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By seizures, glucocorticoids and microRNAs
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6. General Discussion

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(Patho)physiological Regulation of Adult Hippocampal Neurogenesis
by Seizures, Glucocorticoids, and microRNAs

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**Different subsets of newborn granule cells: a possible role in epileptogenesis?**

*Pascal Bielefeld, Erwin A. van Vliet, Jan Gorter, Paul J. Lucassen, Carlos P. Fitzsimons.*

*European Journal of Neuroscience, 2014*

&

**MicroRNA-mediated regulation of adult hippocampal neurogenesis and implications for hippocampus-dependent cognition**

*Pascal Bielefeld, Ben Pustjens, Marijn Schouten, Carlos P. Fitzsimons.*

*In: ESSENTIALS OF NONCODING RNA IN NEUROSCIENCE: ONTOGENETICS, PLASTICITY OF THE VERTEBRATE BRAIN, 2016*

&

**Transcription factor oscillations in neural stem cells: implications for accurate control of gene expression**

*Pascal Bielefeld, Marijn Schouten, Paul J. Lucassen, Carlos P. Fitzsimons.*

*Neurogenesis, 2016*
Summary and Conclusions

This thesis specifically focused on the direct regulation of NSCs; a crucial process for the generation of new neurons throughout life in the mammalian brain. Mammalian species are constantly exposed to environmental challenges for which adaptation of brain function through e.g. neuronal modulation and plasticity is crucial. AHN constitutes one of the recent, most intensively studied forms of structural plasticity. It has long been proposed that newborn neurons are needed in certain types of hippocampus-dependent memory functions, and therefore would relate to cognitive capacity of mammalian species[1–5]. While there is evidence for the involvement of newborn GCs in these functions, a unified theoretical framework for adult neurogenesis has not been reached yet and will likely require more experimental data[6].

The capacity of the adult hippocampal stem cell pool to generate new neurons while maintaining itself is not indefinite, and this capacity decreases with age. To prevent an early exhaustion of the adult hippocampal stem cell pool, AHN must be tightly regulated and several cell intrinsic and extrinsic signaling pathways have been identified that regulate AHN[6,7]. Deregulation of AHN is a hallmark of many brain pathologies, including epilepsy, although its exact role in these pathologies remains mostly unclear[8–12]. Here, we aimed to identify new regulatory pathways of AHN in both health and disease, focusing on epigenetic mechanisms.

Chapter 1 provides a thorough literature overview on the role and regulation of AHN under both baseline conditions and in the case of pathology, in particular epileptic seizures. Epileptic seizures are one of the first well-described deregulators of AHN, affecting numerous aspects of AHN, such as proliferation, apoptotic selection of newborn cells, neuronal differentiation, synaptic integration, and a depletion of the stem cell pool through direct conversion of hippocampal NSCs into astrocytes[8–10,13–15]. Although much effort has been put in understanding how seizures exactly derail AHN to such an extensive level, the answer to this question remains unclear. In this respect, microRNAs have become of interest, and we here provided an extensive overview of all microRNAs up to date involved in the regulation of AHN, followed by a comprehensive overview of the effects of epileptic seizures on AHN-regulating microRNAs. We show that many AHN-regulating microRNAs are affected by epileptic seizures, and discuss the effects this deregulation might have on the neurogenic cascade[16].

When comparing existing mouse microRNA profiling studies related to epileptic seizures, great differences arise between studies. One of the sources of this variability may be the large variety in epilepsy models used in different studies, and the time points studied. This has been a longstanding issue in the preclinical epilepsy field, and in our opinion warrants more attention. Each model has its own advantages and disadvantages, and may differ considerably from the human condition. Therefore, they are best used under specific conditions and to reflect specific questions. However, even with these considerations, improvements can be made to enhance experimental reproducibility.

Classically, the chemoconvulsant Kainic Acid (KA) has been administered in repeated small doses intraperitoneally until SE occurs. This protocol has been designed to compensate inter-individual variability in the sensitivity to KA and to reduce the high mortality associated with high KA doses[17,18]. In chapter 2, we described a standardized, cross-laboratory effort to optimize this model, making use of intrahippocampal administration of the KA. The advantage of this model is that the exact dosage of KA reaching the
Since profiling data on the effects of epileptic seizures on AHN are complex due to the aforementioned reasons, and a dataset specifically on the DG at 3 days post SE was lacking, we carried out our own multi-step profiling study matching the experimental window of our interest. We increased specificity in the identification of factors involved in the early deregulation of AHN by epileptic seizures by microdissecting the dentate gyrus 3 days post SE induction and using it for transcriptomics, proteomics, and microRNAomics. **Chapter 3** described our approach and the datasets obtained, highlighting the severe deregulatory effects that Kainic acid-induced seizures have on cells in the DG.

Using these datasets we have identified a set of critically deregulated microRNAs that warrant further analysis, among these are miRNA-124 and miRNA-137. Previous work from our lab has identified these two miRNAs to have cooperative functions, each enhancing functionality of the other\(^\text{19}\). Furthermore, these miRNAs share numerous targets that are critically involved in the regulation of AHN, including BCL2L13, a proapoptotic protein that we have identified as regulator of NSPC apoptosis/differentiation; and members of the Notch signaling pathway, which regulates symmetric versus asymmetric division of NSCs\(^\text{20,21}\). We then asked whether these two miRNAs were involved in the regulation of NSC fate, one of the characteristic changes found after epileptic seizures. In **chapter 4** we used an antagonir-based approach to counteract the seizure-induced miRNA overexpression post SE development, and were able to (partially) rescue several seizure-induced changes in NSC fate, such as the conversion into reactive astrocytes, and the loss of conversion into Type B NSCs.

