Disease-associated modulation of adult hippocampal neurogenesis

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ABSTRACT

Adult neurogenesis has been the focus of over 1500 articles in the past 10 years. Evidence for the continuous production of new neurons in the adult brain has raised hopes for new therapeutic approaches. On the other hand, the generation of new neurons is modulated in several neurological diseases and disorders, suggesting the involvement of the adult neurogenesis in their pathogenesis. Therefore, a better understanding of the disease-associated modulation of adult neurogenesis is essential for determining the most effective therapeutic strategy.

The purpose of this doctoral project was to investigate long-term adult hippocampal neurogenesis changes in two disease models. BrdU labeling in combination with various cellular markers, and genetic fate-mapping approach were used to reach this goal.

In the first experiment, the impact of the BeAN strain of the Theiler's virus on hippocampal cell proliferation and neuronal progenitors was evaluated in two mouse strains which differ in the disease course. It was shown that Theiler's murine encephalomyelitis virus can exert delayed effects on the hippocampal neurogenesis with long-term changes evident 90 days following the infection. The hippocampal changes proved to depend on strain susceptibility and might have been affected by microglial cells.

In the second experiment, hippocampal neurogenesis was analyzed based on genetic fate mapping of transgenic animals in the amygdala-kindling model of epilepsy. The number of new granule neurons added to the dentate gyrus was increased in kindled animals. A prior seizure history proved to be sufficient to induce a long-term net effect on neuron addition and an ongoing occurrence of seizures did not further increase the number of new neurons. Hypertrophic astrocytes were observed in the kindled animals suggesting that seizures result in structural changes of astrocytes that could be detected long after the termination of the insults.

The results of the experiments indicated the importance of methodological considerations in chronic studies of neurogenesis.

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CHAPTER 1: General Introduction

1.1 Outline of the thesis

The generation of new neurons continues in the neurogenic niches during adulthood in most of the vertebrate brains studied so far including humans (Barker et al., 2011). Studies show that production of new neurons occurs in at least two areas of the adult brain: the subventricular zone of the lateral walls of the ventricles, and the subgranular zone of the dentate gyrus of the hippocampal formation. Adult hippocampal neurogenesis is of particular interest considering its suggested role in learning, memory and cognition in humans (Snyder and Cameron, 2012).

Neurogenesis is a highly regulated multistep process comprising proliferation, differentiation, maturation, survival and functional integration of the newborn neurons into the existing neuronal network (Jhaveri et al., 2012). Adult neurogenesis comprises the entire set of events leading to generation of neuronal development beginning with the division of a precursor cell and ending with the survival of a mature, integrated, functioning new neuron (Sohur et al., 2006; Bergami and Berninger, 2012). Disease-associated modulation of adult hippocampal neurogenesis is a double-edged sword that may contribute to the regeneration of the damaged tissue on one hand, and to the pathophysiology of neurological and psychiatric diseases on the other (Kaneko and Sawamoto, 2009). Studying the alterations of neurogenesis associated with different diseases provides information for a better understanding of the disease that may lead to development of new therapeutic strategies.

The present dissertation aims to give new insights into the disease-associated modulation of adult hippocampal neurogenesis. So far, our knowledge about the impact of viral infections on adult hippocampal neurogenesis is limited. Considering the role of viral infections as a major cause of central nervous system diseases as well as their role in generation of acute seizures and their contribution to the induction of epileptogenesis associated with chronic recurrent seizures, additional studies seem necessary for a better understanding of their effect on adult hippocampal neurogenesis. Multiple studies report quantitative and qualitative evidence for the impact of acute seizures in a naïve brain and long-term consequences of epileptogenesis on the adult hippocampal neurogenesis (Parent and Kron, 2012). However, the long-term net effect of these changes on the hippocampal network remains an open question.

Chapter 1 provides a general introduction on the neuroanatomy of the hippocampal formation, adult hippocampal neurogenesis and its role in the function of the hippocampus. Next, the methodological approaches applied in this thesis for

investigating the production of new neurons are explained briefly. The chapter continues by introducing the Theiler's murine encephalitis virus as an example of a viral infection that is used frequently in neurodegenerative studies, and a detailed description of the amygdala kindling model of temporal lobe epilepsy. The chapter ends with stating the aims of this thesis and the approach in studying the disease-associated modulation of adult hippocampal neurogenesis.

Chapter 2 and 3 consist of the published papers by the author of this thesis that addressed the specific aims described earlier. The author of this doctoral thesis contributed to the Chapter 2: "Impact of Theiler's virus infection on hippocampal progenitor cells: differential effects in two mouse strains" by participating in the experimental part of the study, performing cellular studies of adult hippocampal neurogenesis, statistical analysis, and writing major parts of the manuscript. Contribution of this author to the Chapter 3: "Long-term genetic fate mapping of adult generated neurons in a mouse model of epilepsy" included planning and performing the animals experiment, cellular and statistical analysis, and writing major parts of the manuscript. The references for each paper are listed in the end of the chapter.

Chapter 4 provides a general discussion with an overview of the main findings of this dissertation. Interesting findings from both of the studies are discussed in more details.

1.2 Adult neurogenesis

Adult neurogenesis is the process of generating functional neurons from neural stem cells in the adult brain that can integrate in the preexisting network (Ming and Song, 2005; Mongiat and Schnider, 2011). Production of new neurons has been observed in many vertebrates including humans (Gould, 2007; Barker et al., 2011; Sawada and Sawamoto, 2012). Under physiological conditions, new neurons are generated in restricted regions of the human brain: subventricular zone (SVZ) and subgranular zone (SGZ) (Fig.1) (Mongiat and Schnider, 2011). Both of these structures appear to be important in the temporary storage of memory and it is suggested that adding new neurons may be beneficial in processing new information, however, the details remain under debate (Lledo et al., 2006; Aimone et al., 2011).

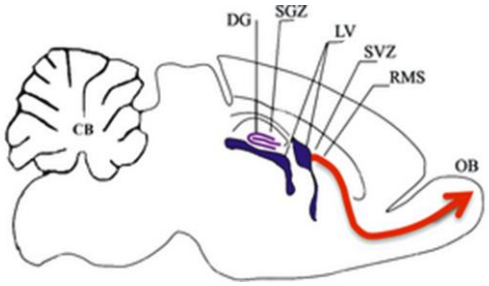

Fig. 1: The schematic diagram represents a sagittal section of mouse brain showing the two neurogenic niches: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampal formation and the subventricular zone (SVZ) of the lateral ventricle (LV). The rostral migratory stream (RMS) is shown in red and CB represents cerebellum. Modified from b et al., 2013.

Neural stem cells (NSCs) have characteristics of astrocytes, though only a fraction of specialized astrocytes in the postnatal brain function as stem cells (Bergström and Forsberg-Nilsson, 2012). NSCs are distinguished by their capability of self-renewal through symmetric cell division, and generating neurons and glial cells through asymmetric division (Götz and Huttner, 2005; Ming and Song, 2011; Ballenchi et al., 2013). The term "progenitors" is usually used when the mitotic cells become lineage-restricted (Emsley et al., 2005). Together, NCSc and progenitors are broadly defined as "precursors" (Emsley et al., 2005). The majority of NSCs from the embryologic stage lose their self-renewal and multipotency capacities with time, however, neurogenesis continues in the lateral ganglionic eminence of lateral walls of ventricles that develops to the SVZ, and the hippocampal sulcus that becomes the SGZ of the hippocampus (Bergström and Forsberg-Nilsson, 2012; Song et al., 2012; Ballenchi et al., 2013).

The SVZ and SGZ are considered as "neurogenic niches" that support neurogenesis with the presence of NSCs and a specific microenvironment that is

permissive for generating new neurons by providing nourishment, structural support, protection and allows preserving the functional integrity of the existing circuitry while the new neurons modify the local neuronal network (Balu and Lucki, 2009; Bergström and Forsberg-Nilsson, 2012; Song et al., 2012; Ballenchi et al., 2013). The neurogenic niche is composed of astrocytes, blood vessels, microglia, ependymal cells, and extracellular matrix proteins and proteoglycans (Palmer et al., 2000; Alvarez-Buylla and Lim, 2004; Bergström and Forsberg-Nilsson, 2012). These microenvironment structures add to the complexity of the neurogenesis process and are involved in its physiological and pathological modulation (Suh et al., 2009; Yao et al., 2012).

