Epigenetic control of hippocampal stem cells: modulation by hyperactivation, glucocorticoids and aging

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Summary and general discussion

1. Aims of this thesis

Adult hippocampal neurogenesis (AHN), i.e. the birth of new neurons from stem cells in the adult hippocampus, is now a widely accepted phenomenon[1,2]. Yet, the characterization of its regulation by cell extrinsic signals as well as cell intrinsic mechanisms remains incomplete[3-5] (see **chapter 1** and **2**). Extrinsic factors like kainic acid (KA), glucocorticoids (CORT) or the process of aging, condition the neurogenic outcome primarily through modulating the initial number, proliferation and apoptosis of the neural precursor cells[3] (see **chapter 1** and **2**). Adult neural precursor cell (NPC) numbers are determined by levels of quiescence, proliferation, selection by apoptosis and eventual neuronal differentiation into new functional neurons[6,7] (Figure 1).

The main objective of this thesis was to identify the relatively unexplored (epigenetic) molecular mechanisms in NPCs in response to these three aforementioned factors. To address this main objective, I focused on the following subquestions in the different chapters of this thesis:

In **chapter 1** we reviewed the literature on age-related epigenetic mechanisms that may underlie the regulation of proliferation, differentiation and synaptic integration of adult-generated hippocampal neurons. We focused on small non-coding RNAs and hypothesized that an interplay exits between the age-dependent alterations in basal levels of circulating steroid hormones and small non-coding RNAs in neurogenic cells.

In **chapter 2**, we discuss stress-associated changes in neuronal plasticity and their respective epigenetic mediators, focusing on differences between the effects of acute and chronic stress. We hypothesize that lasting consequences of stress require a carefully coordinated (epigenetic) molecular control, such as those attributed by (cooperative) microRNA action(s). Furthermore we describe future avenues for studying this cooperative microRNA action.

In **chapter 3**, we characterized the molecular (epigenetic) mechanisms that contribute to some of the mitochondrial functions that are relevant for apoptosis. We studied this shortly after kainic acid administration, a condition that induces neuronal hyperactivation in the hippocampal dentate gyrus where NPCs reside.

In **chapter 4** we developed a working protocol to analyze dendritic spines at a super-resolution level. The application of structured illumination microscopy (SIM) allowed us to obtain a more detailed representation of the subtle morphological changes of dendritic spines, exceeding the performace of regular confocal microscopy.

In **chapter 5**, we studied how differences in basal glucocorticoid level and rhythmicity affect NPC proliferation, glucocorticoid responsiveness and promoter methylation status. Our hypothesis was that basal pulsatile CORT rhythms control the epigenetic state of gene promoters involved in the cell cycle, whereas e.g. a prolonged elevation in CORT exposure attenuates these gene promoters, thereby affecting the stem cell response to subsequent CORT challenges.

In **chapter 6** we characterized GR expression levels in NPCs and its association with an age-related decline in specific NPC populations. Our hypothesis was that NPC populations expressing GR would have slower decay kinetics than those lacking the GR.

2. Summary

"Epigenetic control of hippocampal stem cells"
Adult tissues preserve characteristic populations of self-renewing cells, which can give rise to various specialized cell types, and the brain is not an exception to this rule. The identification of NPCs present in several areas of the adult brain has challenged conservative ideas regarding the regenerative capacity of the brain, creating a research field dedicated to unraveling the mechanisms of adult NPC self-renewal and differentiation. Research over the past 50 years[1,2] has revealed that NPC can give rise to different types of brain cells: neurons, astrocytes and oligodendrocytes and recent observations have demonstrated that molecular (epigenetic) mechanisms play a central role in the regulation of NPC self-renewal and differentiation under physiological and pathological conditions[3,4].

The neural hyperactivity induced by KA administration strongly affects AHN, stimulating NPC proliferation, altering neuronal/glial differentiation and change apoptosis rates in the hippocampus, eventually depleting the pool of resident NPCs[8-12] (Figure 2). Although there are alleviating effects described of epigenetic modulatory drugs on KA-induced AHN, it has thus

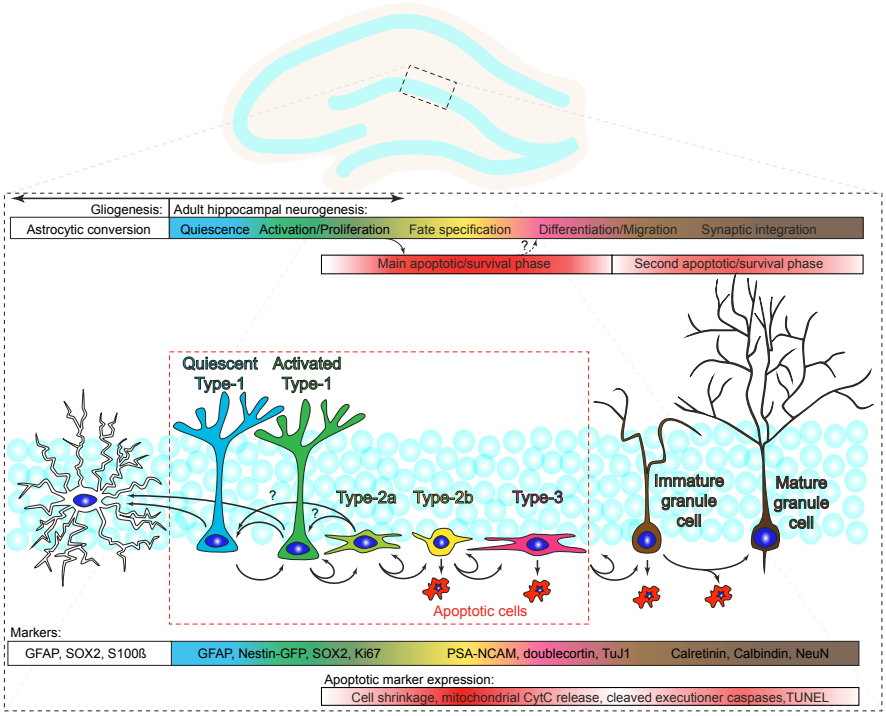

Figure 1 - Schematic representation of the adult hippocampus, showing the neurogenic cascade with critical phases and cell type-specific marker expression.
The red dashed boxed area highlights the cell-types most studied in this thesis. Arrows toward other cell-types indicate possible transition between cell types originating from type-1 NPC and arrows toward the same cell indicate self-renewal potential.

far remained largely unclear which epigenetic alterations occur in NPCs following seizures[9]. This is of relevance given the hyperexcitable properties of specifically this population in the dentate gyrus, and their strategic location within the trisynaptic hippocampal network. Here, we focused on preceding events that could possibly contribute to, or prepare for the hyperexcitable nature of the newborn neurons, and studied microRNA-mediated epigenetic control of apoptosis, a relatively unexplored phenotype in terms of microRNA-mediated control (**chapter 1**). In **chapter 2** we discuss a concept for studying synergistic or cooperative action of multiple microRNAs, and applied an improved version of this method in **chapter 3**. We particularly focused on coordinated microRNA action through microRNA cooperativity, and found KA treatment to induce a coordinated microRNA profile response in the dentate gyrus (**chapter 3**), which modulates apoptotic effector genes like BCL2L13 and caspase-3 in NPCs, thereby favoring differentiation over apoptosis. Overexpression of miR-124 further induced increases in differentiation, highlighting the involvement of microRNA-mediated epigenetic regulation of NPC differentiation (Figure 3).

