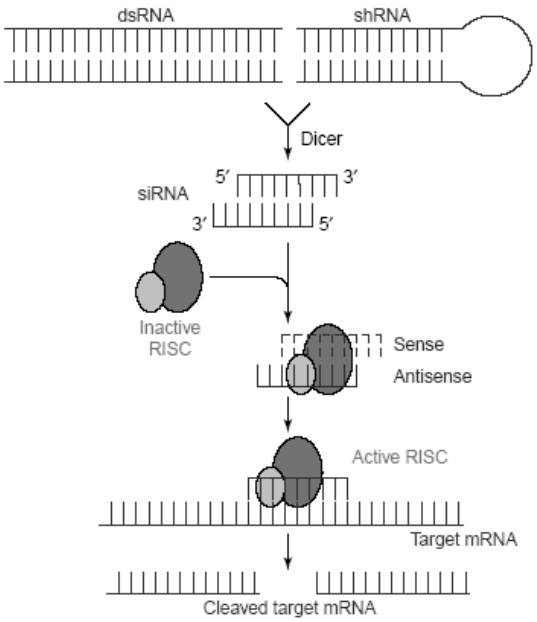

Figure 1.6. Mechanism of RNA-interference. *Upon entering a cell, the double-stranded RNA molecules that trigger RNAi are cut into small fragments by the RNAse called Dicer. The small fragments then serve as guides, leading the cell's RNAi machinery to mRNAs that match the genetic sequence of the fragments. 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].

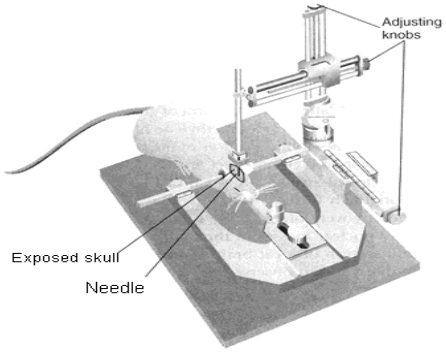

Figure 1.7 Stereotactic delivery in the mouse brain. *Using a stereotact, precise and localized injections can be placed in the brain. Using the frame, the injection needle can be placed at the specified coordinates with a precision of 0.05 mm.*

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.