Besides a clear deregulation of epigenetic factors upon pathology, such as epileptic seizures, epigenetic regulation also plays a crucial role in the maintenance of AHN under physiological circumstances\(^\text{22}\). In **chapter 5**, we characterized how differences in glucocorticoid levels and their normal rhythmicity affect NSPC proliferation, GC responsiveness, and methylation of specific promoters of genes crucial for AHN. We demonstrate a clear role for glucocorticoid oscillations in maintaining AHN throughout life and in the differentiation of newborn cells into mature new neurons. Furthermore, we studied the effect of ageing on the neural stem cell pool, focusing on the critical role of the glucocorticoid receptor in maintaining AHN throughout life. Our results indicate that GR activation by physiological GC oscillations, which increase in amplitude with age, prevents the activation of NSC in the aging hippocampus.

**Emerging concepts**

1. **Epigenetic control of hippocampal NSCs**

As discussed in **chapter 1**, many steps in the neurogenic cascade are under strict epigenetic regulation, either indirectly by chromatin modifications\(^\text{23–28}\), regulating gene expression, or directly by miRNAs. Such epigenetic regulation allows for a tight temporal and spatial control of NSC maintenance, NSC activation, NSC fate decisions, NSC division,
Chapter 6

and subsequent steps in the neurogenic cascade. In this thesis we focus on the early steps in the neurogenic cascade, i.e. NSC activation and fate decisions.

1.2 A role for miRNAs in the regulation of complex biological systems

MiRNAs are small non-coding RNA molecules composed of approximately 22 nucleotides that play a role in RNA silencing and post-transcriptional regulation of gene expression. Gene silencing may take place via mRNA degradation or inhibition of mRNA translation, hence miRNAs can only exert their function when the appropriate target mRNA is expressed as well. Given the biological characteristics of miRNAs, such as their potent ability to (transiently) repress mRNA translation, their rapid turnover, and their relative high conservation between species, microRNAs have been hypothesized to play crucial roles in regulating the switch between different temporal stages of AHN.

In addition to their intrinsic biological characteristics, miRNAs add another level of complexity to gene expression regulation via the possibility of coordinated action. Classically, studies focusing on understanding the role of miRNAs in the regulation of gene expression address the role of single miRNAs and their targets. However, regulation of a single transduction pathway comprises numerous proteins, each of which is under control of specific miRNAs. How the action of multiple miRNAs on one common target is regulated remains poorly understood. We have previously identified one such cooperative mechanism, where two miRNAs have synergistic functions on the regulation of one common target. Besides this classic form of cooperative function, miRNAs offer a wide array of possibilities to control signaling pathways by converging on several targets within one transduction cascade (Figure 1). This complex regulation of numerous biological cascades by cooperative action of miRNAs has been hypothesized to underlie the complex regulation of biological processes, such as neurogenesis. Several examples of how miRNAs regulate crucial processes in the neurogenic cascade will be discussed in the next section.

1.3 The interplay in epigenetic control of hippocampal NSCs by miRNA-124 & miRNA-137 and transcription factors

In chapter 3 we described the identification of a KA-induced miRNA profile specifically in the DG 3 days post seizure induction. Using this dataset we identified two significantly deregulated miRNAs that have regulatory functions in AHN: miRNA-124 and miRNA-137. In chapter 4, we show the involvement of these two miRNAs (124 & 137) in the induction of aberrant AHN upon seizure induction. Interestingly, these two miRNAs have been implicated in aberrant AHN before, though in different processes. Schouten et al showed that miRNA-124 and -137 cooperatively regulate the expression of Bcl2L13 in NSPCs. By doing so, the normally pro-apoptotic Bcl2L13 protein now drives (precocious) neuronal differentiation instead of apoptosis, which is hypothesized to be one of the probable reasons for the survival of many unfit neurons upon seizure induction.

Furthermore, both miRNA-124 and -137 target several transcription factors that play crucial roles in regulating AHN. Both miRNA-124 and -137 target members of the Notch signaling pathway, which is crucial in regulating symmetric versus asymmetric division of NSCs and maintaining NSC quiescence. Though we have not experimentally confirmed the involved targets of miR-124 and -137, we do show in chapter 4 that the induction of seizures results in changes in NSC fate decisions, possibly linked to a shift from asymmetric to symmetric division of NSCs. Furthermore, this phenotype could be
Figure 1. Schematic representation of coordinated microRNA action at different levels. (A) Conventionally, individual microRNAs 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 alterations 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 process. Adapted from Barca-Mayo et al.\textsuperscript{31} and previously published in\textsuperscript{146}.
rescued by administration of antagomirs against miRNA-124 and -137.

Besides regulating Notch signaling, miRNA-124 also directly targets the GR, another transcription factor with significant roles in regulating AHN\textsuperscript{133}. In chapter 5 we described a crucial role for the GR in regulating and maintaining NSCs throughout age, preventing activation of quiescent NSCs and subsequent exhaustion of the NSC pool at later ages. Furthermore, pioneering work from Stavreva \textit{et al.} already suggested that oscillatory exposure of the GR to CORT induces subsequent oscillatory pulses of gene expression, thereby preventing the accumulation of gene transcripts and providing spatio-temporal control of gene expression\textsuperscript{38}.

Besides regulation of AHN at a physiological level, the GR has also been implicated in the induction of aberrant AHN under pathological conditions. We show in chapter 5 that the oscillatory exposure to its ligand, CORT, is crucial for AHN. The progeny of NSCs that have been exposed to altered CORT regimens, in this case chronic steady elevated CORT levels, show significant alterations in gene promoter methylation profiles, suggesting that a loss of rhythmicity in the exposure to CORT results in lasting epigenetic changes carried over to the progeny.

Furthermore, earlier work from Fitzsimons \textit{et al.} already showed a crucial role for the GR in regulating other aspects of the neurogenic cascade: differentiation and maturation of newborn neurons\textsuperscript{60}. Using a retroviral approach to knockdown the GR in proliferative NSPCs, they showed that a lack of GR results in increased differentiation rates, an increase in the number of mushroom spines, and an ectopic location of immature neurons.