Some of the results obtained in **chapter 3** required magnifications with a significantly higher resolution than conventional confocal microscopy. By applying SIM we were able to detect individual mitochondria and record their KA-induced release of cytochrome-C, which could be attenuated by BCL2L13 levels.

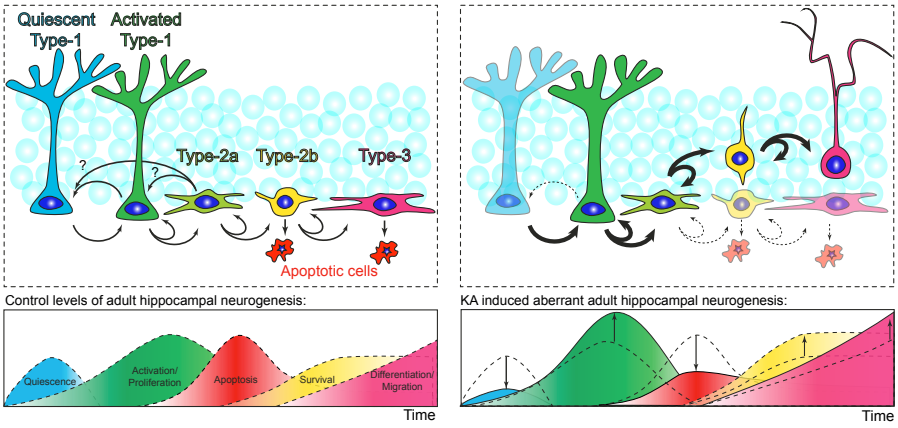

Figure 2 - Schematic representation of the initial stages of the neurogenic cascade in control animals (left) or after KA-induced alterations (right).
Note that KA induces thickening of the primary processes[12] of the type-1 cells and ectopic localization and accelerated differentiation of the type-2 cells (**chapter 3**). A lighter color shade indicates a lower abudance of this cell-type. Arrows toward other cell-types indicate possible transitions between cells of the neurogenic progeny originating from Type-1 cells and arrows toward the same cell indicate self-renewal potential. Thicker arrows indicate induction and dashed ones inhibition of cell transition/proliferation.

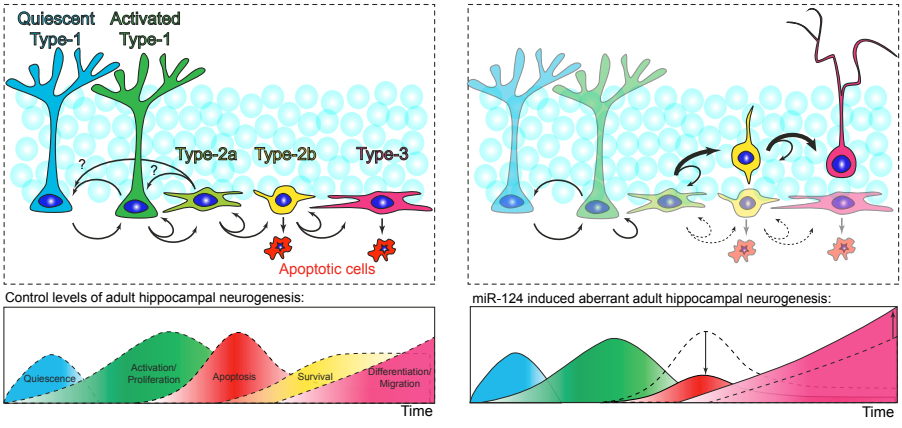

Figure 3 - Schematic representation of the initial stages of the neurogenic cascade in control animals (left) or after miR-124 induced alterations (right).
miR-124 induces ectopic localization, reduced apoptosis and morphological alterations of type-2 cells and DCX+ immature neurons (**chapter 3**). A lighter color shade indicates a lower abudance of this cell-type. Arrows toward other cell-types indicate possible transitions between cells of the neurogenic progeny originating from Type-1 cells and arrows toward the same cell indicate self-renewal potential. Thicker arrows indicate induction and dashed ones inhibition of cell transition/proliferation.

Along these lines, measuring the physical dimensions of spines with confocal microscopy can also be challenging. Accordingly, in **chapter 4** we present a working protocol for the detection of dendritic spines using SIM and found a significantly higher resolution than conventional confocal microscopy allowing for better measurements of e.g. head and neck diameter of the spines.

The results presented Fitzsimons *et al.*[13] point towards the GR regulating differentiation (Figure 4). Although GR activation induces alterations in DNA methylation in NPCs[14], it remained unclear whether it is merely the levels and/or duration of NPC GR activation that triggers this response, or rather the deviation from, or absence of its basal pulsatile release pattern. We therefore focused on the relatively unexplored field of CORT rhythmicity, and addressed in particular possible programming effects through altered gene promoter methylation. Moreover, it was also unknown whether lasting GC-induced alterations in DNA methylation affect promoter methylation of genes that are specifically involved in NPC differentiation. In **chapter 5**, these questions were addressed and we found differential effects on proliferation, quiescence (Figure 5) and on alterations in promoter methylation. Deviations from the basal pulsatile pattern of GC exposure lastingly induced hyper- or hypomethylation of a significant number of promoters, some of which were involved in controlling differentiation, and in a functional network controlling Wnt signaling through dickkopf factors.

Previously reported observations indicate that the Wnt signaling is critically involved in NPC maintanance vs. differentiation decisions[15]. When we studied the expression of the Wnt target gene CCND1, we indeed found changes suggestive of an altered Wnt signaling pathway in the progeny of NPCs that had been initially exposed to CORT (**chapter 6**). Since these results were accompanied by alterations in proliferation, they suggested a CORT rhythm-dependent modulation of the Wnt pathway that is carried on in NPC progeny, and thus reflects a novel epigenetic mechanism.

Since GR-knockdown in proliferative NPCs accelerated their subsequent differentiation[13], and since the majority of NPCs that remain present in the aged DG express GR[16] (**chapter 6**), we asked what the age of onset of this effect is and whether the presence, or absence, of GRs on NPC populations could predict the decay kinetics with age of the NPC subpopulations. The strongest differences in decay kinetics were found between 3 and 6 months, for the type-2b NPC populations with a predicted "protective" involvement of the GR, as well as a striking persistence with age of the type-1 quiescent NPCs that express GR up to 10 months of age (Figure 6). Since decay kinetics can be influenced by levels of quiescence, proliferation, apoptosis and differentiation, these results might be explained by findings from a.o. Fitzsimons *et al.*[13] and **chapter 6**, with the GR controlling NPC quiescence, proliferation, apoptosis and differentiation[17].

3. Cell extrinsic and subsequent intrinsic regulation of AHN levels: novel concepts.

The identification of novel mechanisms that control the phenotype of adult NPCs is closely linked to some new concepts emerging in molecular stem cell biology. Novel techniques that allow a higher accuracy or resolution help to obtain a more detailed representation of the biological system studied and were thus used throughout this thesis. Importantly, the responsivity of NPCs to both glucocorticoid exposure and hyperactivation can, at least in theory, be either directly mediated through the expression of the respective receptors and/or indirectly through a transmitted intracellular response, or one mediated via the neurogenic niche. I have applied a number of strategies to study particularly the direct effects. The isolation of NPCs and subsequently exposing them to specific receptor a- or anta-gonists provides an appealing *in vitro* model for studying their direct effects. These technical and other experimental approaches were used to dissect and study the following novel concepts in AHN.