The multistep process of neurogenesis involves the proliferation, differentiation, maturation, survival and integration of new neurons from precursors, and is tightly regulated by extrinsic and intrinsic factors and by the existing neuronal network activity (Jhaveri et al., 2012; Song et al., 2012). Neurons produced in the anterior portion of the SVZ form chain-like cell aggregates and migrate along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into GABAergic granule cells and periglomerular interneurons that are involved in olfactory processing (Curtis et al., 2012; Mongiat and Schnider, 2011; Yao et al., 2012; Sawada and Sawamoto, 2013). Neurons generated in the SGZ migrate a short distance into the granule cell layer (GCL) of the DG and mature into glutamatergic granule neurons and become part of the hippocampus circuitry (Balu and Lucki, 2009). Production of new neurons declines with aging in both SVZ and SGZ (Lazarov et al., 2010).

NSCs have been found in other regions of the brain and neurogenesis has been demonstrated in these areas in various models of neurodegenerative diseases and brain injuries, however, there is an ongoing debate on whether and to what extent active neurogenesis occurs under physiological conditions in these areas (Emsley et al., 2005; Gould, 2007; Taupin, 2007; Song et al., 2012; Yao et al., 2012). Interestingly, when neural stem cells from non-neurogenic areas are transplanted into a neurogenic area, their neurogenerative potential can be utilized which implies the importance of the regulatory environmental cues (Ehninger and Kempermann, 2008).

1.3 Adult hippocampal neurogenesis

The hippocampal formation is located under the cerebral cortex and consists of the entorhinal cortex (EC), hippocampus, dentate gyrus (DG) and subiculum complex (Fig. 2) (Amaral et al., 2007; Curtis et al., 2012). This structure is well known for its role in multiple higher order functions including emotion, cognition, behavior and memory (Andersen et al., 2007; Curtis et al. 2012; Clark et al., 2013). The hippocampus is also associated in various psychiatric conditions including depression (Danzer, 2012). Adult hippocampal neurogenesis is proposed to affect storing of new memories, cognition and mood regulation (Zhao et al., 2008; Deng et al., 2010; Vivar and van Praag; 2013).

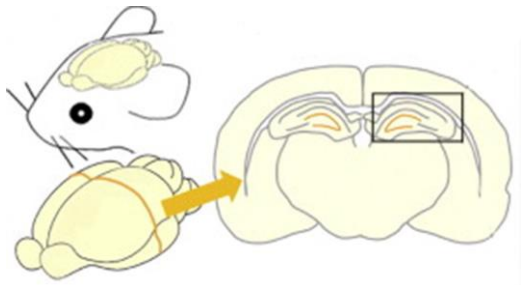

Fig. 2: The location of the hippocampal formation in the adult rodent brain. Modified from Kaneko and Sawamoto, 2009.

The DG is observed as a narrow concave lamina that envelops the CA4 segment of the hippocampus (Fig. 3) (Duvernoy et al., 2005; Curtis et al., 2012). Three layers form the DG: molecular, granular and polymorphic layers (Toni and Sultan, 2011). The molecular layer is the closest layer to the cortical surface and consists mainly of the dendrites of the granular neurons (Toni and Sultan, 2011). The granular layer that forms a V-shaped structure contains the small, round-shaped nuclei of the densely packed granular neurons (Duvernoy et al., 2005; Toni and Sultan, 2011). The main input of mature granule cells is excitatory glutamatergic projections of layer II neurons of medial and lateral portions of the EC that form the perforant path axons innervating the dendritic spines in the distal two-thirds of the apical dendrites (Fig. 3) (Amaral et al., 2007; Zhao and Overstreet-Wadiche, 2008; Bergami and Berninger, 2012). All dentate granule cells project their unmyelinated axons known as the mossy fibers to the CA3 part of the hippocampus (Fig. 3 (Amaral et al., 2007; Jacobson and Marcus, 2008). The axon trajectory is partially correlated with the position of the parent cell body (Amaral et al., 2007). The polymorphic layer, also referred to as the hilus, unites with the CA4 area of the hippocampus and is crossed by mossy fibers (Duvernoy et al., 2005; Jacobson and Marcus, 2008). The subgranular zone (SGZ) is a narrow band

of cells lying between the granular and polymorphic layers (Song et al., 2012). The SGZ is not a distinct layer but rather a sporadic collection of neuronal stem cells and progenitor clusters lining the deep aspect of the granule cell layer (Andersen et al., 2007; Ming et al., 2011).

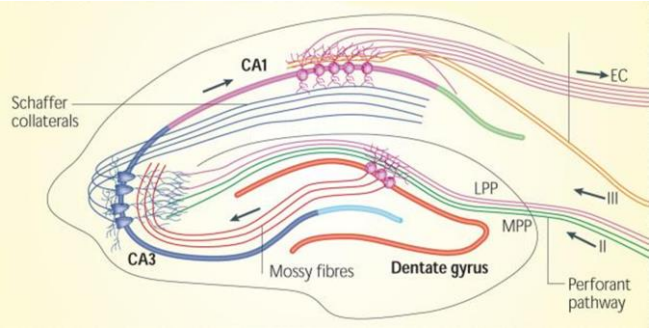

Fig. 3: An illustration of hippocampal circuitry showing the location and a simplified connectivity map of the DG to the hippocampus and lateral and medial perforant pathways (LPP and MPP). Modified from Deng et al., 2010.

The development of newborn adult neurons can be divided into five phases: 1. stem cell maintenance, activation and fate specification; 2. expansion of intermediate neural progenitors; 3. migration and initial pruning of newborn granule cells; 4. maturation and functional integration of newborn neurons, and 5. late-phase maturation and maintenance of adult-born neurons (Song et al., 2012).

Recent studies suggest existence of a heterogeneous pool of neural precursors in the adult rodent hippocampus (Bonaguidi et al., 2012; Jhaveri et al., 2012; Kim et al., 2012; Song et al., 2012). In the most accepted model of adult neurogenesis, the neural stem cell is a quiescent radial glial fibrillary acidic protein (GFAP)-positive cell (Alvarez-Buylla and Lim, 2004; Bergström and Forsberg-Nilsson, 2012; (Jhaveri et al, 2012). Radial cells are characterized by their pyramidal cell body that is located in the SGZ and extends a long radial glia-like process to the molecular layer that branches there (Jhaveri et al., 2012; Song et al., 2012). Non-radial stem cells have also been described in the SGZ (Ma et al., 2005; Lugert et al., 2010; Bergström and Forsberg-Nilsson, 2012; Bonaguidi et al., 2012; Jhaveri et al., 2012; Song et al., 2012). Non-radial precursors express the intermediate filament protein Nestin and the Sry-related HMG-box transcription factor (Sox2) like the radial glia-like neurons, but they do not express GFAP (Lugert et al., 2010; Song et al., 2012). Non-radial cells show a circular soma with very short horizontal processes and divide in large numbers (Lugert et al., 2010; Kim et al., 2012). Some suggest that these two populations are lineage-related but direct evidence is currently missing (Lavado et al., 2010; Hsieh, 2012; Jhaveri et al., 2012). A tight balance between these two cell pools is essential to maintain tissue

integrity and preserve regenerative capacity of the adult hippocampus (Jhaveri et al., 2012). Proliferating radial and non-radial precursors are collectively known as Type 1 cells and can generate neurons, astrocytes, and oligodendrocytes (Fig. 4) (Lugert et al., 2010; Song et al., 2012; Yao et al., 2012; Vivar and van Praag, 2013). The different fates of the NSCs are mediated by a complex system of morphogens including Notch, sonic hedgehog (Shh), wingless-type MMTV integration site family (Wnt), fibroblast growth factor (FGF) and bone morphogenic proteins (BMPs), growth factors, surrounding cells, transcription factors, epigenetic mechanisms and neuronal activity particularly mediated by N-methyl-d-aspartate (NMDA) receptor of GABAergic input (Ming and Song, 2011; Toni and Sultan, 2011; Yao et al., 2012).

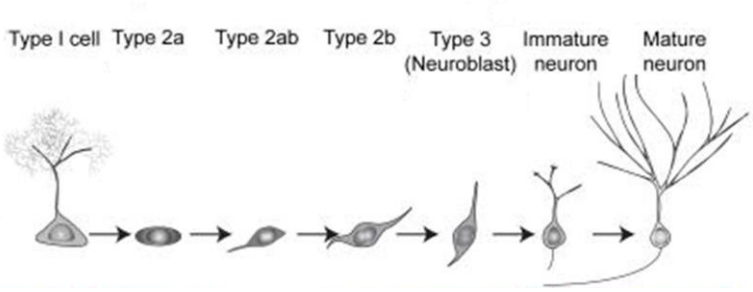

Fig. 4: Adult hippocampal neurogenesis process. Modified from Bonaguidi et al., 2012.