Interestingly, these are all phenotypic characteristics of newborn neurons that are also observed under seizure conditions. The relationship between stress exposure (CORT) and seizure induction has been a longstanding, and heavily debated, topic\textsuperscript{83}. Different animal models of acute stress show anticonvulsant effects\textsuperscript{84–86}, while chronic stress exposure in adult life seems to increase seizure susceptibility\textsuperscript{87,88}. Chronic CORT exposure results in downregulation of the GR, a situation that is mimicked by the retroviral GR knockdown approach by Fitzsimons \textit{et al.} Interestingly, the data from Fitzsimons \textit{et al.} shows several characteristic hallmarks of “pro-epileptic” immature neurons, as described above\textsuperscript{60}.

Based on these observations, we proposed in chapter 1 that the glucocorticoid receptor could be used as target to control newborn granule cell populations with aberrant properties\textsuperscript{145}. Although we have been not been able to further investigate this hypothesis in the context of epileptic seizures, in chapter 5 we demonstrate that GR inhibition induced by intrahippocampal injection of specific siRNAs results in a significant increase in proliferation of Type A NSCs in the dentate gyrus. Furthermore, in senescent SAMP8 mice, an animal model that may share some characteristics with chronic epilepsy, such as a strong depletion of the NSC pool\textsuperscript{144}, GR inhibition results in a significant increase in dendritic arborization and the number of dendritic spines in newborn neurons, a

**Future Directions (1)**

In chapter 4 we showed that epileptic seizures cause a shift in NSC fate decisions resulting in the induction of reactive NSCs and a loss of Type B NSCs, at the cost of Type A NSCs; a phenotype that can be (partially) rescued by administration of antagoniR-124 and -137. The next step in identifying the exact role of these two miRs would be to identify the targets by which they regulate NSC fate decisions.
phenotype that resembles several characteristic hallmarks of “pro-epileptic” immature neurons, as described above\textsuperscript{60}.

2. A role for transcription factor oscillations in gene expression regulation in adult NSCs

Many studies aiming to understand transcription factor action assess target gene responses by a steady level of stimulation. However, steady inputs are infrequently observed under natural conditions and, at the cellular level, many signaling pathways have been optimized to result in a dynamic fluctuation in transcription factor activity. This suggests that a biologically accurate regulation of gene expression requires, or at least benefits from, an oscillatory mode of action in many cases\textsuperscript{32–35}. Oscillating signaling pathways and downstream transcription factors may present “circadian” lower-frequency oscillations over the day/night period and/or ‘ultradian’ higher-frequency oscillations in the order of hours. Furthermore, they frequently present a common architecture consisting of negative feedback loops that introduce time delays responsible for their oscillatory activity\textsuperscript{43}. Conceptually, this necessary time delay could occur due to several mechanisms, such as: 1) the inclusion of a biological process that takes a minimum amount of time (e.g. transcription, translation, synthesis); 2) the inclusion of many such intermediate steps, with each step adding to the overall time delay; 3) the inclusion of a threshold concentration that must be reached before a molecule becomes biologically active, resulting in an on/off-like response; 4) the inclusion of a degradation/sequestration step, where the activity of a molecule is delayed by the formation of a saturated complex\textsuperscript{33} (Figure 2A-C). Often, these mechanisms are combined within signaling pathways that induce oscillatory transcriptional activity.

A delayed negative feedback loop is defined as a negative feedback loop with a time delay due to the inclusion of intermediate processes between the product and the repressor\textsuperscript{36}. Delayed negative feedback loops are often required to induce oscillations in transcription factor activity. For example, in the circadian clock, longer delayed negative feedback loops are central for the reaction to regular external inputs such as light or feeding. The reaction to faster, more irregular signals, such as cellular stress, frequently requires faster (lower period) ultradian oscillations. These oscillations in activity are observed in well-characterized signaling pathways and downstream transcription factors such as NF-kB, p53, Wnt and Notch signaling, and the glucocorticoid receptor (GR)\textsuperscript{33,37,38}. Interestingly, double negative feedback loops are common between transcription factors and microRNAs and have been frequently described in NSPCs\textsuperscript{39,40}.

2.2 The Notch signaling pathway

The Notch signaling pathway is perhaps the best characterized oscillatory pathway in NSPCs. It promotes cell proliferation and maintenance, favoring a non-differentiated cellular state in the developing and adult brain\textsuperscript{41–43}. Proteins of the Notch family are normally activated by cell-to-cell contact and act as transmembrane receptors for specific ligands expressed in neighboring cells, such as Delta-like 1 (Dll1). Upon activation, Notch proteins are proteolytically cleaved and release their Notch intracellular domain (NICD)\textsuperscript{44}. NICD then translocates to the nucleus to form the CSL (or RBP-J in mice) transcriptional complex, which activates the transcription of responsive genes of the Hes/Hey family, such as Hes1. For an extensive description of the canonical Notch signaling pathway we refer to previous reviews\textsuperscript{41,42}.
Levels of Hes1 oscillate in mouse embryonic NSPCs and progenitors, and this oscillatory expression promotes proliferation. On the contrary, sustained (non-oscillatory) Hes1 expression is associated with an inhibition of NSC proliferation and neurogenesis in the developing central nervous system. Therefore, it has been suggested that oscillatory versus sustained expression of Notch target genes may distinguish active and quiescent NSC pools.

In particular, sustained Hes1 expression inhibits the expression of proneural genes, Notch ligands and cell cycle regulators, suggesting that Hes1 oscillations are of key importance for their concerted function in neural progenitors, in which Hes1 oscillations coordinate self-renewal and differentiation. The mechanisms regulating oscillations in Notch signaling are not fully understood but evidence indicates that Hes1 oscillations are regulated by negative feedback loops with delayed timing. Hes1 represses its own expression by direct binding to its own promoter, leading to a rapid downregulation of Hes1 mRNA and protein levels and upregulation of Dll1, thereby generating oscillations in Hes1 expression in NSPCs, which regulate their maintenance. Further studies have demonstrated that Hes1 is engaged in double negative feedback loops involving specific microRNAs as well. Specifically, in the case of Notch signaling in neural progenitors, miR-9 inputs into the Hes1 ultradian oscillator system to introduce a secondary negative feedback loop that controls the emergence and timing of alternative cell states in NSPCs. Thus, the Notch pathway provides a relevant example demonstrating that different delayed negative feedback loops can be engaged in the generation of oscillatory behavior in transcription factor activity.

