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(Patho)physiological regulation of adult hippocampal neurogenesis

By seizures, glucocorticoids and microRNAs

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1.

General Introduction

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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.

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&

MiRNA-mediated regulation of adult hippocampal neurogenesis; implications for epilepsy.

Pascal Bielefeld, Catherine Mooney, David C. Henshall, Carlos P. Fitzsimons.

Brain Plasticity, 2016

Introduction

Adult neurogenesis is the process in which new neurons are derived from neural stem cells (NSCs) throughout adult life. It takes place in a few areas of the mammalian brain, such as the olfactory bulb, the subventricular zone (SVZ), and the Subgranular Zone (SGZ) of the Dentate Gyrus (DG) of the hippocampus, where it is termed Adult Hippocampal Neurogenesis (AHN). Although most knowledge about AHN comes from preclinical animal studies, it was recently shown that the adult human hippocampus retains a neurogenic capacity as well, with about one third of all granule cells being replaced throughout life at a turnover rate of 1.75% per year, which resembles the neurogenic capacity in the middle-aged mouse^{1,2}.

AHN can be divided into several distinct stages (Fig. 1A), each of which is under specific control by cell-intrinsic gene expression programs and environmental factors. This allows tight temporal and spatial regulation of the neurogenic process within a restricted microenvironment and according to the needs of the organism^{3,4} (Fig. 1B). Upon stimulation, quiescent neural stem cells (NSCs) in the SGZ re-enter the cell cycle and become proliferative NSCs. These activated NSCs have the potential to both self-renew and give rise to early amplifying neural progenitors (aNPCs), thereby maintaining the NSC pool, while expanding the progenitor pool. The final fate of activated NSCs is still debated as it has been shown that activated NSCs terminally differentiate into astrocytes after multiple rounds of asymmetric division⁵, while another study suggests that NSCs can re-enter quiescence after several rounds of asymmetric division and only be later activated again⁶, which is thought to act as an intrinsic mechanism to maintain the NSC pool.

Newly generated aNPCs undergo multiple rounds of symmetric division, expanding the neurogenic pool and giving rise to mature neurons. Soon after their birth many aNPCs are depleted by apoptosis, which may prevent an excess production of new and/or unfit neurons^{7,8}. The surviving aNPCs give rise to neuroblasts, which, after neuronal differentiation, migration, and maturation, will integrate into the preexisting DG network, taking in total approximately 4 weeks⁹⁻¹¹. The neurogenic capacity of NSCs in the adult DG is not infinite and decreases throughout life, mainly due to decreases in proliferation and a loss of NSCs, possibly through astrocytic conversion⁵.

Though the exact role of AHN is still debated, it has become clear that newborn granule cells play a key role in hippocampus-dependent cognitive capacity^{10,12-15}, while it has also been hypothesized to have intrinsic therapeutic functions, responding to a local loss of neurons by e.g. disease conditions or in response to pharmacological treatment¹⁶⁻²³.

Thesis Aim 1

The decrease in hippocampal neurogenesis throughout ageing has long been hypothesized to underlie cognitive deficits that occur at late age. Interestingly, at late age the hippocampus still contains neural stem cells, though these are not proliferative. As described before, it is thought that NSCs can undergo several rounds of activation and proliferation before they terminally differentiate into astrocytes and lose their neurogenic capacity. In **chapter 5** we try to answer the question why some NSCs remain at late age, while others disappear. We address the heterogeneity of the hippocampal NSC pool, and possible functional explanations why some populations remain present throughout ageing whereas others disappear.

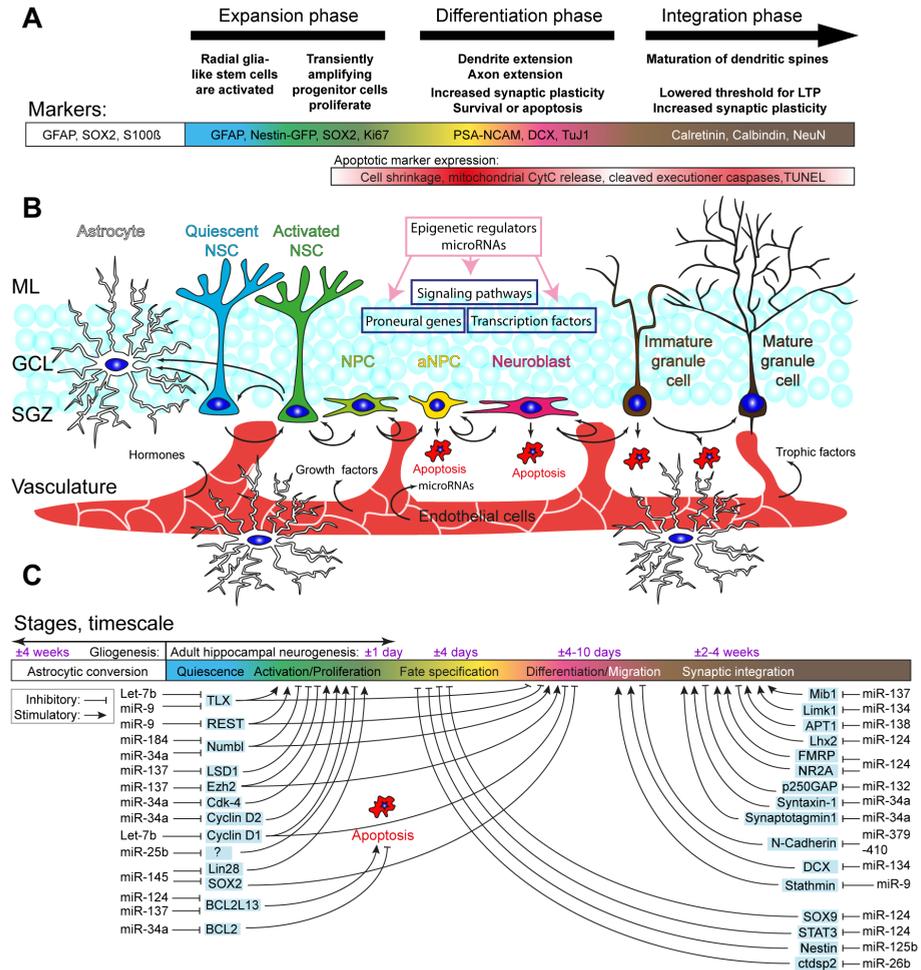


Figure 1. Schematic overview of the hippocampal neurogenic niche, the different processes underlying AHN, and its regulation by microRNAs.

A) Overview of the different stages of AHN. Each cell type can be identified by a combination of presence and absence of markers, combined with morphological cellular features.

B) Overview of the neurogenic niche and the transition of a NSC into a mature neuron. The complexity of the neurogenic niche, consisting of multiple cell types in close association with the vasculature, allows for both local and distant cell communication. Distant cell communication occurs via factors released in the bloodstream, such as cell-extrinsic miRNAs, growth factors (VEGF and bFGF), hormones, and trophic factors (BDNF). Other cell-intrinsic factors, such as miRNAs, TLX signaling, notch signaling, and REST (purple boxes), and cell extrinsic factors such as HDACs, DNA methylation, and miRNAs (pink box), complete the coordinated regulated of AHN.

C) MiRNAs regulate various key pathways important in AHN. Depicted are miRNAs of which a clear link with neurogenesis has been identified, together with their targets through which the miRNAs may exert their effects.

1.1 Regulation of AHN

As mentioned before, AHN is controlled by a wide array of cell-intrinsic and cell-extrinsic factors. This complex interaction becomes clear when studying the fate of neural stem/progenitor cells (NSPCs) upon transplantation to ectopic locations. NSPCs derived from the SGZ will give rise to astrocytes when transplanted into non-neurogenic areas of the brain, while NSPCs derived from the SVZ or from the spinal cord will give rise to granule cells when transplanted into the SGZ^{24,25}. These observations indicate that the impact of the local environment, or neurogenic niche, is crucial for neuronal development.

The hippocampal neurogenic niche comprises many different cell types, such as the NSPCs, neuroblasts and their progeny, mature granule cells, but also astrocytes, GABAergic interneurons, microglia, macrophages, and endothelial cells connecting NSCs and their progeny to the vasculature (Fig. 1B)^{26,27}. The local vasculature and its associated extracellular matrix provide a means for both local cell-cell interaction, i.e. via β -Catenin, which regulates mitotic spindle orientation^{28,29}, and distant cell-extrinsic regulation of AHN, thereby carrying and binding both pro-neurogenic growth factors and trophic factors released by both neuronal and non-neuronal cells, such as VEGF, bFGF, and BDNF^{30–34}. Together, all these elements provide the hippocampus with a finely tuned microenvironment permissive for adult neurogenesis.

While the cellular and vascular composition of the niche provides a crucial structural organization, the local responsiveness of NSPCs and their progeny is also under tight molecular control, e.g. by epigenetic regulation of chromatin that renders cells permissive to the regulation of gene transcription^{35–37}. Epigenetic control of AHN is a combination of NSPC intrinsic mechanisms and extrinsic regulation by non-NSPC cells within and even outside of the neurogenic niche³⁸. Conceptually, both temporal and spatial control of gene expression are crucial for progression of the different stages of AHN and for maintaining NSPC pluripotency and proliferative capacity³⁹. Furthermore, cell fate specification and neuronal differentiation are partly determined by removing epigenetic repressive marks of NSPC differentiation-related genes, whereas non-cell lineage specific genes are permanently silenced^{40–42}. In short, epigenetic (transient) gene repression or silencing is key in controlling the switch from proliferation to neuronal differentiation in NSPCs³⁸.