3.1 Non-apoptotic functions of "pro-apoptotic" proteins.

In **chapter 3** and by Fitzsimons *et al.*[13], is shown that mitochondria-related proteins like active/cleaved caspase-3, can also be detected during the (initiation of) NPC differentiation under specific conditions. This is interesting since active caspase-3 is classically considered a pro-apoptosis

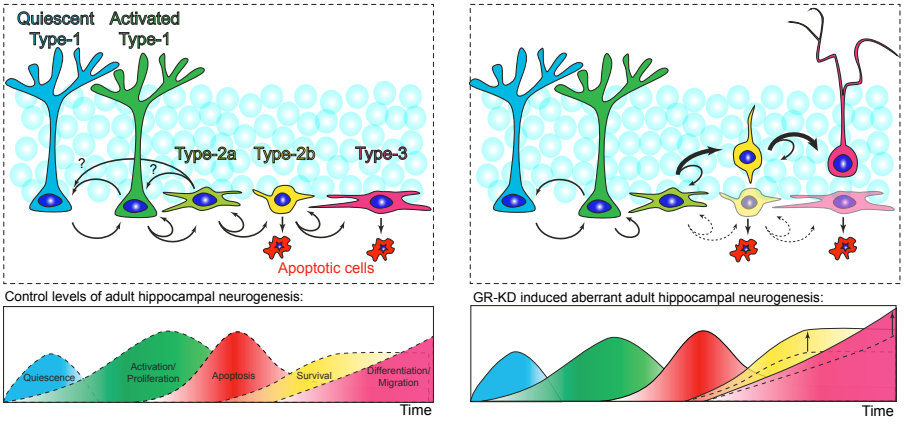

Figure 4 - Schematic representation of the initial stages of the neurogenic cascade in control animals (left) or after GR-KD induced alterations (right).
As shown by Fitzsimons *et al.*[13], GR-KD induces ectopic localization and accelerated differentiation of newborn cells. A lighter color shade indicates a lower abudance of this cell-type. Arrows toward other cell-types indicate possible transitions between cells of the neurogenic progeny originating from Type-1 cells and arrows toward the same cell indicate self-renewal potential. Thicker arrows indicate induction and dashed ones inhibition of cell transition/proliferation.

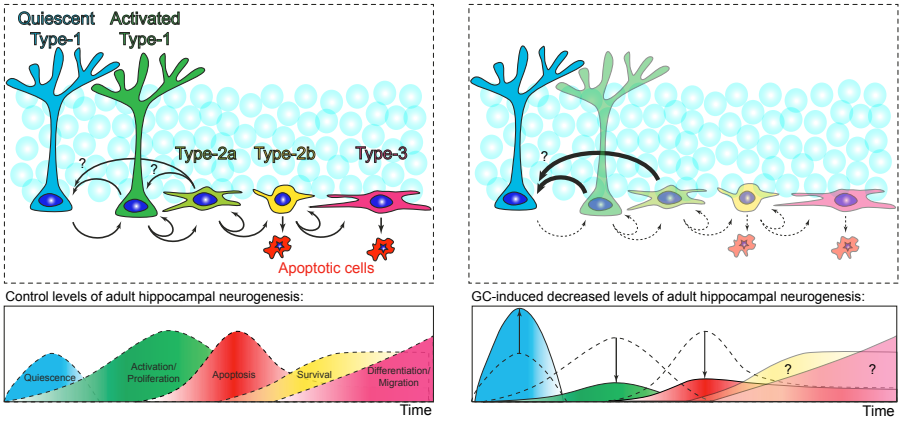

Figure 5 - Schematic representation of the initial stages of the neurogenic cascade in control animals (left) or after GC-induced alterations (right).
GCs induce lower levels of NPC proliferation and increased quiescence (**chapter 5**). A lighter color shade indicates a lower abudance of this cell-type. Arrows toward other cell-types indicate possible transitions between cells of the neurogenic progeny originating from Type-1 cells and arrows toward the same cell indicate self-renewal potential. Thicker arrows indicate induction and dashed ones inhibition of cell transition/proliferation.

marker[18,19], caspase-3 activity can be modulated by cell extrinsic factors like KA-induced hyperactivation (**chapter 3**) and glucocorticoids[13]. Expression of activated caspases also occurs in non-apoptotic neurons[20-22] and even exerts important functions in synaptic plasticity[23] and in learning and memory processes[24], indicating their functions are not exclusively restricted to apoptosis. In fact, NPC differentiation even depends on "pro-apoptotic" cleaved-caspase-3 protein[25]. Time-wise, the main period of apoptosis within AHN seems to coincide with the initiation of differentiation[26], suggesting a critical time window during which active caspase-3-related cell-fate selection can result in either apoptosis or differentiation. Some of the data described in **chapter 3** suggests that both caspase-3 activity dependent differentiation and apoptosis are coupled spatio-temporally, with levels of active caspase-3 determining either cellular phenotype. Indeed, previous literature has proposed a mechanistic link between caspase-3 dependent apoptosis and differentiation[27]. Accordingly, the physiological relevance of fine-tuning of pro-apoptotic proteins upstream of caspase-3 such as BCL2L13 and cytochrome-C (**chapter 3**) and the GR[13] converge not only on apoptosis but also on differentiation and as such need to be interpreted carefully.

While we have identified a mechanism partially responsible for the KA-induced modulation of caspase-3 activity (**chapter 3**), it remained unclear how glucocorticoids modulate caspase-3 activity in NPCs[13]. Regarding the stress-associated seizure hypothesis[28,29], the seemingly opposing effects of KA-induced hyperactivation and glucocorticoid-induced GR activation on NPC caspase-3 activity, may provide an interesting mechanistic link for future research. Such experiments might show possible neuroprotection by GR-activation during KA-induced seizures, through modulation of caspase-3 activity levels.

As a first hint for a link between glucocorticoid signaling and hyperactivation, Fitzsimons *et al.* found that the newborn neurons in which GR expression is knocked down[13], display a similar morphological phenotype as NPCs that are hyperactivated by seizures (**chapter 3**). This includes an accelerated differentiation and migration into the granular cell layer, and an ectopic location[30,31], with the former phenotype linked to caspase-3 activity levels. Also, Fitzsimons *et al.* found increases in the number of mushroom spines in newborn neurons with a GR knock down as seen in seizure-associated aberrant neurogenesis[13,32]. It could be suggested that KA and glucocorticoids are also able to modulate caspase-3 activity in other brain cell types besides NPCs and thus potentially their dendritic spine morphology.

3.2 microRNA cooperativity

Gene expression studies are classically performed by means of transcriptomic profiling. However, mRNA expression levels do not always translate into protein expression in a linear fashion[33,34] (**chapter 3**). Accordingly, protein level measurements are still indispensible to understand how functional changes can arise from gene expression changes. They can sometimes even reveal a microRNA-mediated post-transcriptional level of protein expression regulation[35]. Following this line of reasoning, the collective transcriptomic, proteomic and microRNAomic approach we applied in **chapter 3** allowed for an integrated view of the changes induced by KA, thereby circumventing most of the aforementioned issues regarding gene expression analysis at the mRNA level. In addition, such an integrative approach permits the identification of the potential cooperative action, e.g. of multiple microRNAs, when combined with microRNA-mRNA binding prediction algorithms, as reviewed in **chapter 2** and applied in **chapter 3**.