2.3 Glucocorticoid receptor signaling

Glucocorticoid receptor signaling has well-characterized effects on neural stem cells. The most commonly reported observation is a marked inhibition of proliferation induced by activation of the GR by its natural or synthetic agonists. Further research has established that glucocorticoids also regulate other cellular functions in NSPCs, such as survival, senescence, cell fate and differentiation. All in all, the available experimental evidence indicates that glucocorticoids regulate multiple cellular functions in NSPCs through the activation of the GR. For a more extensive discussion of this evidence we refer the reader to a recent review. As a central component of the hypothalamus-pituitary-adrenal hormonal axis, signaling mediated by the ligand-activated transcription factor GR is regulated at multiple levels, including: 1) the hormone (ligand) synthesis level in the adrenals; 2) the hormone access, binding and activation of the GR; 3) the receptor translocation from the cytosol to the nucleus; 4) the transcription efficiency of target genes resulting from the interaction with transcriptional coregulators and other transcription factors and 5) the negative feedback regulation of releasing factors at the central hypothalamus and pituitary. A thorough description of GR signaling and its complex regulation is beyond the scope of this discussion and we refer to a previous review.

Glucocorticoid signaling involves circadian and ultradian oscillations that regulate multiple organismal and cellular functions to coordinate e.g. energy availability and stress responsivity. Ultradian and circadian CORT rhythms are intrinsically linked, as ultradian pulses are a necessary component of circadian oscillations. Ultradian oscillations in glucocorticoids have the highest amplitude around awakening, which then declines, effectively contributing to a sinusoid curve, characteristic of most circadian rhythms. Circadian and ultradian glucocorticoid oscillations have likely evolved to help adaptation...
Several mechanisms may underlie the generation of oscillations in GR-mediated transcriptional activity. At the organism level, the glucocorticoid lipophilicity prevents it from being stored in membrane vesicles. Thereby, glucocorticoids need to be synthesized de novo in the adrenals, in response to ACTH stimulation. This crucial step introduces a built-in time-delay in the hypothalamus-pituitary-adrenal negative feedback loop, which then presents intrinsic oscillatory activity. To the presence of ultradian...
oscillations, all the tissues tested, including the hippocampus where adult NSPCs reside, are exposed to a pulsatile GR activation, which has considerable consequences for the activation of responsive genes.\textsuperscript{68–70}

At the cellular level, other mechanisms take place that contribute to GR-dependent oscillatory activation of target genes: 1) NSPCs express specialized microtubule-associated proteins that tightly control GR cytosol-to-nucleus translocation.\textsuperscript{71} This translocation step possibly introduces another time-delay within GR-mediated transcription of target genes, that may favor oscillatory behavior; 2) Ultradian glucocorticoid oscillations induce cyclic GR-mediated transcriptional regulation, both \textit{in vitro} and in animal models. This “responsive gene pulsing” is driven by rapid GR exchange at DNA response elements and by intranuclear GR recycling through the chaperone machinery, which promotes GR activation/reactivation in response to the ultradian hormone release.\textsuperscript{38} This GR recycling introduces another built-in time-delay, which may favor oscillatory behavior, and serves pulsatile and constant hormone stimulation to induce unique, treatment-specific patterns of gene and regulatory element activation; 3) The GR is targeted by microRNAs such as miR-124 and miR-433, with the latter dampening GR signaling and impacting on circadian rhythms.\textsuperscript{73,74} miR-124 expression is in turn regulated by glucocorticoids through GR-binding elements in its promoter region, thereby generating a miR-124/GR negative feedback loop.\textsuperscript{40,75} Thus, the GR signaling pathway presents a well-characterized example of oscillatory transcriptional regulation in which several processes interact with each other, i.e. the inclusion of several negative feedback loops, many intermediate steps, a threshold concentration that must be reached before the GR biologically active (ligand affinity), and saturated sequestration/degradation steps. Together, this provides built-in time delays that characterize model oscillatory systems.

2.4 Mimicking alterations in oscillatory behavior

These data suggest that oscillations in transcription factor activity in NSPCs, and their functional consequences, may be much more widespread than hitherto demonstrated. Indicatively, a recent study has demonstrated that 43% of all coding genes show transcriptional oscillations in mice.\textsuperscript{76} We propose that one of the reasons why a further characterization of transcription factor oscillations in NSPCs has not been fully achieved yet, may lie in the technical limitations associated with measuring and mimicking oscillatory activity in an appropriate manner, which requires real-time imaging of individual cells and/or complex incubations with the adequate ligand, in the case of ligand-induced transcription factors such as the GR.\textsuperscript{38,77}

Future Directions (2)

In \textit{chapter 5} we showed the role of the GR and the importance of the oscillatory behavior of its ligand, CORT, in regulating NSC activation, NSC maintenance, and differentiation of its progeny. Interestingly, the GR is also regulated by miR-124, and knockdown of the GR results in cellular phenotypes similar to those seen under seizure conditions, where miR-124 is upregulated. One interesting hypothesis arising from our data is that the GR may be involved in the induction of aberrant AHN seen upon seizure induction. It will be interesting to see whether epileptic seizures affect GR expression via miR-124, and whether this loss of GR expression could play a role in the increased activation of NSCs, the aberrant differentiation of new neurons, and depletion of the NSC pool.
General Discussion

Studies of transcription factor action frequently measure gene responses after long-term stimulation or exogenous (over)expression. However, the mechanisms and examples we discuss here suggest that such treatments may not provide a complete and accurate view of their physiological activity. This concern may have relevance not only for studies assessing physiological transcription factor activity but also for the treatment of common human conditions such as chronic inflammatory diseases and neoplasias. In these cases, for example, patients are treated with high doses of synthetic GR ligands, ignoring the importance of dynamic oscillatory activity. Furthermore, chronic sustained treatment with the GR agonist prednisolone represses the circadian oscillation of clock gene expression in mouse and prenatal exposure to excess glucocorticoids induces depression-like behavior and impaired adult hippocampal neurogenesis in old mice, which correlates with the absence of circadian oscillations in hippocampal clock gene expression.