Epigenetic mechanisms known to play key roles in the regulation of AHN include, among others, chromatin modifications. Chromatin can exist in an open transcribed state or a closed and silent state, regulated by DNA methylation and histone modifications, such as acetylation and methylation. Histone methylation by DNA methyltransferases (DNMTs) and (de)acetylation by histone acetyl transferases (HATs) and histone deacetylases (HDACs), two of the most common forms of chromatin modification, provide a first layer of molecular control over gene expression, rendering the genome (in)accessible to the transcription machinery. Another classic epigenetic regulatory mechanism active in adult neurogenesis is DNA methylation, in which cellular DNA can be covalently modified, usually but not exclusively at locations rich in CpG dinucleotides, through methylation of the carbon group at the fifth position on the pyrimidine ring of the cytosine residue⁴³. These and other levels of epigenetic control of neurogenesis have recently been extensively reviewed⁴⁴. Chromatin can be further silenced by repressor proteins, including the Polycomb group proteins, resulting in complete silencing of a locus⁴⁵.

More recently, non-coding RNAs such as microRNAs (miRNAs) have been shown to play a central role in epigenetic regulation of NSPC, as well^{46–49}. MiRNAs are small, non-coding

RNA molecules composed of approximately 22 nucleotides that are transcribed from endogenous hairpin-shaped transcripts by RNA polymerase II or III. Their transcripts are called pri-miRNAs and will be cleaved into pre-miRNAs in the nucleus by the Drosha/DGCR8 complex. After exporting the pre-miRNA from the nucleus it is once again cleaved, this time by the Dicer/TRBP complex, to its mature functional miRNA length⁵⁰. Recently, several other miRNA biogenesis pathways have been identified, including Drosha and Dicer independent pathways. A complete summary of miRNA biogenesis pathways has recently been published⁵¹. Interestingly, although outside the scope of this review, components of these miRNA biogenesis pathways, such as Drosha, Dicer and DGCR8, also have direct, miRNA-independent, effects on gene expression⁵². Furthermore, Drosha restricts the potency of adult hippocampal NSPCs *in vivo* by targeting NFIB through a miRNA-independent pathway⁵³.

MiRNAs can regulate gene expression post-transcriptionally via either mRNA degradation or inhibition of mRNA translation by binding to the 3'UTR of their target mRNA. Hence, miRNAs can only exert their function when the specific substrate mRNA is available as well, introducing another layer of spatial and temporal complexity to gene expression regulation⁵⁴. Furthermore, numerous proteins involved in the epigenetic machinery are targets of specific miRNAs and vice versa, creating a complex multi-layered control of gene transcription⁵⁵. Due to several biological characteristics of miRNAs, such as their potent ability to (transiently) repress gene expression, their relative high conservation between species, and their rapid turnover, it has been hypothesized that miRNAs might be key regulators of AHN³⁸.

Local temporal control of gene expression is key in regulating the switches between different distinct stages of AHN. Down regulating non-lineage specific genes and removal of repressive marks on the chromatin of lineage-specific genes are excessively abundant forms of AHN regulation, both of which are likely candidates for miRNA regulation. Interestingly, several studies have demonstrated in other tissues that miRNAs are not only found intracellularly, but are also present in the extracellular compartment where they mediate cell-to-cell communication, among others via exosomes⁵⁶⁻⁵⁸. In recent years, numerous miRNAs have been characterized that play crucial roles in stage transitions of AHN by regulating key epigenetic regulators, such as HDACs, Polycombgroup proteins, and REST, but also play important roles in the apoptotic selection and functional maturation of immature neurons (Fig. 1C, Table 1). In order to discuss the complex role miRNAs play in regulating AHN, we here first review stage-specific regulation of neurogenesis by miRNAs.

1.1.2 Neural stem cell maintenance and quiescence

To preserve the neurogenic capacity throughout life, it is critical that NSCs retain both their proliferative and self-renewal capacities. A fine balance between the two is needed, as each NSC has a limited number of proliferation rounds before it terminally differentiates and loses its stem cell capacity⁵. Several miRNAs have been identified that play a crucial role in the regulation of NSC maintenance (Figure 1C). Interestingly, the role of miRNAs in the regulation of astrocytic specification of NSCs remains less well characterized.

A key pathway in maintaining neurogenic capacity is the Notch signaling pathway, which drives asymmetric division, rendering both NSCs and aNPCs⁵⁹. Activation of Notch signaling drives proliferation, and through asymmetric expression of its repressor protein

Numbl, two distinct daughter cells are derived from one single NSC⁶⁰. Absence of Numbl in NSCs results in increased proliferation and symmetric division, 'exhausting' the NSC pool. Both miR-184 and miR-34a regulate Numbl expression by binding to its 3'UTR, driving symmetric division^{61,62}. Similarly, high expression levels of miR-184 are associated with long-term impaired neurogenesis. MiR-34a also indirectly regulates Notch signaling as it targets two downstream proteins of Notch, NeuroD1 and Mash1⁶².

Another pathway implicated in stem cell maintenance throughout ageing is the Insulin/IGF pathway. FoxO3, a component of the Insulin/IGF pathway, directly regulates the expression of the miR106b-25 cluster. The main miRNA from this cluster studied in relation to neurogenesis is miR-25. Expression of miR-25 results in increased proliferation of NSPCs, possibly depleting the NSPC pool⁶³.

Besides maintaining the NSC pool through controlling proliferation rates and cell division, miRNAs may also be involved in maintaining NSC quiescence. Although this phenomenon has not yet been shown with respect to AHN and evidence from other stem cell niches is scarce, there are several interesting findings pointing towards roles for miRNAs in controlling quiescence. One study using muscle stem cells in which Dicer was conditionally knocked out showed a significant increase in proliferation rate, indicating a transition from quiescence to activation. Further analysis of miRNA expression differences between quiescent and activated stem cells yielded several candidates, from which miRNA-489 turned out to be a crucial mediator of stem cell quiescence⁶⁴.

1.1.3 NSPC Proliferation versus differentiation

As mentioned before, both NSCs and aNPCs can undergo several rounds of proliferation, thereby amplifying the NSPC pool before giving rise to new neurons or astrocytes. Several miRNAs and their targets tightly regulate this switch from the proliferative state towards differentiation.

One key regulator of proliferation is the nuclear receptor TLX. TLX activates the Wnt/B-catenin pathway and is crucial for NSPCs to maintain their self-renewal and proliferative capacities^{65,66}. NSPCs expressing TLX can proliferate, self-renew, and differentiate into all neuronal lineages, while NSPCs devoid of TLX fail to proliferate at all. Reintroducing TLX into NSPCs rescues their proliferative potential⁶⁷. Expression of TLX is controlled by several miRNAs, indicating its importance in this complex regulatory system. TLX expression is regulated by miR-9, which is highly abundant in the adult brain. At the same time, TLX controls miR-9 expression through a negative feedback loop, ensuring tight control over NSPC proliferation⁶⁸. Overexpression of miR-9 decreases levels of TLX and increases (premature) neuronal differentiation, while miR-9 knockdown increases NPC proliferation⁶⁸.

TLX forms a second regulatory loop with miR-137, which stimulates proliferation by repressing LSD1, a co-repressor of TLX, while repressing differentiation by down regulating Ezh2, a histone methyltransferase and part of the Polycombgroup proteins^{69,70}. Like miR-9, miR-137 expression levels are tightly regulated via closed regulatory loops involving TLX and its co-repressor LSD1.

A third miRNA that regulates TLX expression is Let-7b, which inhibits proliferation and drives differentiation through direct targeting of the 3'UTR of the TLX receptor⁷¹. Let-7b knockdown increases proliferation, while overexpression of Let-7b in NSCs decreases

proliferation and accelerates neuronal differentiation⁷². Let-7b also regulates Cyclin D1, an effector downstream of TLX, important for cell cycle progression. Down regulation of Cyclin D1 by Let-7b inhibits progression into the S-phase of the cell cycle, lengthening the G1-phase and thereby stimulating the transition into differentiation⁷³.

A fourth miRNA that indirectly regulates TLX expression is miR-145. MiR-145 regulates Sox2, a transcription factor crucial for stem cells to maintain pluripotency and self-renewal capacity, and Lin28, a well known suppressor of Let-7 biogenesis⁷⁴. Expression of miR-145 increases during differentiation of NPCs and thus drives differentiation indirectly via TLX regulation.

These observations indicate that TLX may work as a ‘hub’ in the miRNA-mediated regulation of NSPC proliferation and transition into differentiation. Furthermore, the complex regulation of its expression by several miRNAs, which are themselves again regulated by TLX expression through negative feedback loops, provides an optimal mechanism for a fast and transient switch in gene expression that is needed to drive NPCs out of their proliferative state and into differentiation, without losing proliferative capacity on the long term.

Finally, miR-125b also regulates the transition between proliferation and differentiation. miR-125b targets Nestin, an intermediate filament protein expressed in NSPCs, but not in neurons⁷⁵. Overexpression of miR125-b results in decreased proliferation and increased differentiation of NSPCs. These effects can be rescued by introducing mutations in binding sites for miR125-b present on the Nestin mRNA 3’UTR. Besides this direct regulation of differentiation, miR-125b also targets numerous repressors of neuronal genes, mainly effectors of the ERK signaling pathway involved in the effect of retinoic acid, highlighting a role for miR-125b in neuronal differentiation⁷⁶.