Type 1 cells likely give rise to Type 2 intermediate progenitors under the influence of FGF-2, Shh., Wnt7a and vascular endothelial growth factor (VEGF) (Vivar and van Praag; 2013). A subset of Type 2 cells show a simple morphology with few horizontal processes and are highly proliferative (Type 2a) (Fig. 4) (Bergami and Berninger, 2012; Vivar and van Praag, 2013). Most of the expansion of the pool of the newly born cells occurs during this stage (Ehninger and Kempermann, 2008). These cells have the potential to differentiate into neurons and astrocytes (Hsieh, 2012). At this stage, the pace of neuronal development is under the influence of depolarizing action of local GABA through tonic activation of non-synaptic receptors (Mongiat and Schnider, 2012). Type 2b cells differentiate along a neuronal lineage within 3 days (Fig. 4) (Ehninger and Kempermann, 2008; Kim et al., 2012). In an early critical period, a significant loss of newborn progeny can be observed 4 days after birth due to apoptotic mechanisms (Kim et al., 2012). Therefore, the proliferative activity of precursor cells is not a good predictor of the net neurogenesis (Ehninger and Kempermann, 2008). One week following the production of the cells, one of the neurites becomes thicker and grows toward the molecular layer and starts to receive functional GABAergic synaptic inputs that initially have an excitatory influence on

the immature neurons (Ehninger and Kempermann, 2008; Bergami and Berninger, 2012; Kim et al., 2012). This depolarization serves as a trophic mechanism and promotes differentiation, migration and maturation of the newborn neurons (Kim et al., 2012). Meanwhile, another neurite elongates and directs toward the hilus suggesting acquisition of a vertical orientation and an axon-dendrite polarity, while other processes remain headed toward the hilus (Bergami and Berninger, 2012; Song et al., 2012).

Type 2b cells that express doublecortin (DCX) migrate a short distance into the inner GCL and progress to immature Type 3 neurons (neuroblasts) that express Prox1, a marker for the granule cells of DG and other neuronal lineage markers and lack glial markers (Fig. 4) (Ehninger and Kempermann, 2008; Lugert et al., 2010; Kim et al., 2012; Song et al., 2012; Vivar and van Praag, 2013). Type 3 cells show variable morphology reflecting the developmental transition from neuroblasts to postmitotic neurons (Ehninger and Kempermann, 2008). In two weeks, an axon is clearly distinguishable in the CA3 area and the main dendrite undergoes branching (Bergami and Berninger, 2012; Kim et al., 2012). Mossy fibers begin to form synapses with pyramidal cells during this period (Vivar and van Praag, 2013). About a week later, protrusions of filopodia and spines start to grow in close proximity of pre-existing synapses of the environment, while the cell body enlarges (Ehninger and Kempermann, 2008; Bergami and Berninger, 2012). Adult-born neurons do not migrate randomly in the GCL, instead, they migrate radially from the SGZ and the majority remain within the inner third of the GCL and thereby contribute to the heterogeneous layering plan of it, which is believed to play a role in separation between events (Kempermann et al., 2003; Barker et al., 2011; Mongiat and Schnider, 2011).

After three to four weeks, new neurons start to look similar to the mature neurons; dendrites reach to the external border of the molecular layer and increases in dendritic arborization and spine numbers continue, while the axon terminals are refined and mossy fiber boutons mature in the hilus (Fig. 5) (Bergami and Berninger, 2012; Kim et al., 2012). Excitatory glutamatergic input onto the new granule neurons is largely completed and GABA becomes hyperpolarizing (Ehninger and Kempermann, 2008; Vivar and van Praag; 2013). More than 50% of adult-born neurons are eliminated due to programmed cell death within the first month of their generation (Cameron et al., 1993). Despite similar resting potentials, newborn immature neurons have a higher input resistance and smaller membrane capacitance compared to mature granule cells

and can undergo long-term potentiation more easily (Toni et al., 2007; Ehninger and Kempermann, 2008; Kim et al., 2012; Vivar and van Praag, 2013). Since immature neurons are more readily excitable, they may be preferentially activated and involved in the representation of a particular event (Deng et al., 2010; Barker et al., 2011; Yassa and Stark, 2011). Therefore, neurons at a similar maturation stage may be linked together for the representation of a particular event at a specific time point, while cells that are distinct in age can get linked to represent events further apart in time (Barker et al., 2011). Thus, the adult- born cells may play a role in pattern separation and pattern integration (Barker et al., 2011).

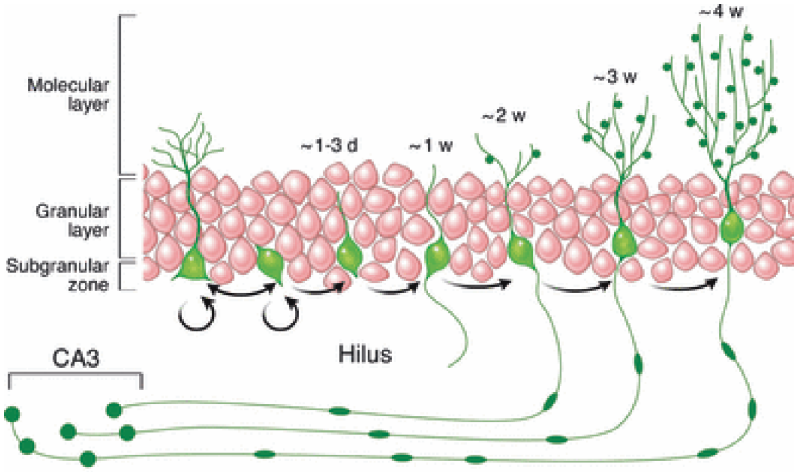

Fig. 5: Timeline showing the morphological development of adult-born hippocampal neurons. Modified from Toni and Sultan, 2011.

By the age of 2 months, synaptogenesis is completed and dendritic arborization reaches the maximum of complexity and the axon appears fully differentiated (Bergami and Berninger, 2012; Song et al., 2012). Full maturation of the spines may take around 6 months (Toni et al., 2011; Vivar and van Praag, 2013). It is reported that the morphological plasticity of adult newborn neurons for parameters such as dendritic branching and soma size is greater than pre-existing granule neurons (Lemaire et al., 2012; Vivar and van Praag, 2013).

After adult-born neurons establish stable synapses, they can survive for at least 6 to 11 months in rodents and remain a part of the mature dentate circuitry (Kempermann et al., 2003; Kim et al., 2012). Hippocampal neurogenesis leads to the addition of new neurons to the existing circuits of the DG rather than replacing the old granule cells (Imayoshi et al., 2008; Deng et al., 2010). New neurons make up to 10% of the DG granule cells in about 6 months into adulthood of mice and thereafter reach a saturation level probably due to decrease of neurogenesis in aged mice (Mori et al., 2006; Imayoshi et al., 2008,2009).

Various studies show that the new neurons are important components of networks involved in cognitive processes and the regulation of emotional status, and that the continued addition of neurons might be required for allowing continual storage and indexing of new information (Zhao et al., 2008; Encinas et al., 2009; Lazarov et al., 2010; Aimone & Gage, 2011; Inokuchi, 2012). On the other hand, this addition contributes to a relatively minor fraction of the total neuronal population and remains stable after reaching a plateau, which may be important in the stability of memory processing (Ninkovic et al., 2007; Imayoshi et al., 2008). Adult-generated neurons mature slower than the neonatal neurons particularly in primates and humans (Bergami & Berninger, 2012; Curtis et al., 2012). This might be a specific adaptation for proper maturation and integration of the newborn neurons into the existing circuit (Bergami & Berninger, 2012; Curtis et al., 2012).

Although the characteristics of the hippocampal neurogenesis process have been primarily studied in rodents, the course of adult human hippocampal neurogenesis shows patterns that are similar to that of rodents (Eriksson et al., 1998; Knoth et al., 2010).

1.4 Methodological approaches to identify newborn neurons

Ezra Allen provided the first evidence that new neurons could be born in the adult mammalian brain by discovering mitotic figures in lateral ventricles of rats up to 120 days of age (Allen, 1912). These results were neglected until 1960s when Joseph Altman published a series of papers in which he used thymidine autoradiography to demonstrate that new neurons can be found in the hippocampus and olfactory bulb of young and adult rats (Altman & Das, 1965; Altman et al., 1969). Later, Michael Kaplan used electron microscopy to show that tritium labeled thymidine cells in the DG and olfactory bulb of adult rat exhibit the ultrastructural characteristics of neurons (Kaplan & Bell, 1984).