3. Disturbed regulation of AHN by epileptic seizures

3.1 Deregulation of NSC fate by epileptic seizures

Studies on AHN in the context of epileptic seizures have mostly been focusing on the aberrant differentiation and integration of newborn neurons after the induction of SE. In chapter 4, we studied the effect of epileptic seizures on NSCs directly, focusing on KA-induced NSC fate changes. Under physiological conditions Type A NSCs mainly generate NPCs, which give rise to newborn neurons, and to a smaller extent generate Type B NSCs, which produce new astrocytes.

We show for the first time that non-convulsive epileptic seizures induced by 2.22 mM KA significantly affect NSCs fate decisions. Upon exposure to epileptic seizures, NSCs show an increased direct conversion into reactive astrocytes, a process first identified by Sierra et al. under convulsive epileptic conditions induced by 20 mM KA. While convulsive seizures result in a complete conversion of all NSCs into reactive astrocytes, we here showed a partial conversion into reactive NSCs, indicating a possible seizure intensity/KA dose-dependent effect. Simultaneously, we showed a significant decrease in the conversion of Type A NSCs into Type B NSCs. Since Type B NSCs normally differentiate into new mature astrocytes, it is an interesting question whether epileptic seizures could alter astrocyte functionality as well, a process less-well studied in the context of non-convulsive seizures. According to our data, it seems that the generation of astrocytes after exposure to non-convulsive seizures has shifted from Type B NSC-dependent astrocyte generation towards the direct generation of reactive astrocytes from Type A NSCs. One outstanding question is to what extent these two different populations of astrocytes differ from each other, and what the functional consequences of this shift might be for the hippocampal network.

Future Directions (3)

Astrocytes play key roles in regulating homeostasis in the hippocampus. Type A NSCs normally produce astrocytes through the induction of Type B NSCs, which differentiate into mature astrocytes. We show in chapter 4 that non-convulsive seizures induce reactive NSCs, at the cost of Type B NSCs. Though both finally generate astrocytes, one interesting and outstanding question is to what extent these two populations of astrocytes differ, and what their impact on the hippocampal network is.
Moreover, the direct conversion of Type A NSCs into reactive astrocytes has been proposed to be the driving force behind the exhaustion of the hippocampal neural stem cell pool\textsuperscript{15}. Several studies have already shown that the neurogenic capacity decreases with age, as the number of (activated) hippocampal NSCs decreases over time\textsuperscript{91,92}. Furthermore, it has been established that each NSC holds a limited self-renewal potential limiting the number of rounds of activation it can go through to generate new neurons before NSCs terminally differentiate into astrocytes\textsuperscript{93}. Hyperactivation of NSCs, for instance by epileptic seizures, significantly speeds up this process resulting in exhaustion of NSCs at younger age. This increased exhaustion of NSCs, together with the increase in direct conversion of Type A NSCs into reactive astrocytes, may significantly affect the neurogenic capacity of the hippocampus\textsuperscript{15}.

3.2 Seizure intensity as a possible modulator of aberrant AHN

Besides affecting NSCs directly, as we have shown in chapter 4, epileptic seizures are also classic moderators of NSPC proliferation, newborn neuron differentiation, and integration. We have already extensively discussed these alterations in chapter 1. To briefly summarize; the induction of SE results in increased proliferation of NSPCs, decreased NSPC apoptosis, altered maturation of newborn neurons accompanied by changes in dendritic spines and excitability, and ectopic localization resulting in aberrant integration into the hippocampal network. All these changes may be involved in the process of epileptogenesis, resulting in chronic epilepsy at a later time point.

The first hints for a possible seizure-intensity dependent effect on neurogenesis came from the work of Mohapel \textit{et al}\textsuperscript{93}. They showed that the intensity of electrical stimulation-induced SE determines the long-term effects on newborn granule cell survival, but not on the initial granule cell proliferation. It was shown before that apoptosis also plays a major role in AHN regulation, with the majority of newborn granule cells undergoing apoptosis\textsuperscript{94}. Furthermore, it has been shown that caspase-mediated apoptosis is triggered after SE, and that the strength of this response determines granule cell survival\textsuperscript{95–97}, and that the initial SE event may be the main trigger for neuronal cell death, not the subsequent spontaneous seizures\textsuperscript{98}. The study by Mohapel \textit{et al}\textsuperscript{93} followed up on these data and showed that animals in the group with the highest seizure severity exhibited a bigger loss of newborn granule cells, and generally a more severe DG pathology based on the extent of neuronal cell loss. Interestingly, animals exhibiting less severe seizures showed a higher rate of newborn granule cell survival, and less DG pathology.

A second interesting \textit{in vivo} study in this context was presented by Hung \textit{et al}\textsuperscript{99}, who showed an association between the number of newborn (ectopic) granule cells and the duration of initial convulsive seizures in rats treated with pilocarpine. Based on this, it could be hypothesized that the intensity and possibly the duration of initial seizures determine the morphological and functional outcome of newborn granule cells. A more severe initial insult could result in a population of granule cells exhibiting the aberrant morphological alterations mentioned before, where less severe, initial insults would not.

Given the recent model by Schneider-Mizell\textsuperscript{100}, indicating that the addition of a single newborn neuron to a previous-established network can severely deregulate network functioning if conditions are not optimal, one could hypothesize that the integration of a small subset of aberrant granule cells with particular characteristics is pro-epileptogenic. However, as became apparent by the work of Murphy \textit{et al} not all newborn neurons seem to mature and differentiate in the same manner after the occurrence of seizures\textsuperscript{13}. Their
work very nicely showed that only a small percentage (approximately 10%) of all newborn neurons born after SE induction develop to be hyperexcitable, and therefore thought to drive the characteristic hyperexcitable state of the hippocampal network, while the majority of newborn neurons show signs of decreased excitability. These findings form the framework for the seizure intensity-dependent hypothesis raised by us in chapter 1.