1.1.4 Cell fate specification

Under normal physiological conditions, most cells derived from adult hippocampal NSCs will differentiate into neurons, while only a small subset will give rise to astrocytes. This balance is tightly maintained, and determines the neurogenic capacity of the stem cell pool. The default differentiation path of NPCs is neuronal, while the induction of astrocytic differentiation requires the active inhibition of proneuronal genes. This active process is regulated by microRNAs, since Dicer knockout results in impaired astrogenesis^{77–79}.

DNA methylation is a main driving force behind the repression of the astrocytic fate³⁵, but recent studies show that also methylation-independent pathways are regulated by miRNAs. Most work done on cell fate specification comes from studies on miR-124, a key player in neuronal versus astrocytic cell fate determination. miR-124 is absent in NSPCs, while expression levels increase in neuroblasts and remain high in differentiated neurons. One of the targets of miR-124 is SOX9, a transcription factor crucial for gliogenesis present in both NSPCs and astrocytes, but not in neuroblasts and neurons⁸⁰. Overexpression of a 3’UTR deficient SOX9 inhibits neuronal differentiation, while a knockdown of miR-124 results in increased ectopic SOX9 expression in neuroblasts. Importantly, in the subventricular zone stem cell niche, miR-124-mediated repression of SOX-9 is important for neuronal fate determination⁸¹. A second pathway regulated by miR-124 is the STAT3 pathway, which plays a role in terminal differentiation⁸². Upon phosphorylation, STAT3 inhibits terminal neuronal differentiation, and drives cells towards a glial lineage^{83,84}. Overexpression of miR-124 reduces STAT3 phosphorylation, driving NSPCs towards the

neuronal lineage⁸². Furthermore, overexpression of miR-124 in HeLa cells, a non-neuronal cell line, results in the expression of pro-neuronal genes, while silencing non-neuronal genes⁸⁵. All together, these data indicate that miR-124 is a crucial regulator of cell fate determination, stimulating neuronal fate by suppressing glial lineage-specific genes.

MiR-9, which controls proliferation through the TLX receptor, also targets several other genes that play crucial roles in neuronal differentiation, such as Foxg1 and Gsh286. MiR-9 also regulates several members of the REST complex, which acts as a transcriptional repressor of neuronal genes, stimulating neuronal differentiation⁸⁷.

Another miRNA involved in cell fate determination is miR-26b, which is specifically expressed in NSPCs. MiR-26b targets *ctdsp2*, an important component of the REST complex, which inhibits the transcription of neuronal genes⁸⁸.

1.1.5. Apoptotic selection of newborn neurons

A majority of the cells generated by adult hippocampal NSPCs are depleted through apoptotic selection, thereby regulating neurogenesis levels. Apoptotic selection of NPCs is thought to serve as a quality control as well, selecting out 'unfit' cells^{7,8}. The first indications for a role for miRNAs in controlling apoptosis comes from *in vivo* Dicer knockout studies, although these studies focused on SVZ neurogenesis instead of AHN. Early embryonic knockout of Dicer results in increased proliferation of neural progenitors, however due to failed cell cycle progression upon differentiation, most of these cells undergo apoptosis⁸⁹.

A recent study showed that miR-124 and miR-137 act cooperatively to regulate apoptosis in NSPCs, through fine-tuning expression levels of the pro-apoptotic protein Bcl2L13, upstream of Caspase-3⁹⁰. Acting together on a common target, miR-124 and miR-137 decrease Bcl2L13 expression levels resulting in concomitant decreases of cleaved Caspase-3, which translates into a decrease in apoptosis.

A third miRNA linked to the regulation of apoptosis is miR-34a, which targets the anti-apoptotic Bcl-2 protein, as well as several cell cycle regulators involved in cell cycle progression, such as Cdk-4 and Cyclin D2⁹¹⁻⁹³. Caution must be applied in interpreting these results, as these data all originate from non-neuronal cell type studies; however, both miR-34a and some of its targets are expressed in NSPCs, thereby providing a possible new regulatory mechanism for apoptotic selection mediated by miRNAs in NSPCs.

1.1.6 Migration

Migration of newborn neurons in the DG is crucial for establishing correct new synaptic contacts with the preexisting network. This migration is controlled by several chemoattractants, such as Reelin, and various adhesion proteins like Doublecortin and PSA-NCAM. Several miRNAs have been identified that play a role in neuronal migration in the DG.

MiRNA cluster miR-379-410 regulates N-Cadherin, an adhesion protein found on cellular membranes, and is expressed in NSPCs and migratory immature neurons. Overexpression of this miRNA cluster in NSCs results in increased neuronal migration, which can be rescued by exogenous miRNA-insensitive N-Cadherin overexpression⁹⁴. The brain-specific miR-9 is expressed in NPCs derived from human embryonic stem cells and loss of miR-9

suppressed proliferation and promoted migration of NPCs independently of precocious differentiation, possible by regulating the expression of its target, stathmin⁹⁵.

Recently, miR-19 was identified to play a critical role in neuronal migration. MiR-19 is abundantly expressed in NSPCs, while its expression decreases upon differentiation. Overexpression of miR-19 in NSPCs in the adult DG results in an increase in migration of newborn neurons⁹⁶.

1.1.7 Maturation and integration

After committing to a neuronal fate, neuroblasts will undergo further maturation over the course of several weeks. Immature neurons first migrate horizontally, along the SGZ, followed by tangential migration into the GCL. They start to form axonal and dendritic processes, and develop dendritic spines to allow for neuron-to-neuron communication⁹. The development of strong synaptic connections with afferent neurons is crucial as an absence of synaptic input will generally result in selective apoptosis^{97–99}. Several miRNAs are known to regulate these processes, including dendritic outgrowth and spine formation, which are crucial for maturation and integration of newborn neurons.

MiR-34a and miR-137 negatively regulate neurite outgrowth and dendritic branching, limiting dendritic complexity of newborn neurons^{91,100}. MiR-34a targets synaptotagmin1 and syntaxin-1, two proteins crucial for functional maturation of newborn neurons. Overexpression of miR-34a results in a significant decrease of dendritic complexity, as well as a reduction in synaptic function. Inhibiting miR-34a rescues these functional deficits, while overexpression of synaptotagmin1 was able to partially rescue dendritic morphology⁹¹. MiR-137 regulates Mib1, a protein known for its crucial role in neurodevelopment that acts on Notch signaling and induces apoptosis. Overexpression of miR-137 severely impacts dendritic outgrowth and spine formation, which can be rescued by overexpression of Mib1¹⁰⁰.

While miR34a and miR-137 negatively regulate dendrite outgrowth, miR-124 positively regulates axonal and dendritic branching¹⁰¹. Knocking out Rcnr3, the primary source of miR-124, results in severe neuronal malformations and aberrant axonal sprouting. Lhx2, a primary target of miR-124, mediates these effects. Like miR-124, the miR17-92 cluster drives axonal outgrowth¹⁰². This cluster is specifically expressed in the distal axon of neurons, where it regulates phosphatase and tensin homolog (PTEN), a repressor of the mTOR pathway. Overexpression of this miR cluster results in increased axonal sprouting, which can be rescued by rapamycin treatment, an inhibitor of mTOR.

MiR-125b, miR-134, and miR-138 are known to negatively regulate spine formation^{103–105}. MiR-125b has been linked to regulation of Fragile X Mental Retardation Protein (FRMP) and its overexpression results in severely altered spine morphology. Furthermore, miR-125b also regulates the NR2A subunit of the NMDA receptor, affecting synaptic plasticity of hippocampal neurons¹⁰⁵. Both miR-134 and miR-138 are specifically enriched in dendritic spines, where they exert crucial roles for synaptic functioning. MiR-134 regulates Limk-1, a transcription factor crucial for spinogenesis¹⁰³, while miR-138 regulates APT1, an enzyme responsible for the palmitoylation state of many synaptic proteins¹⁰⁴.

On the contrary, miR-132 is thought to positively regulate spine formation, in an activity-dependent manner by regulating p250GAP expression¹⁰⁶. Its expression is regulated by CREB and is necessary and crucial for spine formation. MiR-132 expression further

Table 1.
Overview of identified microRNAs that regulate (hippocampal) neurogenesis, their targets, their role in neurogenesis, and the origin of identification.