An important development in the neurogenesis studies was the introduction of 5-bromo-3'-deoxyuridine (BrdU) in the 1990s. BrdU is a halogen-based analogue of thymidine that can be incorporated into the DNA of dividing cells and therefore, is used to detect DNA synthetic events during the S-phase of the cell cycle (Taupin, 2007; Cavanagh et al., 2011). BrdU labeling can be detected with immunohistochemical techniques and provides an opportunity to distinguish the new cells based on the co-expression of specific cell type markers and allows stereological estimation of the total number of cells (Gross, 2000; Cavanagh et al., 2011). Gage and colleagues were the first to use this method to show the adult-generated neurons in rats (Kuhn et al., 1996) and in humans (Eriksson et al., 1998). Today, BrdU labeling has become the method of choice to study cell proliferation, however, its interpretation is not trivial (Cavanagh et al., 2011; Langren and Curtis, 2011). BrdU crosses the blood-brain barrier and can be delivered through intracerebroventricular, intavenous, intraperitoneal or oral administration (Taupin, 2007). The most common doses of BrdU are in the range of 50-100 mg/kg, however it has been demonstrated that for a complete saturation higher doses are needed (Cameron and McKay, 2001; Cavanagh et al., 2011). Physiological differences between individuals, strains and species affect the bioavailability, permeability and dose-dependency of BrdU (Cavanagh et al., 2011). The number of labeled cells by BrdU administration may vary depending on the application mode, duration and concentration of applied BrdU, and survival times following the injection (von Bohlen Und Halbach, 2011). BrdU is not a S-phase specific marker but rather a marker of DNA synthesis and thus can label apoptopic cells, gene duplication, abortive cell cycle re-entry, or cells that undergo DNA repair theoretically (Cavanagh et al., 2011; von Bohlen und Halbach, 2011). However, it is likely that the most common doses of BrdU are insufficient to detect such cells

(Cooper-Kuhn and Kuhn, 2002). A major downside of BrdU administration is that it has a short bioavailability and depending on the efficacy of the administration, it labels the dividing cells in a narrow time-window and therefore repeated administrations are needed to optimize labeling which also increases the potential for stressful or toxic effects (Landgren and Curtis, 2011).

The BrdU method can be combined with various markers that are expressed during different stages of neurogenesis for a better understanding of phenotype, cell origin, identity of the proliferating cell, migration, and lineage and cell fate and the identity of the proliferating cell (Cavanagh et al., 2011; von Bohlen und Halbach, 2011). GFAP, Hes5, Nestin, Sox1, Sox2, BLBP, Mash1, Prox1,, NeuroD, DCX, PSA-NCAM, Calretinin, Calbindin and NeuN are expressed in different stages of adult neurogenesis and can be used as markers to identify the cells (Fig. 6) (Lazarov et al., 2010; von Bohlen und Halbach, 2011). In my studies, antibodies against Ki67, DCX, Prox1 and NeuN were used to differentiate the stages of the newly born neurons.

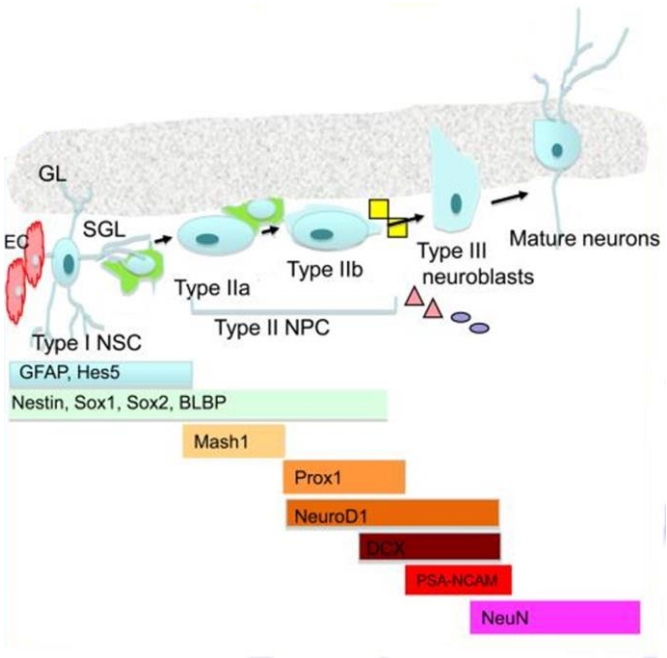

Fig. 6: Expression of various proteins and transcription factors during the morphological and physiological development of NSCs in the GCL. Modified from Lazarov et al., 2010.

Ki67 is an intrinsic protein expressed in all phases of the cell cycle except the resting phase and at the beginning of the G1 phase, and therefore labels approximately 50% more cells than BrdU (Kee et al., 2002; von Bohlen und Halbach, 2011). Ki67 has a short half-life of about 1h and is not detectable during DNA repair processes and is not expressed in the quiescent cells (Taupin, 2007). Ki67 is a useful marker in experiments that do not involve tracing of cells over long periods particularly in humans where the invasiveness of BrdU is a concern (Kee et al., 2002).

Doublecortin (DCX) is a brain-specific microtubule-associated protein. Although its exact function is not fully understood, it is thought to act as a microtubule stabilizer and promotes microtubule polymerization (Fig. 6) (Knoth et al., 2010; von Bohlen und Halbach, 2011). It is detectable in migrating neuroblasts and young neurons both in the central and peripheral nervous system (Gleeson et al., 1999; von Bohlen und Halbach, 2011). DCX expression spans over a long range of the neurogenesis process from the progenitor stage to the postmitotic synaptogenesis stage (von Bohlen und Halbach, 2011). DCX is present in both soma and processes of the newly generated neurons making it a good choice for investigating morphology of neurons (von Bohlen und Halbach, 2011).

Prospero-related homeobox gene 1 (Prox1) is a transcription factor that is upregulated in postmitotic young neurons in the DG and persists in all granule neurons (Fig. 6) (Hodge and Hevner, 2011; von Bohlen und Halbach, 2011). Its expression begins in type 2b cells thus can be used as a marker for the neuronal lineage. Prox1 is important for the maintenance of type 2 cells during adult neurogenesis and regulates maturation (Lavado et al., 2010).

Neuron-specific nuclear protein (NeuN) is observed in most neuronal cell types in the nervous system with the exception of some neuronal populations such as Purkinje cells, mitral cells and cells located in the glomerular layer of the olfactory bulb, but it is not expressed by non-neuronal cells (Fig. 6) (von Bohlen und Halbach, 2011). NeuN is localized mainly in the soma of postmitotic cells and labels newly generated and mature neurons in the hippocampus (von Bohlen und Halbach, 2011).

Although a combinational approach of using morphological criteria together with BrdU labeling and the overlapping expression of a number of genes can successfully lead to the identification of different subtypes of the precursor cells, these methods cannot be used to visualize cells in living tissue (Dhaliwal and Legace, 2011; Jhaveri et al., 2012). Therefore, newer techniques such as genetic manipulation to generate transgenic mice and viral neuronal tracing are becoming more and more common to gain a better understanding of adult neurogenesis.

Genetic manipulation of adult neurogenesis has been achieved by using various methods. One approach is to suppress neurogenesis by engineering diphtheria toxin into the locus of the neuron-specific gene, or by increasing apoptosis by promoting overexpression of suicide genes, or by blocking several signaling cascades (Imayoshi et al., 2011). Several methods have been developed that control the activity of genetically targeted neuronal populations either by chemical triggering, silencing them

using genetic techniques, or using optogenetic tools for stimulation and inhibition (Imayoshi et al., 2011).

The Cre/*lox*P recombination system proved to be a convenient tool for tissue specific or developmental stage-specific modulation of gene expression in knockout and transgenic mice (Kawamoto et al., 2000; Dhaliwal and Lagace, 2011). In this system expression of P1 bacteriophage Cre recombinase is under the control of a cell or tissue specific promoter gene (Dhaliwal and Lagace, 2011; Landgren and Curtis, 2011). Crossing the Cre mouse with a floxed reporter mouse that has a stop codon between *lox*P recognition sequence sites results in a bi-transgenic conditional mouse in which the recombination and expression of the reporter protein is under the control of the promoter (Dhaliwal and Lagace, 2011). Cre recombinase efficaciously excises DNA which is flanked by two directly repeated *lox*P recognition sites and allows the permanent expression of the reporter protein such as enhanced green fluorescent protein (eGFP) (Fig. 7) (Imayoshi et al., 2009). In order to create an inducible system, an estrogen receptor (ER) protein is fused to Cre creating the CreER that allows recombination to be induced at any time through treatment with an estrogen ligand such as tamoxifen (Dhaliwal and Lagace, 2011). CreER protein remains in the cytoplasm until a synthetic estrogen antagonist like tamoxifen is administered which allows recombination by translocating the fusion protein to the nucleus (Landgren and Curtis, 2011). This gives a reliable temporal control over the recombination (Imayoshi et al., 2009). Different modifications have been made to the CreER system to enhance the sensitivity to the estrogen ligand including ERT and ERT2 (Dhaliwal and Lagace, 2011). This system has been successfully used to study postnatal and adult neurogenesis (Carlen et al., 2006; Imayoshi et al., 2006; Mori et al., 2006). Factors such as timing of induction, age of animals, and genomic integration point of the Cre transgene are important in application of this method (Landgren and Curtis, 2011).

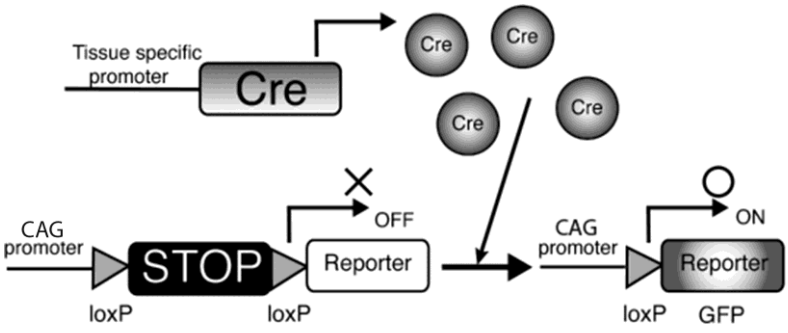

Fig. 7: Fate mapping strategy using Cre- expressing cells crossed with the eGFP reporter lines. Modified from Imayoshi et al., 2009.

Neurotropic viruses are self-amplifying tracers that enter the neurons, find the nucleus and replicate (Lanciego and Wouterlood, 2011). The ability to cross synapses to infect second-order neurons makes viruses a useful tool to study the connectivity in the neuronal network particularly in combinaiton with optogenetic methods (Zhang et al., 2007; Lanciego and Wouterlood, 2011). Viral gene delivery is another method to study adult neurogenesis in which the virus transfects a variety of cells, allowing a greater flexibility than transgenic mice (Landgren and Curtis, 2011). Adeno-associated virus (AAV) has a variety of serotype capsids that determine the cell type specificity that is widely used in stem cell studies (Landgren and Curtis, 2011). Lentiviral vectors enable long-term targeting of the NSCs and can be used in combination with Cre/*loxP* system to allow temporal control (Consiligo et al., 2004; Landgern and Curtis, 2011). The expression of transgenes from a virus requires viral integration into the host genome (Ming and Song, 2005). Retroviruses that lack nuclear import mechanisms are selective for dividing cells because viral integration can only occur when the nuclear membrane breaks down during mitosis (Ming and Song, 2005; Vivar and van Praag, 2013). Marking with viruses requires invasive stereotaxic injection and the labeled population is limited, however, it is a useful tool for birth dating and allows a direct permanent visualization of the newborn neurons (Ming and Song, 2005).

1.5 Disease-associated modulation of adult hippocampal neurogenesis

Adult neurogenesis is a dynamic and finely regulated process that is subject to modulation by various physiological, pathological and pharmacological stimuli (Thompson et al., 2008; Zhao et al., 2008; Kaneko and Sawamoto, 2009; Ming & Song, 2011). Modulation of hippocampal neurogenesis by rewarding or threatening cues may optimize hippocampal function to suit the environment and maximize the chance of survival (Glasper et al., 2012). Experiences that are detrimental to well-being such as stressors reduce the production of new neurons, whereas rewarding experiences including running, learning, sexual experiences, living in enriched environment and intracranial self-stimulation increase the rate of adult neurogenesis in the DG (Glasper et al., 2012).

On the other hand, brain insults and their consequent inflammation may potentiate or block neurogenesis by altering the niche microenvironment (Das and Basu, 2008). The rate of proliferation in the DG can be accelerated under acute pathological conditions such as seizures, cerebral ischemia and neurotoxic lesions (Das and Basu, 2008; Curtis et al., 2012). Infections with viruses and bacteria are mainly associated with neuronal loss and changes in neurogenesis levels while the relative functional impact of the neurotropic viruses and contribution of neuroinflammation to these changes are still unknown (Das and Basu, 2008). Pathological conditions such as epilepsy and seizure activity, traumatic brain injury, ischemia, stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease and depression alternate different stages of neuronal generation in the neurogenic niches in the brain of patients and animal models (Taupin, 2008; Thompson et al., 2008; Kaneko and Sawamoto, 2009; Curtis et al., 2012; Jhaveri et al., 2012; Yao et al., 2012).

Studies of disease-associated neurogenesis alterations not only contribute to a better understanding of the mechanisms of the diseases and co-morbidities, but also provide information for a more accurate modeling and for novel therapeutic approaches (Yao et al., 2012). Despite evidence for modulation of hippocampal neurogenesis following acute insults, the plasticity of the neurogenic niche during chronic disorders remains poorly understood (Pluchino et al., 2008). Therefore, I aimed to study long-term alterations of hippocampal neurogenesis, using two common animal models of diseases: Theiler's murine encephalitis as a model for multiple sclerosis (MS) disease, and the kindling model of epilepsy. It is of utmost interest to uncover how diseases modulate the integration of new neurons into an already functional circuit and how the new circuit contributes to the pathology of disease.

1.6 Theiler's murine encephalitis virus

Theiler's murine encephalitis virus (TMEV) is a non-enveloped single-stranded RNA virus from the *Cardiovirus* genus of the *Picornaviridae* family (Oleszak and Chang, 2004; Tsunoda and Fujinami, 2010; Stewart et al., 2011). TMEV is a natural enteric pathogen of mice, however, in some mouse strains intracerebral inoculation results in a biphasic neurotropic viral infection that leads to the induction of a demyelinating disease with similar pathology to the MS disease (Dal Canto et al., 1996; Lipton et al., 2005; Tsunoda and Fujinami, 2010; Mecha et al., 2013). Two subgroups of TMEV strains are distinguished based on their neurovirulence following intracerebral inoculation of mice: the GDVII subgroup is a highly neurovirulent strain that produces a rapid, fatal acute encephalitis with infection and destruction of a large number of neurons which can result in death within 1 to 2 weeks (Monteyne et al., 1997; Lipton et al., 2005; Tsunoda and Fujinami, 2010). The Theiler's original (TO) subgroup consists of the DA and BeAn strains that cause a chronic biphasic progressive disease starting with an early acute polioencephalomyelitis occurring 3 to 12 days following the infection that lasts 10 to 14 days, and a late chronic inflammatory demyelinating disease developing 30 to 40 days after the infection that eventually can cause the death of the animal (Monteyne et al., 1997; Oleszak and Chang, 2004; Lipton et al., 2005; Tsunoda and Fujinami, 2010). Infection with the TO subgroup results in an immune-mediated persistent infection as well as viral damages of oligodendrocytes and thus demyelination, which provides a highly relevant model for MS disease (Dal Canto et al., 1996; Dal Canto and Rabinowitz, 2004; Lipton et al., 2005).

During the acute phase of infection with TO viruses, the virus infects and damages neurons in the gray matter of the brain and spinal cord resulting in a self-limiting acute polioencephalomyelitic disease characterized by areas of focal necrosis, neuronophagia and mononuclear cell infiltration (Oleszak and Chang, 2004; Dal Canto and Rabinowitz, 2004; Lipton et al., 2005; Mecha et al., 2013). This phase is followed by virus persistence in the white matter of spinal cord and macrophages and to a lesser extent in oligodendrocytes and astrocytes during the chronic phase leading to demyelination that is associated with the development of spastic paralysis of hind limbs (Dal Canto et al., 1996; Monteyne et al., 1997; Dal Canto and Rabinowitz, 2004; Lipton et al., 2005).

Infection with the DA virus results in a higher incidence of demyelinating disease compared to the BeAn strain, whereas the onset of demyelinating disease is more rapid

with the BeAn strain (Zoecklin et al., 2003; Lipton et al., 2005). Also, early acute disease is attenuated in BeAn-infected mice in comparison to DA infection (Oleszak et al., 2004).

Persistence of the infection only occurs in susceptible mouse strains such as SJL/J strain (Monteyne et al., 1997; Oleszak and Chang, 2004). Resistant strains such as C57BL/6 only develop the early acute disease phase with subsequent clearance of the virus in about 2 to 3 weeks, which prevents the development of a late chronic demyelinating disease (Monteyne et al., 1997; Oleszak and Chang, 2004). Differential expression of cytokines in mice strains seems to be responsible for the varying susceptibility to the virus (Chang et al., 2000; Gerhauser et al., 2012). Furthermore, the demyelinating phase of the disease is closely correlated with the presence of inflammatory cells and various immune responses involved in the induction and release of chemokines and cytokines during demyelination and axonal degeneration, while a strong antiviral cytokine response seems to be essential for the elimination of the virus in the early phase (Del Canto et al., 1996; Tsunoda and Fujinami, 2010; Gerhauser et al., 2012).