3.2.1 How to deal with cell-to-cell variation in therapeutic approaches

If only a small percentage of adult-born granule cells would significantly contribute to the epileptogenic process, what may then be the role of the (rest of the) population of adult-born granule cells after seizures, which may show decreased excitability? Clinical observations show that cognitive deficits still occur in patients with subclinical epileptic activity and/or controlled seizures. These findings support our seizure intensity-dependent threshold theory, with low epileptic activity resulting preferentially in the generation of hypoexcitable granule cells that may interfere with normal functions of the DG. If the latter is the case, the seizure severity-dependent threshold theory may provide a framework for understanding the contradictory findings from preclinical work, as well as the clinical observations in epilepsy patients.

To study the contribution of different newborn granule cell populations generated after SE to epileptogenesis, future studies will have to focus on discriminating between their differential intrinsic properties derived from varying seizure intensities. The level of seizure intensity can be regulated in some rodent models of epilepsy, including kindling models and/or SE models (either chemically or electrically induced), and as we have shown in chapter 3 by titrating the dosage of KA combined with focal administration in the hippocampus directly.

In a first step, read-outs currently used to determine granule cell excitability could be used to classify newborn cells in either hypo- or hyperexcitable. If indeed these experiments show a differential induction of granule cell types after seizures of different intensity, “intensity-dependent” seizure models could be used to further study the relative contribution of these populations to epileptogenesis. In this respect, optogenetic techniques could be applied to control (both increase and decrease, depending of the
opsin used) electrophysiological properties of specific hippocampal cell populations in vitro and in vivo\textsuperscript{103,104} (and recently reviewed in\textsuperscript{105}). However, the definition of hypo- or hyperexcitable newborn granule cell populations can only be done a posteriori when the cells have already acquired their aberrant electrical properties. Therefore, an ideal optogenetic intervention would require a distinction between the different types of granule cells with specific markers, either molecular or morphological. As presented in the introduction of this thesis, these markers have not been clearly identified yet and this remains an open question. However, some promising observations have been made. For example, the fact that PTEN deletion results in the generation of hyperexcitable granule cell populations\textsuperscript{106}, suggests that the PTEN or mTOR signaling pathway could be used to direct optogenetic probe expression in specific granule cell populations. Similarly, we have recently shown that in vivo knockdown of the glucocorticoid receptor (GR) in individual newborn granule cells induces significant increases in spontaneous glutamate-receptor-mediated transmission\textsuperscript{60}, suggesting that GR knockdown could be used in combination with optogenetic tools to generate and control newborn granule cell populations with aberrant properties.

In summary, inducing different populations of granule cells using increasing seizure severities, could allow to characterize morphological, molecular, and functional differences between the populations using single cell transcriptomics, which can then be used to specifically target one group of granule cells. Once we are able to specifically target one group of granule cells, either using viral transfections, electrophysiological recordings, optogenetics or pharmacological reagents, interfering with either of the populations will provide us with significant information regarding the role of these specific granule cell populations in the epileptogenic process.

Altogether, more specific targeting of hippocampal granule cells might provide interesting opportunities for preclinical epilepsy studies. If indeed AHN under low seizure activity contributes to the cognitive decline observed in some mesial-temporal lobe epilepsy patients, and AHN under severe seizure intensity contributes to the epileptogenic process, therapeutic interventions targeting AHN may be potentially beneficial in some cases, a possibility that warrants further experimentation.

4. Improved standardized modeling of epileptic seizures using intrahippocampal kainic acid

As discussed in chapter 2, one issue that we are facing when studying the effect of epileptic seizures on the brain are the limitations of each existing epilepsy model. The majority of all studies make use of SE-inducing models that over time generate chronic epileptic animals. These models are very useful when interested in the so-called “latent” stage of epileptogenesis, the period in-between SE and the rise of chronic recurrent epileptic seizures, where supposedly cellular, structural, and functional changes and possible structural reorganizations take place that render the hippocampal network hyperexcitable, and thus prone to develop spontaneous seizures\textsuperscript{107,108}. In order to induce chronic recurrent seizures it is necessary to induce a severe convulsive SE, which understandably goes hand in hand with higher mortality and several other comorbid features, such as severe inflammation and neuronal cell loss. These features make these models less appropriate when the study aims to understand the direct effect of epileptic seizures on specific cell types, as is the case in our chapter 4.

We therefore decided to make use of a novel experimental approach, which was
described in chapter 2. Using the well-established chemoconvulsant Kainic Acid (KA), in combination with a standardized stereotaxic delivery method, which ensures focality and allows for tight regulation of the chemoconvulsant dose, we were able to identify two lower Kainic Acid doses that both have specific effects on the electrophysiological characteristics of the hippocampal network and on the hippocampal neurogenic cascade.

One issue that has not been addressed yet using our experimental approach is the well-characterized inter-strain variability in KA sensitivity. It still remains largely unclear why several mice strains show a higher KA sensitivity compared to other strains. So far, we have only used Nestin-GFP mice on a C57Bl/6J background in our experimental approaches. Traditionally, C57Bl/6J mice are considered relatively resistant to intraperitoneal KA, however, we have been able to successfully elicit epileptic seizures with lower KA doses delivered intrahippocampally, which raises the question whether inter-strain sensitivity to KA might be more a result of the administration pathway than of the KA sensitivity per se. It would be very interesting, and might generate new insights, to test our experimental approach in multiple different mouse strains; to see whether we can bring down the inter-strain variability, which currently raises concerns in the scientific community.