Most studies aimed to understand the role of microRNAs in the regulation of gene expression focus on one individual microRNA and its regulatory effects on one or multiple target mRNA(s). However, most physiological and pathological situations are characterized by coordinated microRNA changes, known as microRNA "signatures". How the action of multiple microRNAs on target regulation is exactly coordinated remains poorly characterized. Recent advances in the understanding of microRNA biology describe e.g. how the coordinated action of microRNA cooperativity (**chapter 2** and **3**) can contribute to target regulation. Along these lines, studies performed by Silber *et al.*[36] and Grimson *et al.*[37] corroborate our findings of cooperative, and synergistic actions of multiple microRNAs on one target. In addition to this coordinated regulation of a

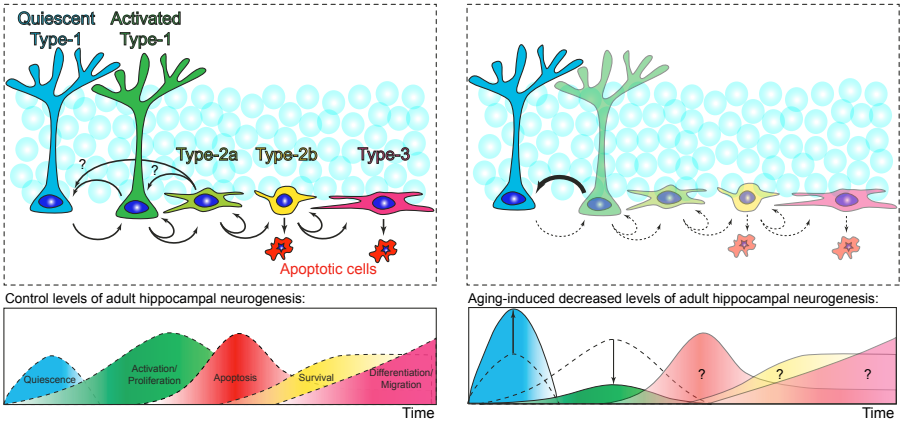

Figure 6 - Schematic representation of the initial stages of the neurogenic cascade in control animals (left) or after aging-induced alterations (right).
Quiescent NPC are overrepresented among the cell-types persisting in the middle aged hippocampus (**chapter 6**). A lighter color shade indicates a lower abudance of this cell-type. Arrows toward other cell-types indicate possible transitions between cells of the neurogenic progeny originating from Type-1 cells and arrows toward the same cell indicate self-renewal potential. Thicker arrows indicate induction and dashed ones inhibition of cell transition/proliferation.

single target mRNA by multiple microRNAs, (multiple) microRNAs also converge on multiple targets and can thereby coordinate cellular functions (Figure 7). Indeed, a recent meta-analysis suggests that multiple higher levels of coordinated microRNA regulation might orchestrate the regulation of complex biological processes, such as e.g. neurogenesis[38]. These data indicate that we are only beginning to appreciate the full complexity of microRNA biology.

3.3 Applying super resolution imaging to study single cell sub-cellular compartments.
Small morphological changes in cellular compartments or organelles can already contribute crucially to the phenotype of a cell. Examples are mitochondria and dendritic spines that are implicated in cellular functions of energy metabolism and synaptic plasticity, respectively, and where deficits in e.g. their structural elements already cause severe cellular dysfunction and related pathologies. We here applied structured illumination microscopy (SIM)[39,40] to to obtain high resolution measurement of dendritic spines on neurons in culture and found that this technique could even identify subtle changes in both the mitochondrial outer membrane and dendritic spine morphology. SIM was applied in **chapter 3** to identify small changes in cytochrome-c localization within or outside mitochondria and in **chapter 5**, we applied SIM to the identification of subtle alterations in dendritic spine morphology. Because of the increase in resolution, the application of super-resolution imaging allows to obtain important information from unknown levels of detail from known biological specimen[41]. One interesting example of the power of applying super resolution imaging for high-content analysis is single cell DNA methylation and hydroxy-methylation shifts during stem cell differentiaton[42]. Although the various develomental stages of NPCs during AHN were generally assumed to reflect rather homogeneous cell subpopulations[43], current studies indicate such cell types are actually more “unique” in terms of their genomic variation[44,45]. This individual genomic variation of newborn cells may occur through e.g. mobile DNA elements. Such retrotransposons can be modulated by cell extrinsic factors and physiologically contribute to neuronal plasticity[46]. Super-resolution imaging techniques combined with clonal lineage tracing to study subcellular compartments such as spines of individual cells might provide a further and more functional readout for the variation present among newborn cells that are derived from the same NPC.

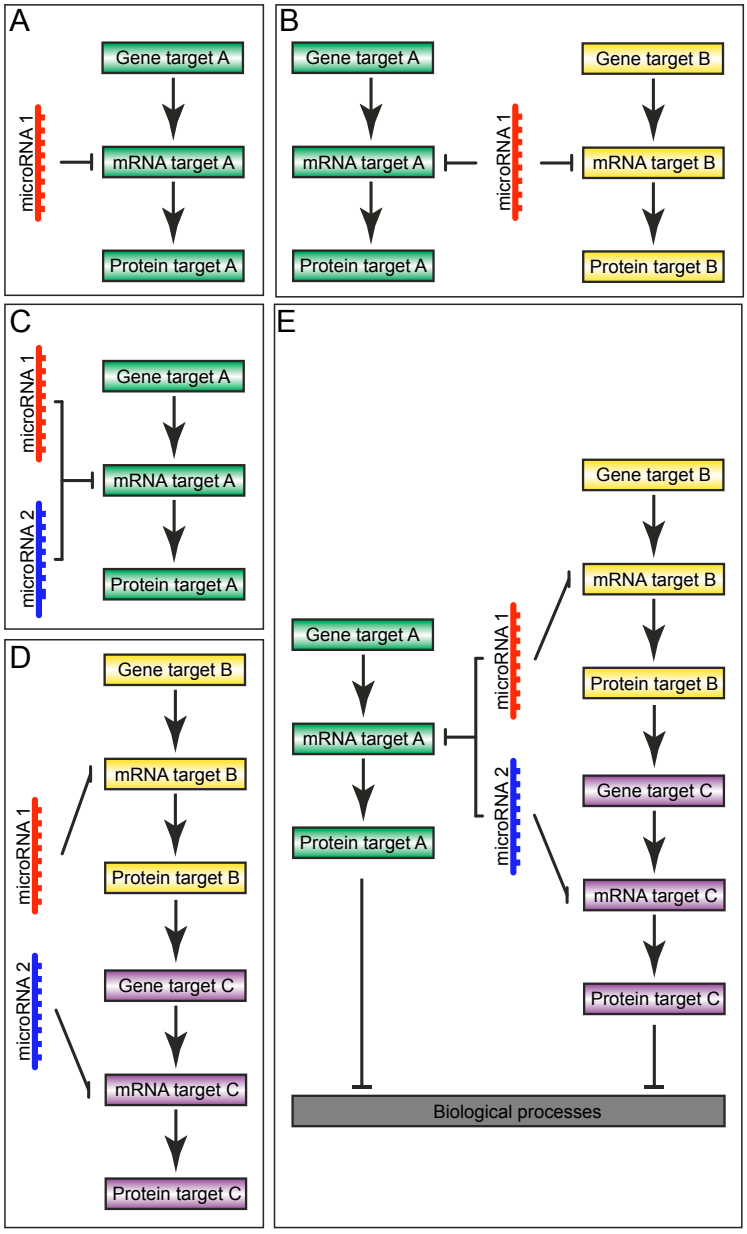

Figure 7 - Schematic representation of coordinated microRNA action at different levels.
(A) Conventionally, individual microRNA are studied by their action on single mRNA targets. (B) Yet, each individual microRNA can have multiple predicted targets and under most characterized physiological and pathological circumstances changes in microRNA expression take place in groups, so called "microRNA signatures". The coordinated action of multiple microRNAs can be achieved through (C) cooperative action on a single target, (D) convergence on a pathway or (E) on an entire biological processes. Adapted from Barca-Mayo *et al.*[38]

