Glucocorticoid receptor knockdown and adult hippocampal neurogenesis

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

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ABSTRACT

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

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Animals

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

Small interference (siRNA) constructs

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

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

Lentiviral vectors

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

Stereotactic surgery

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

Fear conditioning apparatus

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

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

Fear conditioning procedure

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

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

Corticosterone radio immune assay

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

Behavioural assessment

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

Statistical analysis

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

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

RESULTS

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

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

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

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

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

GR knockdown in dentate granule neurons does not affect plasma corticosterone

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

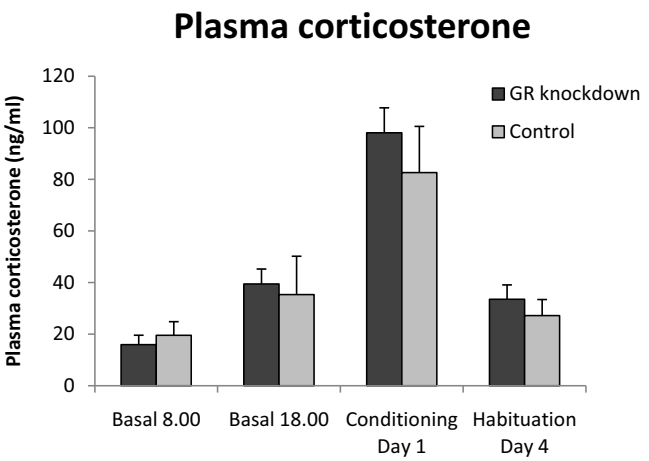

Figure 5.1. Plasma corticosterone concentrations in GR knockdown and control at four time points (mean ± SEM). *Basal morning and evening blood samples were taken two days before the start of fear conditioning. A third blood sample was taken 30 min after the start of conditioning on Day 1 and the fourth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

Freezing during context and cue episodes per testing day

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

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

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

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

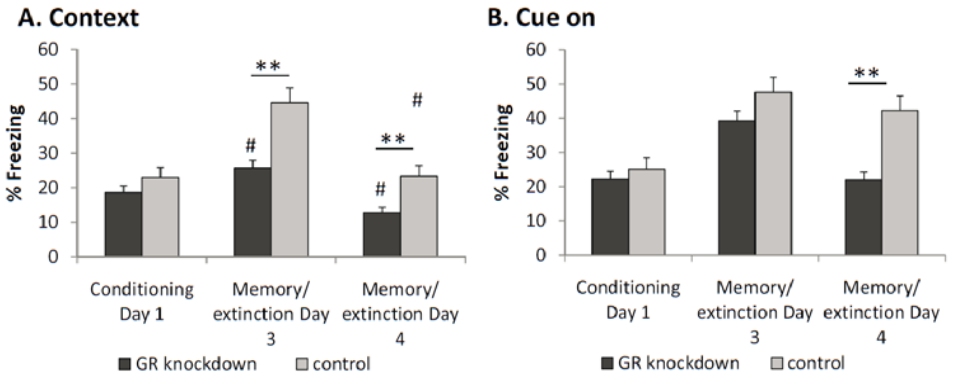

Figure 5.2. Percentage of freezing during context (A) and cue (B) episodes per testing day (mean ± SEM). *(A) freezing to context. B) freezing to cue on. Dark bars: GR knockdown, light bars: control. Between groups ** p< 0.011 GR knockdown compared to control. Within groups # p< 0.01 context freezing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

Progression of freezing behaviour over alternating context and cue episodes

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

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

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

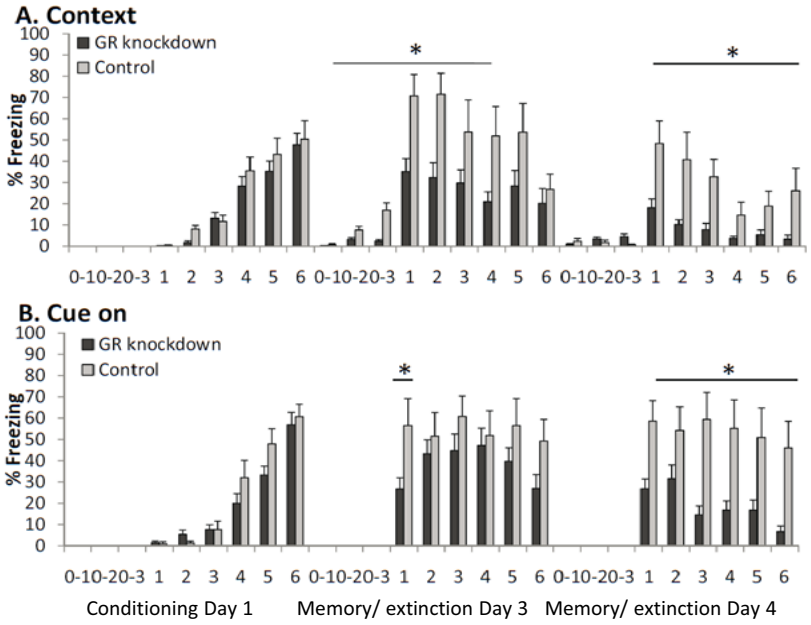

Figure 5.3. Percentage of freezing to context (A) and cue on (B) over the alternating episodes per testing day (mean ± SEM)*. From episode 0-3 onwards paired context and cue on episodes are separated in this graph for better distinction of the freezing patterns between the two groups. Dark bars: GR knockdown;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

Scanning: a more active fear behaviour

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

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

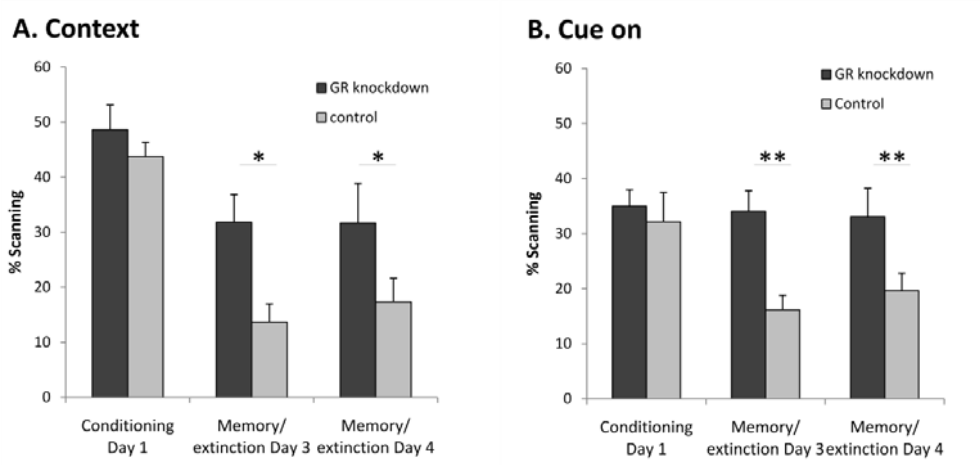

Figure 5.4. Percentage of scanning during context (A) and cue (B) episodes per testing day (mean ± SEM). *Dark bars: GR knockdown, light bars: control. Between groups: * p<0.05 and ** p<0.005 compared to control. Within groups: not significant.*

Factor analysis

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

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

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

DISCUSSION

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

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

GR knockdown in NPCs is not involved in appraisal or acquisition

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Destabilization of memory consolidation in Balb/c mice is adaptive

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

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

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

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

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

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

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

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

Conclusions

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