		MicroRNAs involved in regulation of AHN		
	Target	miRNA function	Origin of Identification	References
miR-184	Numbl	Inhibits differentiation, promotes proliferation, maintenance of the neurogenic stem cell pool	Mouse NSCs	(Liu et al., 2010)
miR-34a	Numbl	Inhibits differentiation, promotes proliferation, maintenance of the neurogenic stem cell pool	Mouse NPCs from embryonic cortex	(Fineberg et al., 2012)
	BCL2	Promotes apoptosis	Mouse cortex/SH-SY5Y cell line	(Wang et al., 2009)
	Cdk-4	Inhibits cell cycle progression	Primary keratinocytes	(Antonini et al., 2010)
	Cyclin D2	Inhibits cell cycle progression	Primary keratinocytes	(Antonini et al., 2010)
	Synaptotagmin1	Inhibits synaptic development	Mouse ES cells	(Agostini et al., 2011)
	Syntaxin-1A	Inhibits synaptic development	Mouse ES cells	(Agostini et al., 2011)
miR-106b/ miR-25 cluster	IGF-signaling	Promotes NSPC proliferation, neuronal differentiation	Mouse primary NSC culture	(Brett et al., 2011)
miR-124	Sox9	Promotes neuronal differentiation	Mouse NSCs	(Stolt et al., 2003)
	STAT3	Promotes neuronal differentiation	Mouse ESCs	(Krichevsky et al., 2006)
	BCL2L13	Inhibits apoptosis	Mouse DG	(Schouten et al., 2015)
	Lhx2	Promotes neurite outgrowth	Mouse hippocampus	(Sanuki et al., 2011)
	Rap2a	Promotes dendritic branching	Mouse NSCs	(Xue et al., 2016)
miR-137	BCL2L13	Inhibits apoptosis	Mouse DG	(Schouten et al., 2015)
	Mib-1	Inhibits dendritic growth and spine formation	Mouse hippocampus & mouse primary neuronal culture	(Smrt et al., 2010)
	LSD1	Promotes proliferation	Embryonic NSCs	(Sun et al., 2011)
	Ezh2	Inhibits differentiation	Adult NSCs	(Szulwach et al., 2010)
miR-9	TLX	Promotes differentiation, inhibits differentiation	Mouse hippocampus	(Zhao et al., 2009)
	REST	Promotes neuronal differentiation	NT2 cell line	(Packer et al., 2008)
	Rap2a	Promotes dendritic branching	Mouse NCSs	(Xue et al., 2016)
	Stathmin	Inhibits migration	Human embryonic NSCs	(Delaloy et al., 2010)
Let-7b	TLX	Inhibits proliferation, promotes differentiation	Mouse NSCs & embryonic mouse brain	(Zhao et al., 2010)
	Cyclin D1	Inhibits cell cycle progression	Embryonic mouse brain	(Zhao et al., 2010)
miR-125b	Nestin	Promotes neuronal differentiation	Rat NSPCs	(Cui et al., 2012)
miR-145	Sox2	Promotes neuronal differentiation	Mouse NSC	(Morgado et al., 2016)
miR-26b	Ctdsp2	Promotes neuronal differentiation	Zebrafish & P19 cells	(Dill et al., 2012)

miR-19	Ragef2	Stimulates migration	Mouse hippocampus & mouse NPCs	(Han et al., 2016)
miR-379/ miR410 cluster	N-Cadherin	Induces migration	Mouse embryonic cortex	(Rago et al., 2014)
miR-134	Limk1	Inhibits spinogenesis	Rat hippocampus	(Schratt et al., 2006)
	Dcx	Inhibits NPC migration	Primary NPCs, primary neurons, mouse embryonic cortex	(Gaughwin et al., 2011)
miR-138	APT1	Inhibits spinogenesis	Mouse hippocampus	(Siegel et al., 2009)
miR-17/ miR92 cluster	PTEN	Induces axonal outgrowth	Rat primary cortical neurons	(Zhang et al., 2013)
miR-132	P250GAP	Promotes spinogenesis	Mouse hippocampus	(Impey et al., 2010)

promotes neurite outgrowth¹⁰⁷ and inhibiting miR-132 results in decreased EPSC frequency and numbers of GluR1-positive spines, indicating that miR-132 may play a key role in both structural and functional spinogenesis^{106,108}.

2.1 Pathological alterations in AHN in the context of epilepsy

In the mammalian hippocampus of young adult animals, newborn granule cells continue to be generated every day. In rodent models of epilepsy, the induction of an initial status epilepticus (SE) increases the number of newborn cells robustly¹⁰⁹, mainly by activating the population of quiescent neural stem cells¹¹⁰, which results in the addition of newborn neurons to the pre-existing network. Huttman *et al* showed in the kainic acid model of epilepsy that seizures also stimulate the proliferation of radial-glia cells, which play an important role in scaffold formation and migration of new neurons¹¹¹. In addition, the adult-generated granule cells born in an ‘epileptic environment’ show several distinct features when compared to new granule cells born in a normal environment, as will be described below.

2.2 Morphological features of adult-born granule cells after seizures

One of the typical features of abnormal adult-born granule cells generated after seizures is their ectopic migration into the DG hilar region rather than into the granule cell layer, which would be their normal destination. These aberrantly located cells have been termed hilar ectopic granule cells (HEGCs) and their first identification in animal models of epilepsy by Parent *et al*¹⁰⁹ was followed by studies showing that HEGCs project to pyramidal CA3 neurons where they form synaptic connections with mossy fiber axons that project into the CA3 area¹¹², and thus form recurrent circuits^{113,114}. Whereas recurrent circuits have always been considered characteristic responses to epileptic seizures, more recent work has shown that recurrent circuits are also formed amongst granule cells under normal conditions, albeit to a lesser extent and possibly only transiently^{115,116}. Hence, the formation of recurrent circuits among granule cells may not be a unique feature of a DG network affected by seizures, but their relative abundance may be increased/stabilized by seizures and could then contribute to the process of epileptogenesis.

The concept that functionally aberrant granule cells contribute to network disturbances and epileptogenesis assumes a successful functional integration and contribution of such cells into hippocampal networks. Scharfman *et al* used pilocarpine-induced SE to

show that HEGCs expressed the immediate-early gene *c-fos* after spontaneous seizures, confirming their active participation in the epileptic circuit¹¹⁷. Moreover, several studies have shown that the number of HEGCs is positively correlated with the number of spontaneous seizures^{118,119}.

One of the functions that has been proposed for the DG relevant in this context, is its function as a hippocampal 'gate keeper'. Due to its high ratio of inhibitory versus excitatory innervation of granule cells^{120–122} the DG limits the amount of excitatory input to, and thereby controls the excitation level of, the downstream CA3 circuit. In fact, the innervation of GABAergic interneurons by granule cells serves as a potent negative feedback mechanism, that can control hippocampal excitability¹²³. Failure of this DG gating function has been observed in several rodent models of epilepsy, and may facilitate epileptiform activity^{124–128}. A shift in the excitation/inhibition ratio could possibly occur as a result of the introduction of adult generated cells with different properties^{129,130}. Moreover, studies have shown that spontaneous temporal seizures originate in the DG, and are characterized by an increased firing frequency prior to seizure onset¹³¹ and by differences in *c-fos* expression over time between brain areas¹³². Interestingly, the dentate granule cells are the first to show *c-fos* expression after seizure onset. However, in freely moving rats that developed seizures after a kainic acid-induced SE, activity in the DG followed, rather than preceded, initiated or contributed to, seizure activity¹³³. Although this suggests that spontaneous seizures may not always originate in the DG, it shows the DG's involvement in seizure progression. Primary causes for the changed excitability in this model were shown to be a local loss of inhibition in association with mossy fiber sprouting^{134,135}.

A second morphological characteristic of new granule neurons born in an epileptic environment is the presence of hilar basal dendrites (HBDs), which normally retract during cell development^{136–138}. Increased numbers of HBDs have been observed in multiple experimental epilepsy models, including the kainic acid-induced SE model¹³⁹ and the pilocarpine-induced SE model^{140,141}. Ribak *et al* were the first to implicate HBDs in recurrent circuits in the hippocampus as they showed that HBDs formed synaptic connections with mossy fibers¹⁴². A recent study further revealed that granule cells exhibiting HBDs are seen already shortly after seizure induction in intrahippocampal kainic acid injection models, when only small numbers of newborn cells are present¹⁴³. This indicates that cells exhibiting HBDs likely arise from granule cells born prior to the insult, and that newborn granule cells are not the only cell type undergoing morphological alterations when seizures are induced.

In addition to these structural changes, mossy fiber sprouting (MFS) is another striking feature of several epilepsy models^{144–146}. MFS comprises axonal sprouting of granule cells towards aberrant locations, mainly the inner molecular layer of the DG. These sprouted axons target the apical dendrites of granule cells, and only a small percentage targets GABAergic interneurons^{139,147}. Although this implies that MFS could contribute to establishing a recurrent excitatory network in the hippocampus, there are also studies showing that mossy fiber sprouting predominantly targets GABAergic interneurons, which gives newborn granule cells a maximal hyperexcitability just after SE induction, when MFS starts to manifest and a hypoexcitatory network starts to develop^{148,149}.

Interestingly, recent work by Pun *et al* provides evidence that the adult generated granule cells play a major role in MFS¹⁵⁰. They used newborn granule cell specific knockout of Phosphatase and Tensin Homolog (PTEN), which resulted in abnormal development of

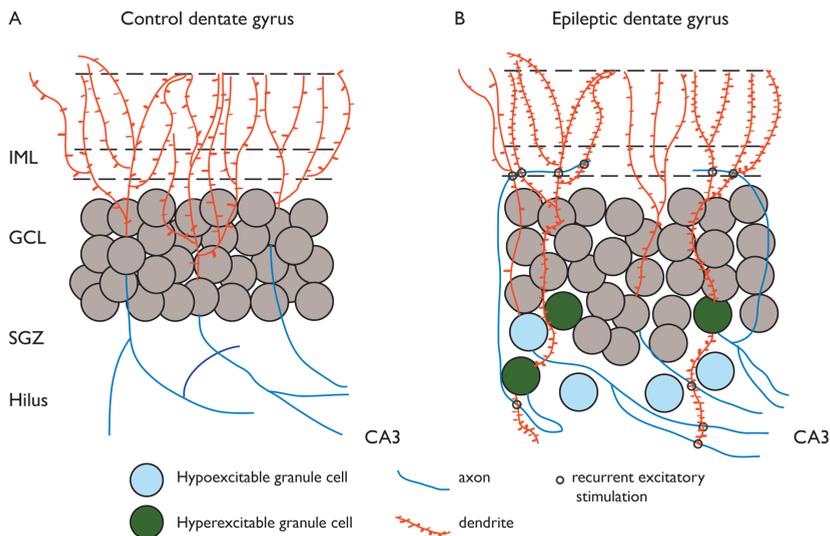


Figure 2. Morphological changes in the epileptogenic DG.