Recently, it has been shown that different strains of TMEV contribute to the development of acute seizures in C57BL/6 mice (Libbey and Fujinami al., 2011). While 50% of C57BL/6 resistant mice that are infected with DA strain of TMEV show spontaneous afebrile seizures during the acute phase, no seizure is observed in the susceptible SJL/J mice (Libbey et al., 2008). Furthermore, TMEV infection with DA strain leads to chronically altered seizure susceptibility that is more pronounced in the C57BL/5 mice that display seizures during the acute phase (Stewart et al., 2010).

1.7 Kindling model of epilepsy

Kindling is one of the best-controlled models of chronic focal epilepsy that is widely used to study complex partial seizures with secondary generalization (McIntyre and Racine, 1986; Adamec, 1990; Kalynchuk, 2000). Graham Goddard first developed this model in 1960s by repeated application of a low-intensity electrical stimulus to forebrain structures (Goddard, 1967; Goddard et al., 1969). Repeated, temporally spaced direct electrical stimulation of various brain structures via chronically implanted depth electrodes leads to persistent lowering of the seizure threshold that results in progressive intensification of both electrographic and behavioral seizures (Racine, 1972a, b; Löscher and Brandt, 2010; Scott et al., 1998). Kindling can be induced by electrical, chemical and acoustic stimulations in various parts of the brains of many species; however, the most frequent model of temporal lobe epilepsy is the daily electrical stimulation of the amygdala (Kalynchuk, 2000; Kupferberg, 2001; Sharma et al., 2007).

Initially, the stimuli are subconvulsive and are only sufficient to evoke epileptic spiking in the EEG record that outlasts the stimulation (afterdischarges) (Hawrylak et al., 1992; Kalynchuk, 2000). With repeated stimulations the afterdischarge threshold decreases and electrographic seizures increase in duration, amplitude, spike frequency, and spike morphology. Furthermore there is a progression of behaviors from mild to severe with successive stimulations (Hawrylak et al., 1992; Morimoto et al., 2004). Kindling convulsions are rated based on Racine classification: Stage 1, immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus; Stage 2, head nodding associated with more severe facial clonus; Stage 3, clonus of one forelimb; Stage 4, bilateral clonus of forelimbs; Stage 5, generalized clonic seizures with rearing and falling (Racine, 1972b). Animals are considered "fully kindled" once the stimulation of the kindling focus results in a bilateral forelimb clonus with rearing and falling. Spontaneous seizures can be achieved if very large number of kindling stimulations are applied (also known as the "over-kindling" model), however, most of the studies use a partial epileptogenesis model in which spontaneous seizures do not readily occur (McIntyre et al., 2002; Morimoto et al., 2004).

The mechanism in the development of kindling is not clear. However, there seems to be a correlation between the development of seizures and alteration in inhibitory circuitry that provokes strong burst responses and lasting hyperexcitability to a seemingly constant initiating stimulus (McIntyre and Racine, 1986; Kalynchuk, 2000; Stafstrom and Sutula, 2005). Repeated network synchronization and seizure-induced

plasticity evolve into morphological reorganization of neurons and neural circuits especially in the hippocampus that leads to functional deficits (Stafstrom and Sutula, 2005). The neurological changes following kindling seem to be permanent since fully kindled animals that have not been stimulated for many months often respond immediately after re-exposure to the original stimulus (Goddard et al., 1969). In the kindling model, altered neuronal response develops in the absence of major morphological damages and inflammation (Morimoto et al., 2004; Wood et al., 2011). In rodents, it has been shown that even a brief single episode of hippocampal electrical stimulation can stimulate proliferation of neurons in the DG (Bengzon et al., 1997).

1.8 Aims of the thesis

The main aim of this doctoral thesis was to study disease-associated modulation of adult hippocampal neurogenesis in long-term. For this purpose, I evaluated the changes of adult generation of neurons in the DG in the TMEV model of the MS disease and in the amygdala-kindling model of epilepsy. It was specifically interesting to investigate the long-term impact of viral infection and repeated seizures as two major brain insults on the production and survival of newborn neurons.

Viruses and bacteria constitute a major source of infections targeting the brain (Das and Basu, 2008). Various neurotropic viruses cause chronic CNS infections including DNA viruses like cytomegalovirus (CMV), RNA viruses like measles virus, Borna disease virus (BDV), lymphocytic choriomeningitis virus (LCMV), canine distemper virus (CDV) and Theiler's murine encephalitis virus (TMEV), and few retroviruses like human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV) (Sharma et al., 2002; Das and Basu, 2008, Kaul, 2008; Tsutsui et al., 2008; Brnic et al., 2012; von Rüden et al., 2012). Encephalitis caused by these viruses may be associated with acute seizures, epileptogenesis and psychiatric co-morbidities (Preux et al., 2005; Libbey et al., 2008). Any injury or insult to the brain has a wide range of consequences always including a neuroinflammatory reaction (Das and Basu, 2008). Microglia, astrocytes and peripheral macrophages and lymphocytes contribute to neuroinflammation by releasing varying numbers of anti- and pro- inflammatory substances, neurotransmitters, chemokines, reactive oxygen species (ROS) and upregulated cytokine and toll-like receptors (Das and Basu, 2008; Gonzalez-Perez et al., 2012). The pro-inflammatory mediators act as double-edged swords by immune protection of the brain on one hand, and initiating a positive feedback inflammatory loop that can lead to neuronal death on the other (Das and Basu, 2008). The interaction between the immune system and the brain can cause major alterations in the neurogenic niche and hence affect neural remodeling and neurogenesis among many other cerebral functions (Das and Basu, 2008; Yoneyama et al., 2011; Gonzalez-Perez et al., 2012). Inflammation has a complex role in adult neurogenesis: The disturbances of the immune system homeostasis lead to detrimental effects in the generation of the neurons, whereas regulated immune activation with the appropriate timing, location, duration and dosing will support generation of neurons (Ekdahl et al., 2009; Schwartz et al., 2009; Huehnchen et al., 2011).

How the viral infections affect hippocampal neurogenesis remains an open question. Recent studies have demonstrated that viruses modulate different stages of

neurogenesis. The HIV envelope protein negatively regulates adult hippocampal neurogenesis by reducing the proliferation of neuronal progenitor cells (Tran and Miller, 2005; Kaul, 2008; Lee et al., 2011). LCMV decreases neuronal progenitors in the DG of neonatal rats, which results in impairment of neurogenesis during adulthood (Sharma et al., 2002). BDV has been shown to impair neurogenesis by interfering with the survival of newly generated neurons (Brnic et al., 2012). CDV seems to additionally modulate neuronal differentiation (von Rüden et al., 2012). However, the mechanisms by which viruses alter neurogenesis are poorly understood and therefore the question remains whether other viral infections modulate adult hippocampal neurogenesis and if they do, what step of the process do they modulate? Do intrinsic factors such as genetic differences and immune responses participate in the outcome of viral infection on neurogenesis? The answer to this question is not only important to understand the pathogenesis of the CNS diseases, but also valuable in describing the animal models more precisely and have a better understanding of the similarities and differences of the model to the condition in human patients.

Therefore, I chose to investigate the differential effect of Theiler's virus infection on hippocampal progenitor cells in two mouse strains in our first study. For this purpose, we inoculated the left hemisphere of five-week old female SJL/J and C57BL/6 mice with the BeAn strain of TMEV virus using a stereotactic apparatus. The animals were clinically checked every week for their overall clinical impairment (total clinical score) based on general appearance, activity level, and gait/paralysis. BrdU was administered twice daily at days 7-11 following the infection to the animals. The brains were collected 14 and 99 days post infection from both strains of mice to investigate differential effects of TMEV in the acute and chronic phase. DCX labeling was evaluated using unbiased stereological cell counting procedures to study the population of neuronal progenitor cells, neuroblasts and immature neurons. BrdU/NeuN double labeling allowed for investigation of proliferation in the hippocampus and the number of mature neurons. Furthermore, we explored the role of microglia by evaluating their morphology and number using CD11b antibody. Additional animals were used for ex vivo study of microglia using cytometry. The phagocytic activity, generation of ROS, and expression of surface proteins (including MHC I, TNF-α and IL-1β) provided information on immunophenotypical and functional characterization of microglia at two time-points following the infection in both strains.

In my second study, we focused on the kindling model of epilepsy. Epilepsy is a common neurological disorder affecting 1-2% of the population (Danzer, 2012). It is clinically defined by the occurrence of two or more unprovoked seizures (Danzer, 2012). Seizure development (= ictogenesis) is the result of an imbalance between neuronal excitation and inhibition leading to the brain exhibiting excessive and hypersynchronous activity (Morimoto et al., 2004; Sharma et al., 2007, Curtis et al., 2012). Mesial temporal lobe epilepsy is a chronic disease characterized by spontaneous, progressive seizures that in many patients is initiated by a traumatic event followed by a latency period before onset of spontaneous seizures (O'Dell et al., 2012). This type of epilepsy originates from mesial temporal structures such as the hippocampus or amygdala and is commonly associated with hippocampal changes including neuron loss and hippocampal lesions, axonal sprouting of the granule neurons of the DG and changes in glial morphology and function (Sharma et al., 2007; Kaneko and Sawamoto, 2009; Löscher and Brandt, 2010, O'Dell et al., 2012). The biochemical pathways leading to these changes remain unclear, however, it has been proposed that changes accumulate over the course of the disease with each insult (O'Dell et al., 2012). Brain inflammation is discussed as a major factor that contributes to determine seizure threshold and its recurrence (Vezzani et al., 2013). Poorly controlled seizures may lead to loss of autonomy and the progressive nature of disease may lead to cognitive decline and other co-morbidities including memory and learning disturbances, anxiety, psychosis and depression (Löscher and Brandt, 2010; Danzer, 2012; O'Dell et al., 2012). Although the mechanisms of co-morbidities are so far incompletely understood, disruption of hippocampal neurogenesis is discussed as a putative common factor (Danzer, 2012).

The impact of seizures on neurogenesis has been widely studied during the initial and the chronic phase of different epilepsy models (Bengzon et al., 1997; Parent et al., 1997, 1998; Hattiangady et al., 2004; Mohapel et al., 2004; Fournier et al., 2010). However, the question remains how the seizures change the hippocampal network in long-term and what the net outcome of alterations of neurogenesis during acute and chronic seizures is.

We used genetic fate mapping for a permanent labeling of newborn neurons to assess the long-term impact and net effect of seizures on neurogenesis. In the GLAST::CreERT2 x CAG-CAT-eGFP mice, administration of tamoxifen induces permanent activation of Cre recombinase in the cells expressing GLAST promoters (astrocytes and neural stem cells) so that the reporter eGFP protein would be produced

and be traceable. We used the kindling model of epilepsy that gave us the opportunity to specifically determine the intensity of stimulations. The 10-16 week old mice were implanted with a bipolar electrode aimed for the right amygdala and stimulated for nine days with a stimulation current 20% above the individual threshold that was determined two weeks following the surgery. After this initial period, the animals that showed at least one generalized seizure were distributed to three groups so that group means of afterdischarge threshold and seizure severity did not differ between the groups. In the chronic phase, a total of eight injections of BrdU were administered to the animals for further investigation on the proliferation and generation of new neurons in the early chronic phase. Animals were stimulated one or three times per week or kept without stimulation for 12 weeks. The control group was implanted with an electrode and was treated like the rest of the animals (same injections) but did not receive any stimulation in the initial and chronic periods. At the end of the experiment, proliferation in the early phase following the initial kindling acquisition phase, generation of new mature neurons during the complete experiment, and the number of proliferating cells and new immature neurons were carefully analyzed.

CHAPTER 2: "Impact of Theiler's virus infection on hippocampal neuronal progenitor cells: differential effects in two mouse strains"

2.1 Abstract

Aims: Disease-associated alterations in hippocampal neurogenesis are discussed as an important factor contributing to long-term consequences of central nervous system diseases. Therefore, the study aimed to determine the impact of Theiler's murine encephalomyelitis virus infection on hippocampal cell proliferation, neuronal progenitor cells and neurogenesis as well as the influence of microglia on respective disease-associated alterations.

Methods: The impact of the infection was evaluated in two mouse strains which differ in the disease course with an acute polioencephalitis followed by virus elimination in C57BL/6 mice and a chronic demyelinating disease in SJL/J mice.

Results: Infection with the low-neurovirulent BeAn strain did not exert significant acute effects regardless of the mouse strain. In the chronic phase, the number of neuronal progenitor cells and early postmitotic neurons was significantly reduced in infected SJL/J mice whereas no long-term alterations were observed in C57BL/6 mice. A contrarious course in microglia activation was observed in the two mouse strains, with an early increase in the number of activated microglia cells in SJL/J mice and a delayed increase in C57BL/6 mice. Quantitative analysis did not confirm a correlation between the number of activated microglia and the number of neuronal progenitor cells and early postmitotic neurons. However, flow cytometric analyses revealed alterations in the functional state of microglial cells which might have affected the generation of neuronal progenitor cells.

Conclusions: Theiler's murine encephalomyelitis virus infection can exert delayed effects on the hippocampal neuronal progenitor population with long-term alterations evident three months following infection. These alterations proved to depend on strain susceptibility and might contribute to detrimental consequences of virus encephalitis such as cognitive impairment.

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The author of this doctoral thesis contributed to this paper by participating in the

experimental part of the study, performing cellular studies of adult hippocampal neurogenesis, statistical analysis, and writing the major parts of the manuscript.

2.2 Introduction

Infection of mice with low-neurovirulent strains of Theiler's murine encephalomyelitis virus (TMEV) causes an acute polioencephalitis with infection of neurons in the grey matter [1-3]. Whereas the acute phase following intracerebral virus injection is comparable in different mouse strains, the genetic background has a major impact on the further disease course [3]. In C57BL/6 mice the immune response results in elimination of the virus from the central nervous system (CNS) within two to three weeks [3-4]. In contrast, SJL/J mice exhibit a biphasic course of the disease during which a chronic demyelination of spinal cord white matter develops in association with virus persistence [3, 5-7]. The clinical and pathophysiological features of this chronic phase show many similarities to the chronic progressive form of multiple sclerosis [3, 8-9]. Moreover, the disease course in susceptible SJL/J mice mimics the findings in other demyelinating diseases such as canine distemper virus infection [10-12].

During acute TMEV-induced polioencephalitis viral antigen positive cells can be detected in the hippocampus associated with hippocampal neuropathology. Taking into consideration that the microenvironment can have a major impact on hippocampal neurogenesis [13] the question arises whether TMEV infection affects cell proliferation and its survival and neuronal differentiation in the hippocampus. Alterations in hippocampal neurogenesis are discussed as an important pathophysiological factor in different CNS diseases [14-16]. Information about its modulation by virus infection is so far limited. However, it is well accepted that brain inflammation, microglia activation and cytokine production can exert pronounced effects on hippocampal neurogenesis [13, 17-18].

Any disease-associated disturbance of the balance in adult hippocampal neurogenesis is of particular interest as it might contribute to the consequences of CNS diseases [15-16, 19]. Experimental evidence exists that human immunodeficiency virus (HIV-1) infection can result in impairment of neurogenesis [20-22]. Subsequent studies revealed that virus-specific mechanisms seem to be involved in the interference of HIV with hippocampal neurogenesis [22-23]. Therefore, it remained questionable whether respective findings can be extended to other virus infections.

Here, we addressed the question whether BeAn TMEV infection differentially affects hippocampal cell proliferation and its survival and the neuronal progenitor cell population in susceptible SJL/J mice and resistant C57BL/6 mice. In view of the

regulatory role of microglia in hippocampal neurogenesis, we additionally analyzed the functional state of the microglial cell population.

2.3 Material and Methods

Animals and virus infection

Five-week-old female SJL/J and C57BL/6 mice (Harlan Laboratories, Eystrup, Germany) were used in this study. Animals were housed in groups using a microisolator cage system (Techniplast, Hohenpeißenberg, Germany). Animals were anesthetized using a combination of medetomidine (0.5 mg/kg, Domitor®; Pfizer, Karlsruhe, Germany) and ketamine (100 mg/kg, Ketamin 10%®; WDT, Garbsen, Germany). Intracerebral injections were performed using a small animal stereotactic instrument (TSE Systems, Bad Homburg, Germany) with a 50 µl syringe and 30 gauge needle (Hamilton, Bonaduz, Switzerland). Mice were inoculated into the left cerebral hemisphere (caudate putamen / striatum) (stereotaxic coordinates in relation to bregma: rostrocaudal +1.0 mm, dorsoventral 4.0 mm, left lateral 2.0 mm) with 1.63 x 10^6 plaque-forming units/mouse of the BeAn strain of TMEV in 20 µl of Dulbecco's Modified Eagle Medium (PAA Laboratories, Cölbe, Germany) with 2% fetal calf serum and 50 µg/kg gentamicin. Non-infected mice (placebos) received 20 µl of the vehicle as described previously [24]. After injection, the needle was left in place for 4 minutes to prevent flow of the fluid along the injection canal. Groups of 14-18 TMEV-infected and non-infected mice were killed 14 and 99 days post infection (dpi). Brain samples were then used for immunohistology or for microglia preparation and flow cytometric analyses. All animal experiments were conducted in accordance with the German Animal Welfare Law and were authorized by the local government (Regierungspräsidium, Hannover, Germany, permission number 33.9-42502-04-09/1770).

Clinical evaluation

Animals were clinically investigated once a week. A score ranging from 0 to 3 was used to categorize general appearance and activity level as previously described [8, 24]. General appearance scores were as follows: 0 = normal appearance; 1 = minimal change in fur; 2 = moderate impairment, with a scruffy appearance and a hunched back; and 3 = severe impairment with a severely hunched back and unkempt fur. Activity level scores were based on spontaneous locomotion behavior during a 1-minute test in an open field (sized 25 x 40 cm without border, located 14 cm above the table surface). Activity was scored as follows: 0 = no change in activity; 1 = mildly decreased spontaneous movement; 2 = moderately reduced spontaneous movements; and 3 = no or minimal spontaneous movement. Gait/paralysis was categorized using a score ranging from 0-4 as follows: 0 = normal gait; 1 = mild disturbance of gait with

inconsistent waddling; 2 = moderate disturbance with consistent waddling gait or hindlimb stiffness; 3 = severe disturbance with hindlimb stiffness or sliding gait and reduced righting response; and 4 = severe gait disturbance and spastic paresis of the hind legs. The overall clinical impairment ('total clinical score') was defined as the sum of the scores (sum ranging from 0 (healthy) to 10 (maximal clinical impairment)).

BrdU labeling

As a thymidine analog 5'Bromodeoxyuridine (BrdU) is incorporated into the DNA during the S phase of the cell cycle. BrdU is available for about 15 minutes following injection and thus labels a proportion of dividing cells which are in the S phase during this period [25]. Mice received a total of ten intraperitoneal injections of 50 mg/kg BrdU at days 7-11 (twice daily with 8 h interval between morning and afternoon administration) following the stereotactic injection of virus or virus-free medium. This timeframe of BrdU injections was chosen based on data demonstrating that the acute phase of brain infection ranges between days 3-12 following TMEV injection with virus replication reaching a maximum at day 5 and then declining until day 12 and with pathological signs of brain infection becoming visible from day 7 on. Thus, with BrdU administration between days 7-11 we guaranteed that we analyzed hippocampal cell proliferation during a phase in which virus replication is still observed and in which inflammation becomes evident [2-3].

Tissue preparation

Brains were sampled at day 14 or 99 following infection as we planned to analyze the impact of the infection on hippocampal cell proliferation, survival as well as on the neuronal progenitor population in susceptible SJL/J mice and resistant C57BL/6 in different phases of the disease course. At the end of the experiment (day 14 or 99 following injection of virus or virus-free medium) mice were anesthetized with chloral hydrate (Applichem, Darmstadt, Germany) and transcardially perfused with 0.01 M phosphate buffered saline followed by 4% paraformaldehyde. The brains were removed and transferred into 30% sucrose and stored at 4 °C. Cerebri were cut in the coronal plane on a cryostat (HM 560; Microm, Walldorf, Germany) at a thickness of 40 µm with 200 µm distance between successive sections of each series. Sections were stored at -80 °C in cryoprotecting solution (glycerol and 0.2 M phosphate buffer, pH 7.4, 1:1 in volume).

Verification of the infection

Following sampling of cerebral sections for the immunostaining of newborn cells, neuronal progenitor cells and microglia, the remaining brainstem tissue and adjacent

cerebellum were used for immunohistochemical detection of TMEV antigen and for evaluation of TMEV-induced lesions. Brainstem and cerebella were re-immersed in 4% formaldehyde for 24 hours and embedded in paraffin wax. Two to 3 µm serial sections were stained with hematoxylin and eosin for detection of inflammatory changes as well as with the Luxol fast blue-Cresyl violet stain (equivalent to Klüver-Barrera method) to label myelin, thereby allowing detection of myelin loss. In hematoxylin and eosin stained sections, the brainstem was evaluated semiquantitatively by light microscopy for the presence of inflammatory cell infiltrates both in the neuroparenchyma and in the perivascular space. Furthermore, loss of brainstem neurons as well as hypercellularity of neuroparenchyma indicating gliosis was assessed. A semiquantitative scoring system was used for evaluation with 0 indicating no change, 1 = mild, 2 = moderate, and 3 = marked change of the respective parameter. Immunhistochemical detection of TMEV antigen was performed using a rabbit polyclonal antibody as previously described [26]. A positive reaction as assessed by light microscopy was characterized by an intense dark brown signal in the cytoplasm, cytoplasmic processes, or the nucleus of various cell types in the brainstem.

Doublecortin immunstaining

Doublecortin labels neuronal progenitor cells, neuroblasts and immature neurons. For simplicity, we use the term "neuronal progenitor cells" to refer to all of the positive cells that were detectable due to the staining. Free-floating 40 µm thick sections were washed, pre-incubated and treated with the primary antiserum overnight at 4 °C (polyclonal goat anti-doublecortin diluted 1:300; Santa Cruz Biotechnology, Santa Cruz, California, USA). Sections were washed and incubated with biotinylated donkey anti-goat immunglobulins (1:500; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA). Subsequently, sections were rinsed in tris-buffered saline and incubated for 60 minutes in horseradish peroxidase-labeled streptavidin (1:4000; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA). Finally, the nickel-intensified diaminobenzidine reaction (0.05% 3,3'-diaminobenzidine from Carl Roth GmbH + Co. KG, Karlsruhe, Germany and 0.01%; nickel ammonium sulfate from Sigma and 0.01% H_2O_2) was performed. All sections were washed, mounted onto glass slides, air dried, dehydrated, and cover-slipped with Entellan® (Merck, Darmstadt, Germany).

BrdU/NeuN Double-Labeling

After washing in tris-buffered saline all sections were incubated in formamide (Sigma, Taufkirchen, Germany) for 2 hours at 65 °C. Then sections were incubated in 2 N HCl for 30 minutes at 37 °C and washed in 0.1 M borate buffer (pH 8.5) for 10 minutes. Blocking was performed as described above using donkey serum. Sections were then incubated in an antibody mixture containing rat anti-BrdU (AbD Serotec, Oxford, UK), 1:30, and anti-Neuronal nuclei (NeuN, Chemicon, Hofheim, Germany), 1:500, at 4 °C overnight. After washing the sections, carbocyanine 3-labeled donkey anti-rat antibodies (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA; 1:1000) and biotinylated donkey anti-mouse antibodies (DAKO, Hamburg, Germany; 1:500) were applied for 60 minutes. The sections were washed again and incubated in carbocyanine 2-labeled streptavidin (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA), 1:2000, for 60 minutes. Finally all sections were washed, mounted onto glass slides, air dried, dehydrated, and cover-slipped with Entellan® (Merck, Darmstadt, Germany).

Microglia immunostaining

For evaluation of microglia activation we analyzed the expression of CD11b using a rat affinity purified anti-mouse CD11b antibody (1:200; eBioscience, San Diego, USA). CD11b is an integrin αM which non-covalently associates with CD18 to form complement receptor-3 (MAC-1). Although it is constitutively expressed on microglia and monocytes, its up-regulation in the brain which occurs in response to insult is considered to be indicative of activation of microglia [27]. Briefly, sections were washed with tris-buffered saline containing 1% Triton (AppliChem, Darmstadt, Germany). Following blocking with bovine serum albumin (BSA), sections were incubated with primary antibody overnight at 4 °C. Sections were then rinsed and incubated for 60 minutes in biotinylated mouse anti-rat immunoglobulins (1:200; eBioscience, San Diego, USA). Endogenous peroxidase activity was blocked with a 1:500 mixture of H_2O_2 (30%) in methanol for 30 minutes. Sections were washed and incubated for 60 minutes in horseradish peroxidase-labeled streptavidin (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA), 1:3000. Finally, the diaminobenzidine reaction was performed as described above.

Immunohistological evaluation

The number of doublecortin-labeled cells in the dentate gyrus of the hippocampus was quantified by unbiased stereological analysis using the computer-assisted imaging system StereoInvestigator 6.0 (Microbrightfield Europe, Magdeburg, Germany). The hardware consists of a Leica DMLB microscope (Leica, Bensheim, