Glucocorticoid receptor knockdown and adult hippocampal neurogenesis

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6

GENERAL DISCUSSION

OVERVIEW

6.0 INTRODUCTION TO GENERAL DISCUSSION

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

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

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

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

6.1 LOCAL APPLICATION OF LV-RNAI-MEDIATED GR KNOCKDOWN

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

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

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

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

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

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

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

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

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

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

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

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

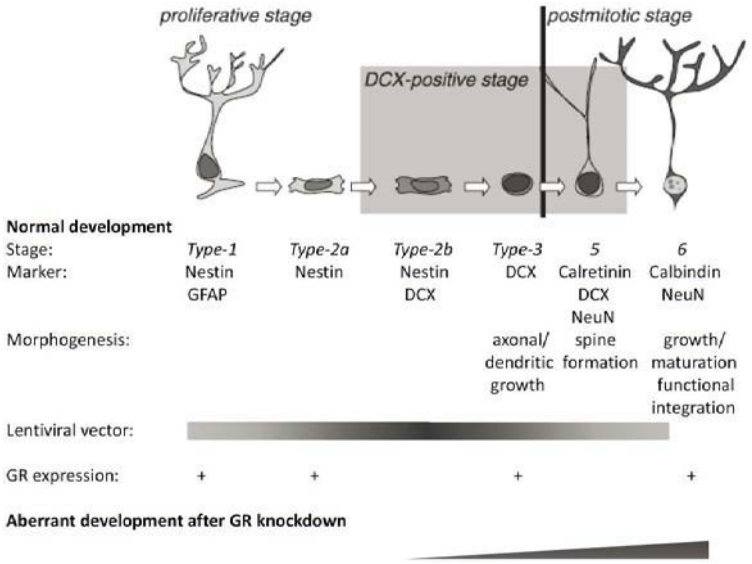

Figure 6.1 Proposed development of newborn neurons in the dentate gyrus (see also CHAPTER 1). *Six stages of neuronal development in the adult hippocampus can be readily identified on the basis of morphology, proliferative ability, and expression of markers such as nestin, GFAP, DCX, calretinin, calbindin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

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

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

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

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

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

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

6.2 GR MAY CONTRIBUTE TO HIPPOCAMPAL PLASTICITY BY MODULATING NEUROGENESIS

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

6.2.1 Are neurogenic alterations a substrate for cognitive processes?

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

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

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

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

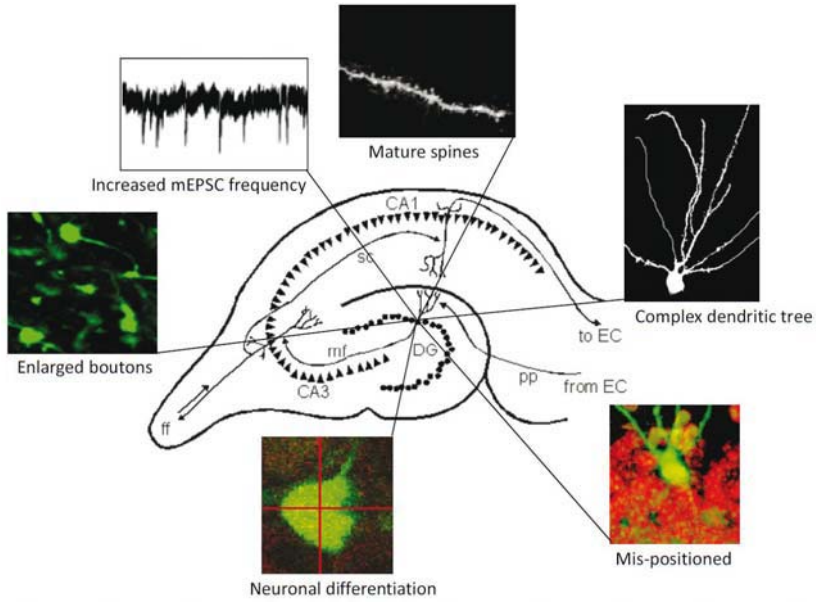

Fig 6.2 GR knockdown- mediated alterations in NPCs of the dentate gyrus (see also CHAPTER 1).
Summary of the main cellular findings in NPCs. Orientation of the dentate gyrus (black dots) and cornu ammonis (black triangles) and their connections with the trisynaptic circuit. Abbreviations: CA1-3 = cornu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

6.2.2 Are GRs modulating cognitive performance by regulating neurogenesis?

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

6.4 PERSPECTIVES

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

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

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

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

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

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

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

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

6.5 CONCLUSIONS

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

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

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

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