Interestingly, our experimental approach has been validated across 3 laboratories and already resulted in interesting new data sets. For example, a very low KA dose (0.074mM), which does not elicit epileptic seizures, but does induce epileptiform-like spiking, as assessed by EEG recordings, still has clear effects on the neurogenic cascade accompanied by a drastically changed DG transcriptome, which both differ dramatically from the situation observed when applying the classical convulsive 20mM KA dose. A second, intermediate, Kainic Acid dose (2.22mM) significantly affects NSPC apoptosis, resulting in increased survival of newborn neurons in the hippocampus. These two studies together provide more evidence that indeed seizure intensity seems to be a crucial factor when looking at the response of the hippocampal neurogenic cascade. Moreover, these data suggest that some characteristic neurogenic responses, such as the short-term increase in proliferation, are independent of the seizure intensity since similar increases in proliferation have been found using all KA doses, while other characteristic features, such as the induction of reactive NSCs, only seems to occur when seizures reach a threshold intensity as reactive NSCs are only observed under seizure-inducing dosages (2.22mM and 20mM).

Going back to the seizure intensity-dependent theory proposed by us in chapter 1, we can hypothesize that neurogenic responses observed under 0.074mM KA have distinctly

**Future Directions (5)**

We show in chapter 2 that varying intensities of epileptic seizures can be modeled using the administration of different doses of intrahippocampal KA. In this thesis we have used this model to address questions concerning the effect of non-convulsive seizures on NSCs. However, it is yet unclear whether these non-convulsive seizures also result in the development of chronic recurrent seizures, one characteristic that has to be met in order to qualify a model a proper epilepsy model. So far, no epilepsy models exist that make use of non-convulsive SE induction, which makes it interesting to see whether a convulsive SE event can be circumvented by local administration of the chemoconvulsant, to establish a new epileptogenesis model with decreased animal suffering.
different functional outcomes from the neurogenic events happening restrictively under seizure conditions. One of the longstanding agreements thus far in scientific literature is that chronic epilepsy will not develop if the initial seizure event, in this case SE, is not strong enough. As mentioned before, this is one of the key arguments for using such high intraperitoneal chemoconvulsant doses resulting in a convulsive SE phenotype and high mortality rates. The search for crucial cellular alterations during epileptogenesis that actually drive disease progression has been one of the main fields of interest for the past decades. Our experimental approach now allows us to differentiate between possible general seizure-induced neurogenic responses elicited by any type of epileptiform activity versus seizure-induced neurogenic responses that are only observed under severe seizure conditions, which potentially might be the critical factors driving the epileptogenic process resulting in chronic epilepsy development.

5. Profiling microRNA signatures upon seizure induction

Since the discovery of miRNAs, and their vast regulatory functions, numerous miRNA profiling studies have been performed to assess the effect epileptic seizures have on the miRNA profile (see table 2 in chapter 1). As we describe in chapter 1, the recent publication of the EpimiRbase provides us with an up to date overview on all miRNA profiling studies that have been carried out in the preclinical epilepsy field. As we mentioned before, this database once more shows the great variability in miRNA profiles observed between different epilepsy models, and different time points of analysis. However, this database also provides us with the opportunity to look for common targets that recur in different epilepsy models, indicating a potentially more critical common role in disease development.

In order to develop strong therapeutic strategies we will first need to optimize the preclinical models used so far. For example, in the case of miRNA profiling of the epileptic brain there is no standardized procedure for tissue selection, resulting in “epilepsy” miRNA profiles from numerous different sources and timepoints. Since we here try to understand the role of miRNAs in AHN, it would be preferable to have miRNA profiles from the DG specifically, according to our approach in chapter 3, instead of whole hippocampus, CA1, or CA3 tissue. An even more sophisticated approach that holds great promise are the recent technological advancements in single cell ‘-omics’110,111. If we could identify miRNA profiles in individual cells from all different cell types in the AHN cascade we could systematically elucidate their roles and we could be one step closer to identifying pathological miRNA expression and a potential miRNA-based therapy. Additionally, long non-coding RNAs (lnRNAs) and circular RNAs (circRNAs), which have been recently discovered as potent regulators of gene expression112,113, can also be profiled using single cell sequencing strategies.

5.2 Future application of microRNAs as potential therapeutic strategy against epileptogenesis/cognitive comorbidity

The complexity by which miRNAs regulate various biological processes related to neurogenesis makes it a daunting challenge to summarize their role in the regulation of stem cells and AHN. The list of miRNAs that regulate AHN keeps on growing (see table 1, chapter 1), and more and more complex levels of miRNA regulation are being identified. In chapter 1 we have summarized some examples of miRNAs and their targets that may function as ‘hubs’ for the regulation of AHN. This concept is in line with recent observations that indicate functional convergence of multiple miRNAs on the same
biological processes, or even on the same mRNA targets\textsuperscript{19,31}. By doing so, miRNAs have the potential to achieve precise temporal and spatial regulation of biological processes. Specifically, the convergence of two or more miRNAs on common targets will significantly reduce the number of potential targets because the number of common targets is lower than the list of potential individual targets in most cases. However, the converging actions of miRNAs remain a poorly understood layer of complexity in miRNA regulation, but some examples involving NSPCs have recently been described\textsuperscript{19}.

5.3 Feasibility of clinically applied miRNA-based therapies

Besides the biological complexity of miRNA functioning, applied clinical RNA therapy poses multiple hurdles that still need to be overcome. First, RNA molecules are generally unstable due to the presence of hydroxyl groups. Since the discovery of siRNAs and their promised therapeutic value several strategies have been developed to stabilize small RNA molecules, which can also be applied to miRNAs. Most of these strategies involve chemical modification of the RNA, such as ribose 2’OH modification\textsuperscript{114} and LNA modification\textsuperscript{115,116} that significantly stabilize ssRNAs through multiple mechanisms. Interestingly, naked small RNA molecules administered using osmotic minipumps or directly by stereotaxic injection into the brain specifically regulate intended targets \textit{in vivo}, shortly after injection in some cases\textsuperscript{117–119}.