3.4 Rhythmicity of "stress" hormones.
Most organisms adjust their physiology to anticipate daily environmental fluctuations[47] mediated by the daily rotation of the earth[48], which causes a cycle with an on average 12h light and 12h dark phase. Some of the earliest chronobiological studies already describe many physiological parameters to show diurnal rhythmic changes that are found to correlate with light exposure, including locomotor activity, body temperature, urine excretion and feeding behavior[47]. Further studies have found that physiological rhythms are dictated by daytime light, one of the external cues called zeitgeber, that is being sensed by specialized cells in the retina, that subsequently project to the suprechiasmatic nucleus (SCN) that entrains the biological rhythm[49-51]. Often referred to as the central clock, the SCN has been shown to control amongst others sleep-wakefulness rhythms[52,53] and activation of the HPA axis by inducing the adrenals to release glucocorticoids in a circadian manner[54,55]. Furthermore, compelling evidence from Balsabore *et al.* demonstrated that glucocorticoids released form the adrenal gland in a cyclic pattern entrain peripheral tissues clocks through glucocorticoid receptor activation and resulting cyclic changes in gene expression[56].

In addition to evidence showing that endogenous glucocorticoids are released in a circadian manner, these powerful hormones are also released in a discrete pulsatile profile and follow an ultradian rhythm that is superimposed on this circadian cycle[57]. Thus far, data has implicated the ultradian pulsatility of CORT release to occur in both humans and rodents, and to originate from a transient HPA axis activation that is initiated by a continuous stimulation of the pituitary by hypothalamic corticotropin releasing hormone[58,59]. The biological role and implications of the ultradian rhythm, however, remain largely unexplored. Pioneering work from Stavreva *et al.* suggests that ultradian CORT pulses induce subsequent pulses of gene expression, thereby preventing the accumulation of gene expression during the active phase of the circadian rhythm[60]. In response to ultradian hormone fluctuations, these pulses of gene expression originate from CORT-bound GRs that cyclically occupy the chromatin at glucocorticoid response element (GRE) containing promoters. They are subsequently released and recycled by chaperones[60,61] (Figure 8). Interestingly, further work from Stavreva *et al.* demonstrated that levels, location and duration of GR occupancy at the chromatin show a striking spatiotemporal relation with both DNase I binding and gene expression, which is again dictated by the rhythm of CORT administration[62]. These data indicate that the ultradian rhythm of CORT release specifically, sends a biologically distinct cell extrinsic signal to cells sensitive to corticosteroids.

The hippocampus is rich in corticosteroid receptors and thereby sensitive to rhythmic changes in CORT. It is also involved in shutting down the activated HPA axis under specific conditions[63,64]. Moreover, hippocampal neurogenesis in the DG has been recently shown to contribute to the negative feedback of the HPA-axis[65]. Within the hippocampus, the DG contains NPCs that are sensitive to rhythms of CORT, and they show diurnal fluctuations in mitotically active NPCs[66]. Interestingly, artificial flattening or 'clamping' the CORT rhythms at peak levels impairs the diurnal rhythm in NPC mitosis[66] (**chapter 5**). In addition, chronic stress induced alterations in CORT rhythmicity[67] also induce reductions in DG proliferation rate, which appear to normalize after a recovery period[68].

As demonstrated by Fitzsimons *et al.*, lowering GR expression in NPCs, and therefore likely their sensitivity to CORT, prematurely triggered their differentiation[13]. This highlights that CORT rhythmicity might act as a cell extrinsic pacemaker affecting differentiation of NPCs and their progeny. Previous results from Bose *et al.* show that a prolonged GR activation leads to lastingly hypomethylated DNA of NPCs, which correlated with a loss in mitogenic capacity[14]. The aforementioned data all suggest that deviations from cyclic CORT exposure gives a relevant cell extrinsic signal that is transmitted into epigenetic signals in NPCs, that subsequently affect both proliferation and differentiation. Our data presented in **chapter 5** support this hypothesis. Furthermore, the large differences in numbers of promoters

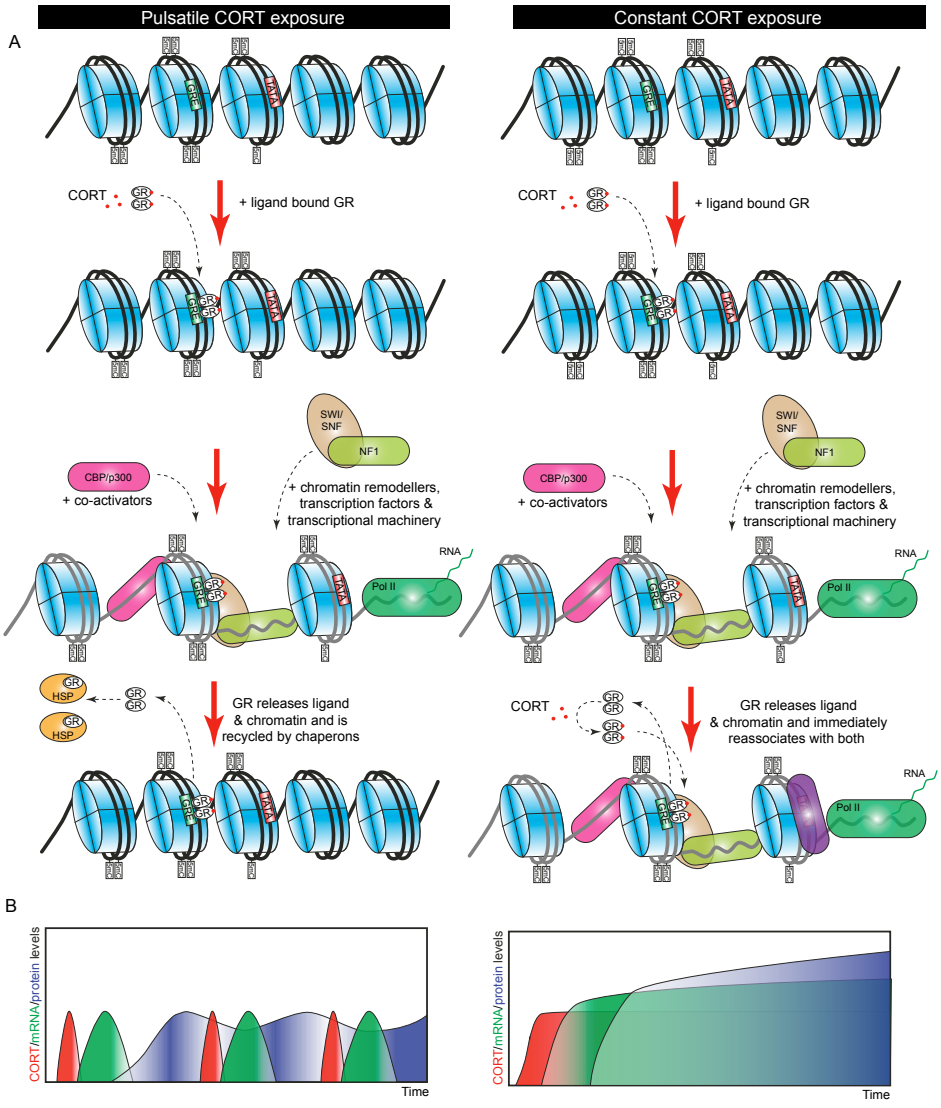

Figure 8 - Exposure to pulsatile or constant CORT rhytms elicit different effects at the level of chromatin and gene expression.
(A) Schematic representation of the events that take place at GREs upon pulsatile (left) or constant (right) CORT exposure (references). (B) Schematic graphs displaying how pulsatile (left) and constant (right) CORT exposure result in different levels of mRNA and protein[60].

that are differentially methylated when "naturalistic" CORT rhythms are compared to a flattened or 'clamped' constant exposure, suggest that not only the levels, location and duration of GR occupancy at the chromatin are critically regulated by CORT rhythms[62], but that also chromatin accessibility at promoter loci is regulated by specific DNA methylation marks (**chapter 5**).