A) Schematic representation of a healthy DG. All granule cells are neatly localized in the granule cell layer (GCL), with healthy dendritic trees and axonal projections into the CA3. All granule cells exhibit normal electrophysiological characteristics.

B) Schematic representation of a DG in an epileptic environment. Several distinct morphological features have been observed in the epileptic DG, including granule cell dispersion/ectopic localization of granule cells in the hilus, Hilar Basal Dendrites, and Mossy Fiber Sprouting resulting in a recurrent excitatory network. Besides morphological alterations, previous work has also shown changes in granule cell excitability. Both granule cells with an increased excitability (green) as well as decreased excitability (blue) have been described. Debate is still ongoing, but it seems that adult-born granule cells are more vulnerable to undergo these morphological changes, as they mature under epileptic conditions.

newborn granule cells. One striking result was the positive correlation between the number of PTEN knockout cells and the occurrence of MFS. Moreover, animals that displayed low numbers of successful PTEN knockout cells and no MFS still exhibited seizures. Similar data supporting the concept that MFS is not needed for seizure development has been shown by Buckmaster and Lew¹⁵¹. Pun *et al* further showed that somatic hypertrophy, but not MFS, might be one of the causal features for seizure development. They showed that all animals that developed seizures also exhibited somatic hypertrophy, and that this hypertrophy coincided with the presence of HBSS, which contribute to the formation of recurrent hippocampal circuitry¹⁵⁰.

Altogether, changes in adult-born granule cells are hallmarks of Temporal Lobe Epilepsy (Fig. 2), including an ectopic location, HBDs, MFS, and the formation of recurrent circuits. These characteristics are not exclusive to the epileptic hippocampus and also occur in the healthy hippocampus, albeit to a significantly lower extent. Therefore, their exact role in epileptogenesis, and whether these changes are cause or consequence of epilepsy development, remains elusive.

2.3 Electrophysiological features of adult-born granule cells after seizures

Once they reach their final maturation state, normal individual adult-born granule cells are functionally indistinguishable from prenatally-born mature granule cells¹⁵². Adult-born granule cells exhibit baseline membrane potentials and functional synaptic inputs comparable to those of the mature granule cells generated during development^{115,153,154}. Findings that epileptic activity increases neurogenesis, combined with the morphological changes in the newborn granule cells, have triggered research into the specific electrophysiological properties of newborn granule cells. Scharfman and colleagues have shown that: 1) seizure activity itself induces integration of newborn neurons into the existing hippocampal network, and 2) these new neurons are comparable to normal granule cells, both in their intrinsic firing properties as well as in their morphology¹¹³. However, the newborn granule cells showed synchronous bursting patterns with CA3 pyramidal neurons, indicating an aberrant functional integration into the existing hippocampal network. Furthermore, bursting is not a normal feature of this otherwise largely silent, cell population and is therefore considered pro-epileptogenic¹¹³.

To investigate a potential role for the newborn cells in epileptogenesis, numerous studies have collected electrophysiological data from granule cells born after epileptic insults, but obtained discrepant results. Granule cells with both increased as well as decreased levels of intrinsic excitability were found, together with either a decreased or an increased synaptic drive on granule cell afferents. Importantly, these data have been collected from a diverse group of granule cells at different (ectopic) locations, often without the age of the individual cell being known. Keeping in mind the fast maturation process of the adult generated hippocampal granule cells, it is important to distinguish between granule cells born prior to the initial insult, and granule cells born during or after the insult. These results will be discussed in more detail below.

2.3.2 Increased excitability of adult-born granule cells after seizures

For granule cells activated by initial seizures to contribute to subsequent epileptogenesis, their intrinsic electrophysiological properties should be abnormal, or they must be integrated in an aberrant manner into the existing network. Zhan and colleagues compared both excitatory and inhibitory post-synaptic currents (EPSCs and IPSCs) between differentially located populations of granule cells, and concluded that HEGCs significantly differ in their properties compared to normotopic granule cells, with HEGCs showing higher EPSCs and lower IPSCs frequencies, together with a less polarized resting membrane potential¹⁵⁵. Such a high ratio of excitatory/inhibitory synaptic function agrees with the concept that HEGCs are hyperexcitable. The hyperexcitable state of the network can also be derived from the fact that under kainate-induced epileptic conditions, only a small percentage of all granule cells' mossy fibers target GABAergic interneurons, whereas under normal conditions, GABAergic neurons are their main target^{139,147}. This way, granule cell activation is more likely to result in hippocampal excitation rather than inhibition and could thereby potentially contribute to epileptogenesis.

Consistent with these findings, Wood *et al* have shown that granule cells born in an epileptic environment have an increased synaptic drive compared to normal granule cells born in a healthy environment¹⁵⁶. They found that synaptic afferents of granule cells in an epileptic environment have a higher firing frequency, as characterized by an increased frequency of EPSCs. Furthermore, Hung *et al* showed an association between the number of newborn granule cells and their ectopic location and epileptogenesis¹⁵⁷.

They used pilocarpine to induce SE, which was halted using diazepam either after 30 minutes or after 2 hours. Only the group that had SE for 2 hours showed an increase in AHN, with a concomitant abnormal dendritic outgrowth and ectopic localization of newborn cells. They also developed spontaneous recurrent seizures, indicating that the induction of neurogenesis might have contributed to the development of progressive seizures. Dashtipour *et al* used electron microscopy to show that ectopic granule cells differ morphologically from normotopic granule cells, showing a threefold increase in the number of synapses on their somata, indicating increased excitability¹⁴⁰.

Altogether, this suggests that at least some subsets of newborn granule cells could be pro-epileptogenic. The shift in excitation/inhibition ratio and their increased excitatory synaptic drive could result in the generation of a distinct (sub)population of hyperexcitable granule cells that can integrate within the existing hippocampal network, mainly via the mossy fiber pathway.

2.3.3 Decreased excitability of adult-born granule cells after seizures

Contrary to the observations discussed before, newborn granule cells may also exhibit a decreased excitatory drive under certain circumstances. Using the electrical stimulation SE model, Jakubs and colleagues observed increased amounts of new granule cells 5.5 weeks after electrical SE induction¹⁹. When these newborn neurons were further characterized electrophysiologically, no intrinsic differences were found as compared to newborn granule cells induced by running, a potent stimulus for AHN¹⁵⁸. However, the authors found a striking decrease of glutamate release probability, together with an increased inhibitory synaptic drive from inhibitory synaptic afferents. Together, this resulted in an overall decreased excitation/inhibition balance¹⁹.

In a follow-up study by the same group, Wood *et al* showed that adult-generated granule cells born after rapid kindling-induced seizures have a decreased intrinsic excitability 8 weeks after seizure induction, but have an increased excitatory drive¹⁵⁶. These contrasting findings suggest that while the newborn cells themselves might in principle be protective, the epileptic environment could potentially overrule this property by increasing the synaptic drive on the granule cell afferents.

Although all these studies focused on newborn granule cells, not all results came from the exact same population of cells, nor were the same epilepsy models used and it is thus likely that these factors may underlie the different findings between these studies. For example, where Zhan *et al* studied ectopic granule cells in the hilus¹⁵⁵, Jakubs *et al* studied newborn granule cells in the molecular layer¹⁹. Using a different model, Wood *et al* indicated that also the biological microenvironment, or local niche, of the adult-born granule cells is important and may eventually determine their functionality, even though the exact factors or elements responsible remain largely unknown¹⁵⁶.

Thus, with literature supporting increases and decreases in the intrinsic excitability of the newborn cells, as well as changes in synaptic drive, a consensus on the exact role of AHN in epileptogenesis is lacking. Two major confounding factors, which contribute to this lack of consensus on this topic, are the use of different epilepsy models, and the different time points selected to analyze cellular excitability after SE induction, and it will be important for future studies to distinguish the differential effects of each SE model on adult hippocampal neurogenesis.

Thesis Aim 2

One of the long-standing issues in preclinical epilepsy work has been the high variability between different epilepsy/seizure models, and in certain models even between animals. Therefore, it is challenging to draw strong conclusions that can be translated to the human situation. One thing that has become clear from numerous studies using different epilepsy models is the typical induction of aberrant neurogenesis after status epilepticus, although the exact degree and functional consequences seem to differ between models.

Systemic delivery of chemoconvulsants induces high variability, resulting in the use of non-standardized dosages. A common approach is to inject several dosages up till the point the animal exhibits severe status epilepticus. Ideally, one would prefer to expose only the brain to one standardized dose in order to decrease variability. In order to standardize SE induction we, together with collaborators in Spain and the USA, have aimed to set up a model that makes use of local intrahippocampal administration of the chemoconvulsant Kainic Acid, which is described in **chapter 2**. This model offers greater control of the exact chemoconvulsant dosage delivered selectively to the area of interest, providing the possibility to study the effects of seizures of increasing severity between animals in a more controlled matter.

3.1 Deregulation of AHN under seizure conditions: miRNAs

Even though AHN is tightly regulated, numerous internal and external factors can compromise the process. This becomes particularly clear when studying the neurogenic process under pathological conditions, such as epilepsy. Epileptic seizures have a strong effect on AHN, all of which have been pooled under the umbrella term “aberrant AHN”¹⁵⁹. Whether these alterations causally underlie disease formation is still a matter of debate, but it has become clear that the alterations in AHN under epileptic conditions strongly affect hippocampal network excitability, thereby creating a pro-epileptic environment, which possibly also underlies comorbid cognitive deficits observed in epilepsy patients¹⁶⁰.

How epileptic seizures can deregulate AHN is topic of intense research, and a plethora of hypotheses have been raised about this in the past. Over the years, miRNAs have gained serious interest as it became clear that most miRNAs known to regulate AHN are also deregulated in case of seizure events or chronic epilepsy. In fact, 60,5% of all 1996 “high confidence” miRs registered in miRBase²¹ have been found dysregulated after epileptic seizures, indicating a strong correlation between miRNA dysregulation and epilepsy. Furthermore, 95,5% of all 45 miRs known to regulate adult hippocampal neurogenesis (AHN) were found dysregulated after epileptic seizures (Fig. 3)¹⁶¹. Here we provide an overview of AHN-regulating miRNAs linked to epilepsy, based on analyses done using the EpimiRBase developed by Mooney *et al*¹⁶². EpimiRBase is a comprehensive manually curated database of over 2,000 miRNA-epilepsy associations, which was developed to address the need for a database to keep track of the rapidly expanding published literature on miRNAs in epilepsy. The June 2016 release of EpimiRBase lists miRNAs from 41 publications including 1207 unique miRNA (1,198 up and 870 down regulated) from three species: human (161), mouse (854) and rat (1,053). The miRNAs are categorized into four study types: expression analysis (41), functional (19), profiling-biofluid (32) and profiling-brain (1,976).

When we used EpimiRBase against a list of all miRNAs known to regulate AHN, this provided numerous matches, again indicating a crucial role for miRNAs in the deregulation of AHN under seizure pathology (Table 2 and Fig. 3). Here we summarize all AHN-regulating miRNAs whose altered expression levels have been found in both rodent and human epilepsy studies.

3.1.2 Stem Cell maintenance and quiescence

MiR-184 and miR-34a, which play a role in stem cell maintenance through regulation of the Notch signaling pathway, as discussed before, are differentially expressed in both rodent and human profiling studies related to epilepsy. Increased miR-184 expression has been found both in chronic human epilepsy patients, as well as in the early stages of epileptogenesis in a rodent model^{163,164}. MiR-34a is commonly found upregulated at different stages of disease development in rodents^{165,166}. Chronic upregulation of these miRNAs leads to depletion of the stem cell pool, one characteristic feature of epilepsy models and a potential cause of comorbid cognitive deficits observed in both rodent and human epilepsy subjects^{5,13,167–169}.

3.1.3 NSPC proliferation and differentiation

As mentioned before, TLX is involved in several negative feedback loops with different miRNAs, including miR-9, miR-137, miR-145, and Let-7b. All 4 miRNAs have also been identified in profiling studies, though the directionality of its expression differs temporally. Expression of the Let-7 family is severely altered by the initial insult. Shortly after SE-induction, Let-7 expression decreases, followed by a severe increase at 24 hours post SE. Following this peak expression Let-7 is again down regulated lasting up till 50 days post SE¹⁷⁰. Unlike Let-7, miR-9 is found up regulated during the chronic stage of the disease¹⁷⁰, while its expression decreases two hours after SE induction³⁹. Finally, expression of miR-137 increases both in acute⁹⁰ and chronic¹⁷⁰ stages of animal SE models, while expression of miR-145 is yet inconclusive as both up and down regulation have been reported. Decreased expression of these miRNAs will result in increased expression of the TLX receptor, thereby driving proliferation over differentiation, partially explaining the increased proliferation rates found in the early stages of epileptogenesis, while chronic

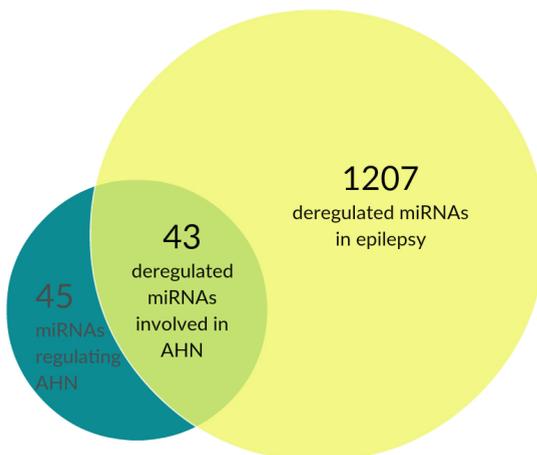


Figure 3. AHN-regulating miRNAs are severely deregulated in epilepsy. We have identified 44 miRNAs that are established regulators of AHN (dark green). Using the EpimiRbase, which lists a total of 1207 deregulated miRNAs in epilepsy (yellow), we identified a total of 42 out of 44 AHN-regulating miRNAs to be deregulated in epilepsy (light green).

Thesis Aim 3

As discussed before, epileptic seizures can be induced using many different approaches. Each animal model has its own characteristics, and thus it can be implied that each method will also have its own miRNA expression profile, which in turn are timepoint-dependent. Since the development of the EpimiRBase, a comprehensive overview of all different miRNA profiles is available and one can better compare the epigenetic effects of all seizure/epilepsy models. We performed a multi-omics-study in mice that received systemic KA. Our aim was to identify a more specific KA-induced miRNA profile that could be used for further interventional strategies using miRNA manipulation. This approach and the obtained dataset is the focus of **chapter 3**.

up regulation of these miRNAs could potentially underlie the diminished proliferation observed in chronic epileptic hippocampi.

3.1.4 Astrocytic conversion of NSCs

It has been recently shown that hippocampal NSCs can directly convert into astrocytes under seizure conditions. Though this phenomenon has not been linked directly to miRNAs yet, it could be argued that miRNA deregulation probably plays a role based on the strong microRNA expression changes associated with epileptic seizures¹⁶¹. In this respect, one possible key player could be miR-124, which is a crucial regulator of NSPC fate determination, through targeting SOX9, a transcription factor critical for gliogenesis that is normally absent in neuroblasts and immature neurons⁸⁰. MiR-124 has been found upregulated under epileptic conditions^{90,171}, providing a possible mechanistic explanation for astrocytic conversion of NSCs.

3.1.5 Apoptotic selection

MiR-124 and miR-137, which cooperatively regulate NPC apoptosis after SE, are both significantly upregulated during the initial stages of epileptogenesis⁹⁰. Furthermore, both miR-124 and miR-137 are also upregulated during the chronic stage of the disease¹⁷¹. Upregulation of both miR-124 and miR-137 inhibits apoptosis of NPCs through down regulation of Bcl2L13, providing a possible explanation for the survival of unfit immature neurons commonly found in the epileptic hippocampus. On the contrary, miR-34a, which down regulates anti-apoptotic Bcl2 levels, is significantly upregulated at different stages of the disease. Increased expression of miR-34a should then increase apoptotic selection under epileptic conditions.

3.1.6 Migration of newborn neurons

The miR-379-410 cluster is also deregulated under epileptic conditions^{90,165,172}. Interestingly, at acute stages expression of this cluster increases, down regulating N-Cadherin and making immature neurons more prone to migrate. During the latent phase expression levels decrease, indicating a clear link between the presence of epileptic seizures and aberrant migration of newborn neurons commonly observed in the epileptic hippocampus.

MiR-9, which promotes migration of immature neurons, is also upregulated under epileptic conditions¹⁶⁵. Together, this points toward a strong effect of seizures on

migration of immature neurons, providing a possible explanation of the characteristic ectopic granule cells commonly found in epileptic hippocampi.

3.1.7 Maturation and integration

Mir-34a and miR-134, which inhibit dendritic branching, are both upregulated in all stages of the disease^{90,165,170,171,173,174}. On the contrary miR-124, which is known to stimulate neurite outgrowth, is downregulated at acute stages of epileptogenesis, but upregulated a few days after SE induction all until the chronic epileptic stage^{90,165,171,173}. The miR-17-92 cluster, which also promotes dendritic outgrowth, is up regulated at all stages of the disease^{165,173}. All together, this points towards a complex regulation of dendritic outgrowth under seizure conditions.

A similar picture arises when looking at the expression levels of miRNAs known to regulate spinogenesis and functional integration. MiR-132, which stimulates spinogenesis, has been found upregulated in all stages of the disease, indicating the occurrence of increased synaptic connectivity^{165,172,173}. In line with these findings expression levels of miR-138, which inhibits spinogenesis, are downregulated during all stages of the disease^{165,173}. On the contrary miR-134, which also inhibits spinogenesis, is upregulated during all stages of the disease^{166,171}.

4.1 Elucidating the functional role of newborn granule cells

One way of unraveling the role of AHN in epileptogenesis is to selectively inhibit the neurogenic process induced by seizure activity. If the newborn granule cells do contribute to epileptogenesis, then blocking neurogenesis should be protective. Polysialylation of NCAM, a neural cell adhesion molecule, is important for successful neurogenesis in the adult brain and can be blocked by administration of endoneuraminidase (endoN)¹⁷⁵. Administration of endoN in the amygdala kindling model indeed decreased hippocampal cell proliferation but did not affect progression of seizures. Moreover, inhibition of polysialylation of NCAM resulted in increased acute seizure susceptibility. Furthermore, the number of HEGCs was significantly decreased in the kindling group that received endoN treatment. This shows that (partially) blocking the ectopic migration of newborn granule cells did not rescue the epileptogenic process, at least in the amygdala kindling model¹⁷⁶. In a follow-up study, administration of endoN did not rescue the development of spontaneous seizures in the same model either¹⁷⁷.

Jung *et al*¹⁷⁸ used continuous cytosine-b-D-arabino-furasonide (Ara-C) infusion, an anti-mitotic agent, to block neuronal proliferation in the pilocarpine-induced SE model. They showed that a continuous infusion of Ara-C during the latent period of SRS development successfully reduced the likelihood of developing spontaneous recurrent seizures, indicating that newborn cells potentially play a critical role in the progression of seizure development after pilocarpine-administration. Strikingly, the reduction in neuronal proliferation did not alter the occurrence of MFS, again indicating that neurogenesis is not required for MFS induction per se.

Another pharmacological way to reduce neurogenesis makes use of serotonin 1A receptor blockade¹⁷⁹. In the pilocarpine model of experimental epilepsy, blocking the serotonin 1A receptor indeed decreased hippocampal neurogenesis after seizures, but failed to mitigate the occurrence of spontaneous recurrent seizures¹⁷⁹. This blockade also failed to rescue MFS, indicating that neurogenesis is not required for MFS development. The

Table 2. Overview of differentially expressed microRNAs in epilepsy known to be involved in neurogenesis, and their (predicted) biological consequence.

miR	Expression during acute stage	Expression during chronic stage	Predicted Biological consequence	References
miR-184	Up	Up	Increased proliferation	(Haenisch et al., 2015; McKiernan et al., 2012)
miR-106b/25 cluster	Up	Up	Increased proliferation and neuronal differentiation	(Gorter et al., 2014; Kretschmann et al., 2015; Roncon et al., 2015)
miR-34a	Up	Up	Increased proliferation, Increased apoptosis	(Gorter et al., 2014; Hu et al., 2012; Jimenez-Mateos and Henshall, 2013)
miR-124	Up	Up	Increased neuronal differentiation, Decreased apoptosis	(Peng et al., 2013; Schouten et al., 2015)
miR-137	Up	Up	Increased proliferation, Decreased apoptosis	(Schouten et al., 2015; Song et al., 2011)
miR-9	Down	Up	Dynamic changes in proliferation and differentiation	(Pichardo-Casas et al., 2012; Song et al., 2011)
Let-7b	Down	Down	Increased proliferation, Decreased differentiation	(Song et al., 2011)
miR-26b	Up	Up	Increased neuronal differentiation	(Kan et al., 2012; Schouten et al., 2015)
miR-145	Up/Down	Up/Down	Changes in neuronal differentiation	(Gorter et al., 2014; Li et al., 2014; Pichardo-Casas et al., 2012)
miR-125b	Up	Up	Increased neuronal differentiation	(Alsharafi and Xiao, 2015; Kretschmann et al., 2015)
miR-19	Up	Up	Increased migration	(Gorter et al., 2014)
miR-379/410 cluster	Up	Up	Increased migration	(Kretschmann et al., 2015; McKiernan et al., 2012; Schouten et al., 2015)
miR-138	Down	Down	Increased spinogenesis	(Kretschmann et al., 2015; Schouten et al., 2015)
miR-134	Up	Up	Decreased spinogenesis	(Peng et al., 2013)
miR-132	Up	Up	Increased spinogenesis	(Gorter et al., 2014; Jimenez-Mateos et al., 2011; Kretschmann et al., 2015)

presence of MFS, notably in the absence of changes in newborn granule cell numbers, has also been observed in earlier studies^{178,180}, suggesting that mossy fiber sprouting and AHN are dissociable and independent of each other. These findings indicate that pathological processes underlying epileptogenesis do not only comprise newborn granule cells. This suggests that MFS may be a crucial morphological alteration in the epileptogenic DG that concerns primarily pre-existing, mature DG neurons. However, the differences observed between these studies could have resulted from the use of different inhibitors of AHN. Indeed, Kron *et al.*¹⁸¹ showed that the developmental stage of granule cells influences their contribution to seizure-induced plasticity, especially MFS. Using the pilocarpine-

induced SE model, they showed that granule cells born 4 weeks before SE display severe MFS 4 weeks post SE induction. Remarkably, granule cells born 4 days after SE did not display MFS 4 weeks post SE, but showed robust MFS 10 weeks post SE. Granule cells born 7 weeks before SE were resistant to injury, indicating that only developing granule cells are prone to seizure-induced plasticity. While both Radley and Jacobs, Parent *et al*, and Kron *et al*^{179–181} used the same epilepsy model (pilocarpine-induced SE), different results were obtained from these different studies; Kron *et al* showed that pre-existing granule cells were resistant to seizure-induced injury¹⁸¹, while the other two studies showed that neurogenesis is not needed for robust MFS to occur^{179,180}.

A method frequently used to block AHN is irradiation, a technique that can halt the generation and expansion of newborn cells for prolonged periods of time. The exact mechanism is complex and multifactorial, but the main effect of photon irradiation seems to be DNA damage, that preferentially targets dividing cells, induces cell cycle arrest and subsequent cell death^{20,182}. As neurons are post-mitotic cells, irradiation has relatively little side effects on them. Besides suppression of neurogenesis, brain irradiation can cause significant inflammation^{183,184}, which can indirectly decrease neurogenesis *in vivo*^{22,185,186}, influence newborn granule cell integration¹⁸⁷ and hippocampal function. Raedt *et al*¹⁸⁸ used an 8 Gy whole brain photon-irradiation protocol, sufficient to prevent neurogenesis, without inflammatory side-effects¹⁸⁹, one day before initiation of a rapid kindling protocol. Irradiation resulted in decreased numbers of newborn granule cells, enhanced excitability during kindling acquisition and an increased seizure severity. However, there was no clear effect on the final establishment of the fully kindled state. Because of the importance of inflammation for the neurogenic process^{185–187}, inflammatory markers were also assessed in both irradiated and control groups. Although inflammatory markers were present, no differences between the irradiated and non-irradiated epileptic animals were found, probably reflecting the baseline-level inflammation shown multiple times as part of the epileptogenic process^{190–192}. As precluding neurogenesis did not halt epileptogenesis, these results indicate that newborn granule cells are not essential for the epileptogenic process. Moreover, as a lack of newborn granule cells results in a more severe epileptic phenotype and increased excitability of the hippocampal neurons during kindling, the study suggests that the newborn granule cells may even exert a possibly protective role¹⁸⁸.

A study by Pekcec *et al* used a focused X-ray-irradiation strategy on the rat hippocampus in combination with the electrical kindling model¹⁹³. While the total dose of 8,2 Gy was comparable to that used by Raedt *et al*¹⁸⁸ the absolute dose on the hippocampus was much higher due to the focal nature of the procedure followed. In this model, irradiation prevented proliferation of new granule cells and its associated increase in HBDs. Although proliferation was successfully halted and the presence of HBDs was decreased, irradiation did not affect the rate of kindling progression. Furthermore, both pre- and post-kindling thresholds proved insensitive to irradiation¹⁹³.

In accordance with Raedt *et al* the results presented by Pekcec *et al* suggest that newborn granule cells born around the induction of SE are not essential for the epileptogenic process in the amygdala kindling model. However, contrary to Raedt *et al* Pekcec *et al* do not support the concept that newborn granule cells might have a protective function. Whereas Raedt *et al* found a significant increase in seizure severity and excitability after global inhibition of AHN, Pekcec's study did not show differences in these parameters between irradiated and non-irradiated groups^{188,193}. One explanation could be the focal administration of irradiation applied to the hippocampus, which

resulted in more concomitant inflammation. This suggests that irradiation studies rarely target the neurogenic process alone and one might therefore argue that the high dose used by Pekcec *et al* may have interfered with secondary processes, for example through inflammation, which was absent in Raedt *et al*.

Overall, although these studies contradict each other to some extent, they also share important features. One could conclude that blocking neurogenesis does not alter progression of epileptogenesis, with the exception of the data by Jung *et al*¹⁷⁸. However, many of these studies showed that interfering with neurogenesis decreased seizure threshold, while also enhanced intrinsic excitability of the granule cells has been found¹⁸⁸. Surprisingly, these changes had no protective effect, which would imply that although the newborn granule cells themselves may have a higher seizure threshold and decreased excitability, they do not play an important role in the epileptogenic process *per se*.

This raises the question whether the intrinsic characteristics of granule cells born after seizures might be, at least in part, under environmental control¹⁵⁶. Candidates for environmental factors are abundant, and several clinical and experimental studies have indicated a role for inflammatory processes in the epileptic hippocampus (for reviews see^{190,191,194,195}). The work of Wood and Jakubs already showed that an inflammatory environment influences the excitability of maturing granule cells^{19,156}. Together with data provided by Monje and Palmer, showing that inflammation interferes with neuronal differentiation *in vivo*^{185,186}, one could assume that inflammatory processes can act as modulators of the neurogenic process in the (pre-) epileptic brain. Jung *et al* showed that normalizing the hippocampal microenvironment, using a Cox-2 inhibitor (celecoxib), rescued the effects of pilocarpine-induced SE¹⁹⁶. Hippocampal cell death was reduced, together with microglia activation and abnormal neurogenesis, attenuating the likelihood of developing spontaneous recurrent seizures. However, the use of a different Cox-inhibitor (SC58236), did not give any beneficial results, and even resulted in increased number of seizures during the second week of treatment when given during the chronic stage of epilepsy¹⁹⁷. Taking into account the study by Kron *et al*, one can hypothesize that mainly the very immature granule cells, i.e. those born close to, during, or just after the initial seizure, will be more susceptible to environmental factors¹⁸¹.

4.2 Integrating different roles of newborn granule cells

In order to provide an explanation for the contradictory findings on the role of newborn granule cells in epileptogenesis, Murphy *et al* hypothesized that the population of newborn granule cells might integrate heterogeneously into the existing network, thereby taking on both protective as well as contributing roles in the process of epileptogenesis¹⁹⁸. They assessed the morphological characteristics of granule cells born after pilocarpine-induced SE. According to their findings, newborn granule cells can be divided into two groups that differ in their final location and excitability. The first group, which comprises the majority of all newborn granule cells, showed a significant reduction in spine density after SE, indicating a decreased excitatory input. However, 10% of all newborn granule cells exhibited increased spine density, combined with the occurrence of long HBDs. The cells with HBDs had more synaptic contacts with sprouted mossy fibers, indicating a larger degree of integration into epileptic hippocampal networks. Although most newborn granule cells exhibit decreased excitability and migrate to their normal location in the granular cell layer where they might play a protective role, a second, smaller population that exhibits increased excitability also integrates into the existing hippocampal network, where they form a recurrent excitatory network. The final outcome for the epileptogenic

process may therefore depend on the relative contribution of these two populations, and their development over time. While this study clearly shows the complexity of the effects of seizures on AHN, it also points to an important implication: the effect of newborn granule cells on the epileptogenic process may not depend on the absolute numbers of newborn granule neurons, but may be determined by the fraction of hyperexcitable granule cells that do form connections with the existing hippocampal network via mossy fiber sprouting. This is in agreement with recent findings demonstrating that a small population of granule cells with altered PTEN expression and mTOR activity can indeed induce seizures¹⁵⁰. A remaining question is then which factors drive and regulate the development of distinct populations of newborn cells derived from the activation of adult NSC present in the hippocampus.

Previous studies have shown that the number/duration of seizures does not influence the net rate of AHN^{199,200}. However, other authors have found that initial seizure duration and severity may be key factors regulating important properties of adult-born granule cells^{201,157}. Granule cells born under severe seizure conditions may be more prone to develop into hyperexcitable cells, whereas cells born under less severe seizure conditions would preferentially generate a granule cell population with decreased excitatory drive (Fig. 4). It is important to realize that although we present this hypothesis using only two granule cell populations (hypo- and hyperexcitable cells), in reality one would expect to see a continuum of cell populations, with high seizure intensity increasing the probability of hyperexcitable cells. Under all seizure conditions hypo- and hyperexcitable cells would co-exist within the DG and the balance between them, driven by the initial seizure intensity, could be one of the factors determining the final outcome of the epileptogenesis process. As groups of adult-born granule cells with different intrinsic properties have been considered often one unique population, this novel concept provides an alternative explanation for the contradictory findings in literature. The majority of adult-born granule cells may be induced under less severe conditions, in a normal microenvironment without inflammation, thereby increasing the chances of survival and their incorporation into the DG network.

It is hypothesized that a population of hyperexcitable granule cells is induced under more severe conditions. This small population of hyperexcitable cells may be damaging to specific cell populations (e.g. calcium binding, or inhibitory neurons) within the hippocampus and could cause a lack of inhibition and contribute to the (continuation of the) epileptogenic process. The idea that the integration of a specific population of

Thesis Aim 4

Seizure severity is an abstract term, commonly used to address length and severity of individual seizure bursts, either based on electrophysiological or behavioral levels. In **chapter 4** we partly address the question how the severity of the first epileptic insult, the Status Epilepticus, affects the neurogenic cascade. We used 3 different dosages of Kainic Acid, administered intrahippocampally allowing for tight dosage, and thus intensity control. Each dose has its own behavioral and electrophysiological characteristics, and we assessed its effect on proliferation, differentiation, astrogliosis, and granule cell dispersion. We show a clear dosage, and thus, seizure severity-dependent effect on all parameters, indicating that seizure-severity is a factor that should be taken along in any experimental setup focused on identifying the effect of seizures on the neurogenic cascade.

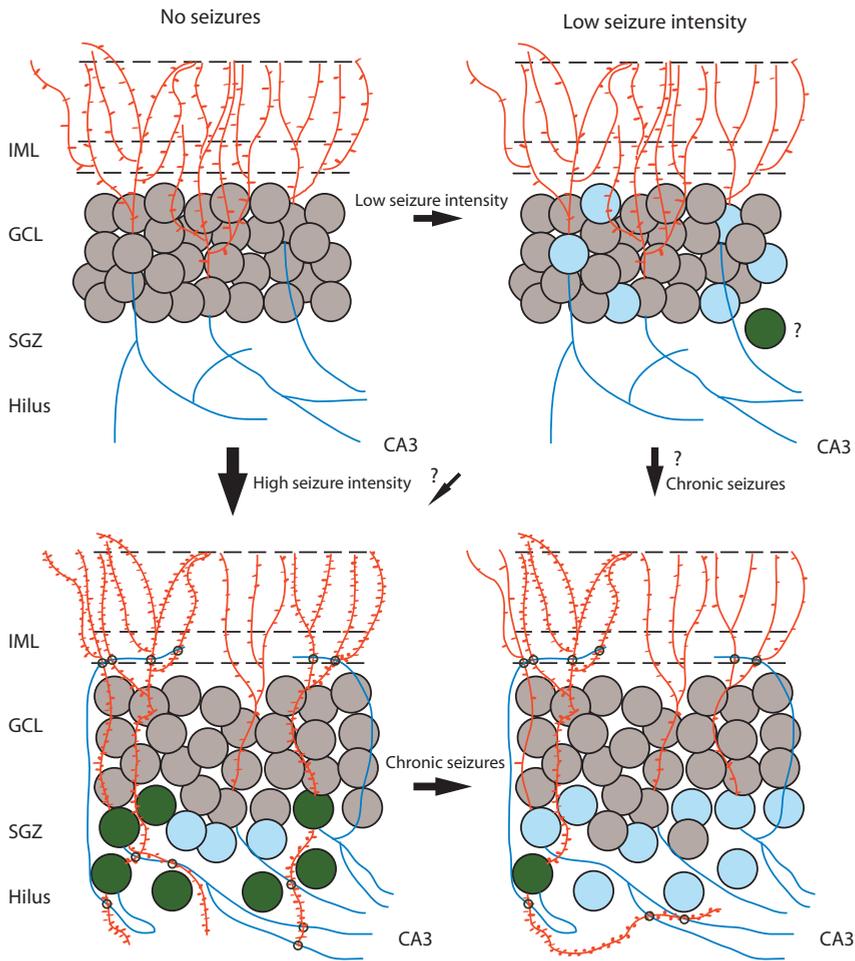


Figure 4. Schematic representation of the seizure intensity-dependent induction of distinct granule cell populations.

Low intensity seizures induce the generation of newborn granule cells. These are characterized by decreased excitatory input (blue soma, less dendritic spines); possibly to counteract overexcitation induced by seizures. Conversely, under high intensity seizures, hyperexcitable granule cells (green soma, more dendritic spines) will arise exhibiting ectopic localization, together with the formation of Hilar Basal Dendrites, Mossy Fiber Sprouting, and the consequential recurrent excitatory networks. In the chronic situation, a relatively large population of granule cells with low excitatory input may survive, as these were generated under less severe conditions. The hyperexcitable granule cells may not survive at the same rates, resulting in a small, but pathologically significant population, located ectopically in the hilus/subgranular zone. Together, these two populations interfere with the healthy granule cells that were already incorporated into the hippocampal network prior to the rise of the seizures, and contribute to the progression of seizures. The epileptogenic process might follow different paths, as indicated by the arrows, possibly resulting in different endpoints. Most preclinical studies however follow the path indicated by the large arrow, inducing high intensity seizures, resulting in chronic seizure occurrence. Studying other possible routes to the chronic stage could provide new interesting data on the exact role of the different subtypes of newborn granule cells.

Thesis Aim 5

As mentioned before, and also partly studied in **chapter 4**, epileptic seizures of varying intensity induce changes in the neurogenic cascade, summarized under the umbrella term “aberrant hippocampal neurogenesis”. The occurrence of aberrant AHN has been shown numerous times, in many different animal models of epileptic seizures, and is thought to be one of the key structural changes in the hippocampal network underlying epileptogenesis. Recently, miRNAs have gained a lot of attention, as epileptic seizures drastically change the miRNA profile in the hippocampus. Over the years, more and more miRNAs have been identified that play a role in AHN, while these miRNAs are also deregulated upon the presence of epileptic seizures. In **chapter 4** we focus on two miRNAs that have been previously identified to be upregulated after epileptic seizures in our lab, miR-124 and miR-137. Our aim was to apply a miR-124 and miR-137 silencing approach after Status Epilepticus onset, thereby aiming to prevent the occurrence of aberrant AHN.

newborn cells with different properties into the hippocampal network contributes to epileptogenesis is supported by recent computational models²⁰², which predict that under non-pathological conditions, newborn cells integrate into the hippocampal network and thereby strengthen it. However, under pathological conditions, newborn hyperexcitable cells are likely to drive the hippocampal network into bursting dynamics, possibly contributing to seizure induction. Therefore, network-level changes caused by the integration of intrinsically different sets of newborn granule cells could create a reorganized environment that induces or intensifies epileptic dynamics. Although attractive, this hypothesis may be difficult to test experimentally.

In this thesis I propose that miRNAs are key players in the regulation of hippocampal neurogenesis in the presence of epileptic seizures, and that they play a crucial role in the formation of the aberrant hippocampal network characteristic for the epileptic hippocampus.

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