A second issue that arises in view of clinical application is the miRNA delivery method, especially in the case of neurological disorders, since the blood-brain barrier normally excludes large polar molecules such as oligonucleotides from entering. MiRNA-based treatments could be administered in several non-invasive ways, bypassing the need for intracranial administration. First, it has recently been shown that miRNAs can be successfully delivered to the brain via intranasal administration\textsuperscript{120}, although the mechanisms through which miRNAs reach their target is still unclear. Secondly, the recent discovery of exosomal trafficking of miRNAs\textsuperscript{121} holds great therapeutic value, since exosomes can cross the blood-brain barrier and thus be administered intravenously\textsuperscript{122}. Another possibility is to time intravenous delivery of the molecules with breaches in the integrity of the blood-brain barrier, such as may occur following brain injury or after prolonged seizures.

With all current modifications of ssRNAs and new delivery methods that will allow non-invasive miRNA administration to the brain, miRNA-based treatments for neurological disorders seem within reach, at least from a technical point of view. Though mainly in cancer research, several clinical trials are already in progress trying to establish miRNA-based therapy\textsuperscript{123–126}.

Besides being a potential therapeutic target, miRNAs also hold great promise as potential biomarkers\textsuperscript{127}. With the recent discovery that miRNAs are excreted in exosomal vesicles and can be detected in blood\textsuperscript{121,128–130} it has now become possible to non-invasively identify altered miRNA expression levels from pathological brain tissue\textsuperscript{131}. Several studies have already obtained miRNA profiles from epileptic patients using blood samples\textsuperscript{130,132}, allowing comparisons with preclinical epilepsy models\textsuperscript{133}.

5.4 MiRNA-based therapeutic strategies in epilepsy

AHN-focused miRNA-based therapies for epilepsy seem rather challenging considering the existence of multiple miRNAs targeting the same biological processes or pathways,
complex regulatory feedback loops within these pathways, and bidirectional expression control in the form of feedback loops. However, several attempts to identify miRNA-based anti-epileptic therapies have been performed, though not primarily focusing on AHN. miR-34a has been the focus in two different rodent epilepsy studies. Silencing miR-34a using antagonirs during SE induction successfully reduced neuronal apoptosis in CA1 and CA3, but this was not assessed in the DG\textsuperscript{134}. Therefore extrapolating these findings to AHN requires caution. A second study administering antagonirs against miR-34a, but now 24 hours post SE induction, did not find any neuroprotective effects, indicating a potential time-dependent treatment window\textsuperscript{135}. More importantly, both studies also failed to show any beneficial effects of antagonir-34a administration of seizure duration and severity.

Probably the most compelling evidence supporting the future feasibility of miRNA-based epilepsy therapy comes from two studies targeting miR-134 expression. As mentioned before, miR-134 is involved in the regulation of spinogenesis, and therefore is an important regulator of excitability and network formation. Administration of antagonir-134 one day before SE induction significantly decreased the proportion of animals developing pilocarpine-induced SE. Furthermore, animals that did develop SE showed a significant delay in seizure onset and a decrease in total seizure power\textsuperscript{136}. In a second study, antagonir-134 administration one hour post SE induction reduced the occurrence of chronic spontaneous seizures by 90\%\textsuperscript{137}. Furthermore, antagonir-134 administration reduced CA3 pyramidal spine density, neuronal cell loss, and astrogliosis, which are all hallmarks of the epileptic hippocampus. Strikingly, these findings have later been replicated using different epilepsy models, showing the robust effect antagonir-134 treatment has on seizure formation\textsuperscript{138}. Like the studies performed on miR-34a, these studies on miR-134 did not assess any AHN-related pathology.

Given the current limited evidence for miRNA-based anti-epileptic treatment, the future for miRNA-based therapies remains still uncertain but is heavily investigated. Though some preclinical evidence points towards successful application of single miRNA-based anti-epileptic approaches, one might argue that the future of miRNA-based therapy comprises multi-miRNA approaches instead, based on the robust regulation of common targets or biological pathways. Fundamental research is providing more and more evidence regarding complex interplay between different miRNAs, raising questions about single-miRNA therapies.

Interestingly, recent challenging experiments have demonstrated that AHN may be associated with cognitive impairments observed in epilepsy\textsuperscript{139}. Cognitive impairment comorbidity among epilepsy patients is high and in many cases it still progresses even when seizures have been controlled\textsuperscript{140–142}. If these cognitive deficits indeed severely rely on the presence of aberrant AHN, and miR-based treatments can rescue cellular alterations observed under pathological conditions, one might argue that miR-based\textit{Future Directions (6)}

Our work in chapter 4 provides a first insight into how miRNA-based therapies might be able to restore AHN after the induction of SE. Although we merely focus on restoring NSC fate decisions after SE onset, the interesting and logical next step is to assess the effects of antagonir-124 & -137 administration of the epileptogenic process in the long term.
therapy against cognitive deficits associated with epilepsy, may be another promising direction for future miR-based therapeutic studies. Thus, most likely, multi-miRNA-based approaches targeting several aspects of AHN will provide promising avenues to successfully rescue disease-associated aberrant AHN, and they could pave the way towards future clinical applications of miRNA-based therapies.

5.4 AHN as a target for miRNA-based therapies in epilepsy

Several studies have already shown a significant role for AHN in the epileptogenic process. Most compelling evidence comes from studies using (genetic) ablation of neurogenesis around the time of SE induction, which results in suppression of chronic recurrent seizures, and prevents associated cognitive comorbidities\textsuperscript{139,143}. These findings suggest that the prevention of aberrant AHN, though in a very crude way, might be sufficient to prevent a single seizure event, or primary insult, to develop into chronic epilepsy. Since full ablation of neurogenesis is rather crude, and cannot be translated to the clinical situation, we must look for other therapeutic strategies that are able to prevent the rise of aberrant AHN. MiRNAs provide a promising tool in this respect, for both practical as well as for mechanistic reasons, as described in the previous sections.
References

that does not respond to fluoxetine.


59. Wong EYH, Herbert J. The corticoid environment: a determining factor for neural progenitors' survival in the adult hippocampus.


146. Schouten M. Epigenetic control of hippocampal stem cells: modulation by hyperactivation, glucocorticoids and aging.