Although aging has been proposed as a strong anti-neurogenic stimulus[80], the levels of late-life AHN may vary depending on particular "life-style" differences such as physical activity and experience[69]. Specifically, with this "neurogenic reserve" theory, Kempermann proposed that an enriched life, that e.g. contains many challenges and learning experiences ("life-long learning") will provide more functional DG plasticity and related cognition later in life under conditions where plasticity is needed[70] (Figure 9). Therefore, a life in which these "life-style" related challenges are absent or neglected, might lead to a quiescent or even senescent NPC population in the DG. Although a lack of NPC proliferation is a prominent feature of age-related alterations in AHN[71,72], the underlying mechanisms remain largely elusive.

As discussed in **chapter 1** and **6**, aging represents a physiological situation in which deviations from CORT rhythmicity occur that might contribute to the induction of NPC quiescence. It has e.g. been reported that in rats, stress-related increased CORT levels superimposed on age-related increased CORT levels, could not further reduce the age-related decline in NPC proliferation[73]. Yet, removal of the age-related flattened CORT rhythm, and thus mitogenic "brake", through adrenalectomy, has been reported to result in a recovery of NPC proliferation[74,75], although probably only transiently[76]. Whether this occurs in accordance with previously reported CORT induced alterations in DNA methylation (**chapter 5**) remains to be tested experimentally. The relative increase in GR expressing NPCs with increasing age we found in **chapter 6** makes it possible to speculate that age-related deviations from basal CORT can also induce changes in NPC DNA methylation. Therefore this may impact stronger on neurogenesis at old age compared to young age, since more NPCs express the GR with increasing age. Accordingly one can conclude that the NPC pool may become increasingly homogeneous with age, and could thus lack molecular flexibility to produce new neurons under persistently elevated CORT. Why this would occur however, remains unclear.

One possible scenario could be an altered regulation of GR expression levels. Studies have reported that miR-124 expression in the brain is altered with age[77], which may occur through an altered GR activation[78] (**chapter 1** and **5**). Although speculative, the cyclic rhythms of CORT do represent a cell extrinsic signal that could possibly activate multiple layers of cell intrinsic molecular mechanisms including DNA methylation and microRNAs, all of which might possibly be involved in self-regulatory loops. Concerning NPC microRNA expression, we could show differential effects of the normal daily rhythm of CORT, and deviations from this on miR-124 levels (**chapter 5**). Accordingly, one likely scenario could be that advancing age presents challenge for the miR-124 mediated GR feedback regulation in NPCs through prolonged deviations of basal CORT rhythmicity, which could favor a of quiescent NPC phenotype at old age.

4. Implications: phenotypical overlaps between KA-induced hyperactivation, microRNA expression and deviations from basal pulsatile glucocorticoid hormone exposure

4.1 NPC quiescence: overlapping effects of aging and deviations from basal CORT pulsatility.

As described by Fitzsimons *et al.*, under non-stressed conditions, a reduction of GR expression, selectively in the newborn cells in the adult DG, is sufficient to alter their differentiation and migration[13] (Figure 4). In **chapter 5** we show that persistently "flattened" CORT rhythms on the one hand alters the adult NPC pool to acquire on the short term a quiescent phenotype, while on the other, this lastingly altered DNA methylation of several promoters, involved

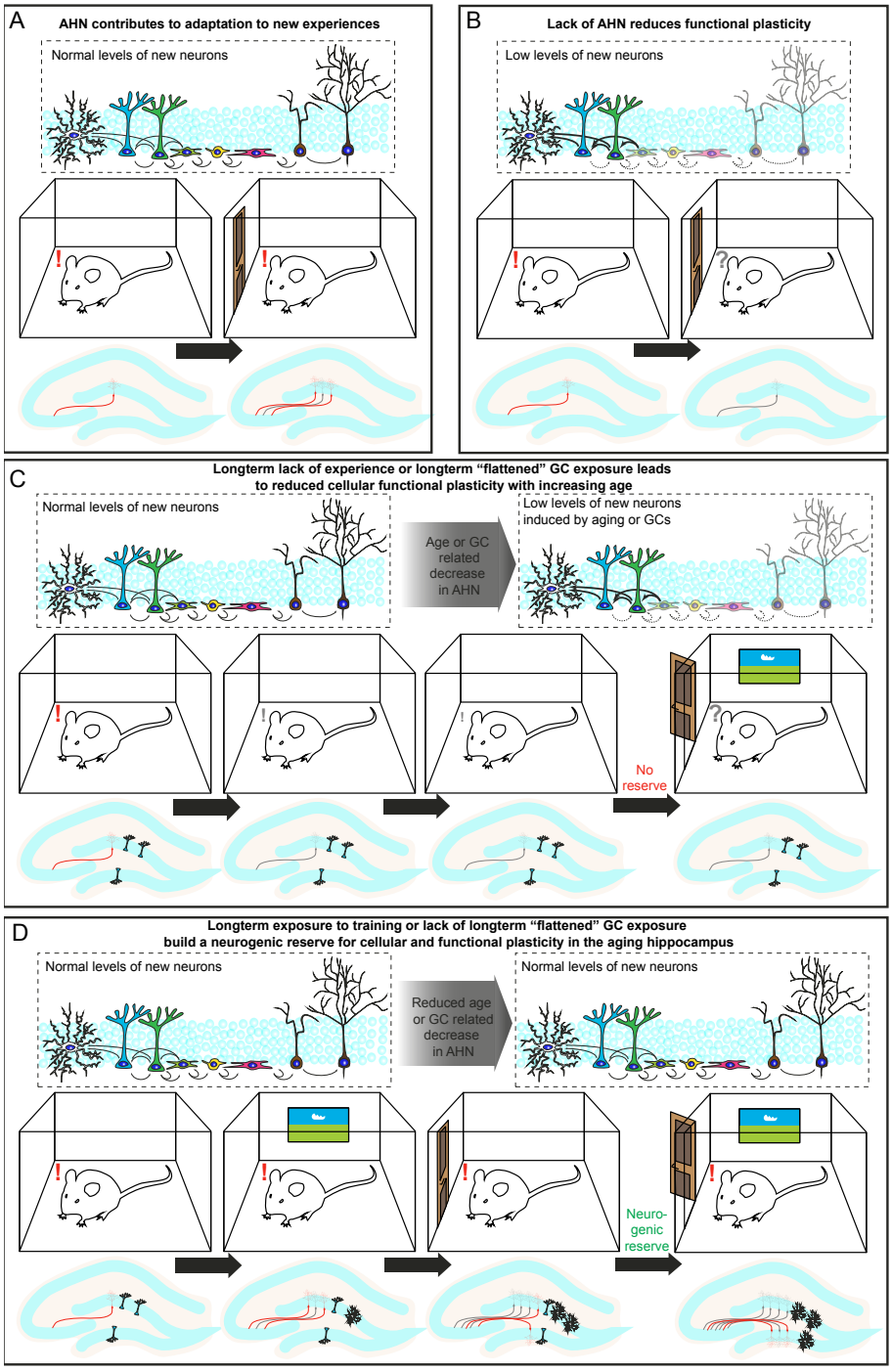

Figure 9 - The "deforestation" hypothesis[6] integrated within Kempermann's "neurogenic reserve" theory[70].
(A) Schematic representation of how AHN contributes to adaptation to new experiences, providing "cognitive flexibility".

Figure 9 - *continued*
(B) Schematic representation of how a lack of AHN hampers functional plasticity in the hippocampus, impacting on learning and memory and thereby reducing "cognitive flexibility". (C) Schematic representation of how life-long lack of training/novel experiences or long-term "flattened" GC exposure may prevent the formation of a neurogenic reserve at old age, yet maintains the quiescent type-1 NPC pool by preventing "deforestation" and driving them into "hibernation". (D) Schematic representation of how long-term exposure to training/novel experience or lack of long-term "flattened" GC exposure may contribute to the formation of a neurogenic reserve at old age, yet considerably depletes the quiescent type-1 NPC pool by stimulating "deforestation".

in differentiation (Figure 5). These data suggest that GR expression in NPCs is crucial in "sensing" basal glucocorticoid pulsatility and contributes to the regulation of NPC differentiation and proliferation rates. Accordingly, conditions that alter the GC rhythm, as observed during aging[79], could alter structural plasticity of the hippocampus. Aging has been associated with a dramatic reduction in AHN through reductions in the numbers of proliferative NPCs[80]. We and others, however, have found NPCs to persist in the aged hippocampus[8,16,81] (Figure 6).

A relatively small number of studies have molecularly characterized aged NPCs and found amongst others increased GR[16] and Dickkopf[82] expression levels. Fitzsimons *et al.* found GR expression levels to regulate AHN through apoptosis/differentiation[13]. As the levels of GR expressing NPCs increase with age[16] (**chapter 6**), this may suggest a potentially causal role. Loss of DKK1 from NPCs in the aged brain restores AHN through increases in both proliferation and differentiation, and counteracts age-related cognitive impairments[82]. Furthermore, Dickkopf1 has been shown to be a critical mediator of CORT induced alterations in NPC proliferation and differentiation[83].

Together, these data suggest a potential molecular interplay between the Wnt signaling antagonist DKK and the GR in the aged NPC population. Previous literature has linked stress-associated GC release to increases in hippocampal DKK expression levels[84] while our own results indicate that deviations from basal GC pulsatility exposure can lastingly hypomethylate promoters from DKK family members (**chapter 5**). Whether this mechanism can also explain the persistent NPC quiescence at old age remains to be addressed experimentally. Additionally, it is unclear whether the age-related alterations in GC rhythmicity per se contribute to the AHN-related cognitive decline, and whether decreases in differentiating newborn cells are implicated. As shown in **chapter 5**, persistent flattening of CORT rhythmicity drives NPCs towards quiescence. Accordingly, one could hypothesize that when this occurs at old age, the remaining type-1 cells are less likely to produce new type-2 cells. Indeed, as described in chapter 7, while at 10 months of age, considerable numbers of type-1 and type-2a cells are still found, type-2b cells were reduced at a significantly higher rate. The type-2b neurogenic cells are of particular interest since they represent the first transitional cell type that lost their astrocytic or glia-like properties and have become committed to the neuronal phenotype. Thus, this age-related decrease in type-2b cells cannot be explained by a loss in type-1 activation and subsequent asymmetric division towards type-2a and subsequently type-2b cells.

Alternatively, this decrease could be due to a decrease in NPC progeny that is committed to the neuronal fate. Indeed, previous literature proposes an age-dependent "commitment-shift" of type-1 and type-2a cells towards the astrocytic fate[81]. As NPCs can thus be rather heterogeneous in terms of cell-fate decisions of their progeny, one could hypothesize that GR expression might direct cell-fate decisions towards the astrocytic phenotype at old age when CORT rhythms are more flattened. Although not tested experimentally yet, the implications of this could help to understand the age-related reduction in newly born neurons. In this respect, a first experiment could be to assess whether the strong reduction in type-2b cells temporally precedes or overlaps with alterations in CORT rhythms. The implications of this study would provide an insight in whether the age-related changes in NPC numbers are either a cause, consequence or unrelated to the age-dependent changes in CORT rhythms.

4.2 Ectopically located immature granule cells: phenotypical overlap between local GR-knock down and miR-124 overexpression.

A number of cell intrinsic factors converging on the mTOR pathway, including the genes DISC1[85], GSK3ß[86] and PTEN[87], have all been related to precocious or accelerated differentiation of immature granule cells. Indicative of this, alterations in the local expression levels of both DISC1 and GSK3ß induce an ectopic positioning of immature granule cells, that end up in higher numbers towards the molecular layer of the DG specifically. Furthermore, after DISC1 knockdown, this ectopic localization only occurred during the hyperpolarizing GABA period of immature granule cells[88]. Modulation of the mTOR pathway in immature granule cells has also been shown to attenuate both the moment, or time window, of differentiation and their electrophysiological properties[88,89].

Fitzsimons *et al.* also observed both phenotypical alterations following local GR knock down[13] (Figure 4), while ectopic localization was induced by miR-124 (**chapter 3** and Figure 3). Whether the latter also alters the electrophysiological properties of the immature granule cells remains to be studied. Furthermore, it remains unclear whether ectopic localization coincides, precedes or follows changes in electrophysiology. Although not tested experimentally, exogenous overexpression of miR-124 likely also affects the expression of numerous other targets than BCL2L13 alone (**chapter 3**), such as BIM[90] and the GR[91]. Therefore, miR-124 could ultimately converge on the regulation of apoptosis from various angles, and ultimately coordinate entire biological pathways or multilevel processes (Figure 7).

We have demonstrated that, in part through regulation of caspase-3 activity, both miR-124 and GR are key modulators of apoptosis and survival/differentiation. Both biological processes share a considerable overlap with the mTOR pathway[92,93] and these data thus suggest a molecular convergence of DISC1, PTEN, GSK3ß, GR and miR-124 on the regulation of apoptosis and differentiation of NPC, possibly through caspase-3. Regulation of caspase-3 activity might be linked to both ectopic localization and electrophysiology of immature granule cells, as caspase-3 activity levels are crucially involved in neuronal electrophysiology[23].

Indeed, previous findings support the possibility of a link between apoptosis and ectopic localization that is mediated through the pro-apoptotic protein BAX[94]. Whereas some of the previously discussed biological processes seem to converge on the regulation of mitochondrial function, a potentially fruitful avenue for further research could thus be the spatiotemporal comparison of mitochondrial proteins of ectopically localized immature granule cells and their normal counterparts within the DG. The implications of this experiment might contribute to the finding of new drugable targets that could prevent ectopic positioning of newborn neurons and thus the generation of aberrant neurogenesis-induced seizures[87].

5. Implications of altered NPCs beyond the initial stages of AHN; a possible involvement in feed forward loops.

While the majority of cell-types studied in this thesis are limited to the initial stages of AHN, some could have implications also for the later stages of AHN (Figure 1). Importantly, in these later stages, newborn neurons integrate and functionally contribute to the pre-existing network, and it is during that period where the initial alterations might manifest themselves most prominently and can lastingly alter AHN and functional hippocampal plasticity. In this section I will discuss and speculate on how the subsequent consequences of our initial manipulations could impact on later end-points of AHN and their longterm implications.

5.1 Aberrant neurogenesis as a driving force in DG excitability and hippocampal epileptoform activity.

One of the classical hallmarks often observed in temporal lobe epilepsy is the rearrangement of the hippocampal network, including a rewiring of the connections of newborn neurons. Although initial seizures induce aberrant integration of newborn neurons, it has, until recently, not been clear whether this was either a consequence or a possible driving force behind the subsequent development of chronic seizures. Although not entirely

without controversy[95], pioneering work from Pun *et al.*[87] and Cho *et al.*[96] describe that the induction of aberrant AHN promotes epileptogenesis. Specifically, these particular newborn cells are responsible for the epileptoform activity of the hippocampus, are ectopically integrated and display phenotypical hallmarks of accelerated differentiation and integration[87]. These observations are strikingly similar to the ones we made in **chapter 3** and by Fitzsimons *et al.*[13] (Figure 3 and 4, respectively). Accordingly, miR-124 overexpression and GR knockdown in newborn neurons specifically could render them to acquire a hyperexcitable phenotype ultimately contributing to hippocampal network rearrangements[11]. This could lower the threshold for the development of subsequent concomitant epileptic insults, and aggravate sequential aberrant neurogenic responses and thus starting a feed forward loop that might shift overall DG excitability and promote epilepsy.

5.2 A driving force in major depression disorder? Stress hormones, a feed forward loop between AHN and subsequent alterations in HPA-axis activity

As described in **chapter 5**, elevated glucocorticoids levels, such as those seen in mouse models of chronic stress[97], elicit an anti-neurogenic effect through inhibition of NPC proliferation (Figure 5). As shown in **chapter 5**, these changes in NPCs induced by "flattened" CORT are not transient but even epigenetically transmitted to their progeny, indicating that termination of an episode of high CORT exposure can have lasting consequences for AHN even if the stress hormone is no longer present. As example of such lasting consequences, we found that a subsequent CORT exposure induced an attenuated response in cell cycle inhibition of NPC progeny (**chapter 6**). Whether this progeny also shows an attenuation in CORT responsiveness at later stages in the neurogenic cascade remains to be tested experimentally. Whereas cell type specific knockdown of the GR lead to impairments in fear-associated contextual memory, the final consequence of alterations in CORT responsiveness of the newborn mature neurons is likely also determined by multiple factors. Either way, a correct regulation of glucocorticoid signaling is critical for multiple stages of AHN and possibly for stress-related behaviors.

Although a possible interplay between glucocorticoid signalling and neurogenesis in the etiology and recovery of depression has been proposed 15 years ago[98], a causal link has not been demonstrated. A major advance came from Snyder *et al.*, that described AHN to buffer stress induced HPA axis activation[65]. Specifically, the genetic ablation of neurogenic cells resulted in a delayed shutdown of the HPA axis upon acute stress and in signs of behavioral despair and anhedonia[65]. Further evidence that supported these observations was provided by Hill *et al.*, showing that artficially increasing levels of hippocampal neurogenesis could reduce the chronic CORT induced anxiety and depressive-like behavior[97]. These data indicate that AHN plays a role in stress or CORT-induced depressive-like behavior, yet a complete explanation of how this takes place, is lacking. These data suggest repeated exposure to chronic stress or prolonged deviations from basal glucocorticoid rhythms can engage feed forward loop in NPCs and their lack of progeny, which in turn can modify the buffering effects of AHN on HPA axis activity[65].

5.3 Saving plasticity for later? Implications of lifelong glucocorticoid exposure, heterogeneity in "deforestation" of the NPC pool and neurogenic reserve.

Based on the numerous reports of CORT induced alterations on the body, glucocorticoids are key factors controlling energy distribution[99]. Moreover, glucocorticoid action often aims to inhibits high-energy processes such as reproduction and growth[100]. Among the latter, we and many others found CORT to induce inhibition of NPC proliferation through entry into G0 and therefore to stimulate NPC quiescence (**chapter 5**).

Nestin-GFP expressing type-1 NPCs can only undergo a limited number of type-2 NPC generating divisions, before committing to an astrocytic fate[81,101]. Accordingly, a recently emerged and much debated hypothesis termed "neural stem cell deforestation" postulates that forced entry into the cell cycle would eventually lead to exhaustion of the type-1 NPC pool with advancing age[6,102]. Following this line of reasoning, one might hypothesize that CORT induced NPC quiescence might have an opposing contribution by promoting these "hibernating" NPCs to persist into old age (Figure 6).

Either hypothesis could be correct, assuming that the type-1 NPC pool cannot be considered a homogeneous pool of cells and would then display differential deforestation sensitivity. In **chapter 6** we noted a striking heterogeneity within the type-1 NPC population, with subsets expressing and lacking GR expression at a young adult age, and differences in age-dependent decay kinetics, or deforestation. These data imply that a considerable untouched neurogenic capacity or reserve might be present in these persisting type-1 NPC even at old age, assuming they are still capable of exiting quiescence which may depend on the identification of the appropriate stimulus. Consistently, pioneering work from Cameron *et al.* demonstrated that removal of the adrenals and thus most CORT transiently restored the production of new neurons several fold even in old age[74]. Related findings suggest that also in other parts of the brain, immature neurons reside that might respond to specific stimuli, could represent a "reservoir" of structural plasticity throughout life[103] and could play a role in a neuronal reserve[104-106].

6. Final remarks

The data presented in this thesis focused on critical cellular and molecular processes in the early stages of AHN. Within a framework of neuronal hyperexcitation associated with epilepsy, hormonal regulation and aging models, we have been able to demonstrate a number of phenotypical changes of NPCs and explain them by changes in specific molecular mechanisms. How these alterations eventually translate into a (functionally) different neurogenic outcome remains to be elucidated. This neurogenic outcome is not trivial, since the birth of new hippocampal neurons has been implicated in the regulation of some cell extrinsic signals themselves. As discussed, DG hyperactivation[87,96] and HPA axis activity can be modulated by AHN[65]. Furthermore, epigenetic alterations in NPC might contribute to a decrease in newborn age-related cognitive decline. Accordingly, future research should address if and how the molecular alterations in NPCs can affect neurogenesis beyond the NPC stage. Primary indications of (possible) attenuations in NPC differentiation have been observed in **chapter 3, 5** and by Fitzsimons *et al.*[13]. Along these lines, exogenous overexpression of miR-124 phenocopied the KA-induced and GR knockdown-induced ectopic positioning and accelerated differentiation of newborn neurons >7 days after treatment. What remains unclear is whether these phenotypical alterations contribute to e.g. abberant hyper-excitable DG network formation or alterations in HPA axis activity. Furthermore, **chapter 6** describes that, although largely quiescent, there is a considerable number of NPC found up into middle age that expressed the GR, highlighting a preserved potential reservoir of structural plasticity.

Although we found multiple molecular mechanisms associated to the phenotypes mentioned here, numerous questions remain to be addressed. Addressing these future directions might help to better understand the contribution of AHN to (psycho)pathologies such as depression, epilepsy and age-related cognitive decline.

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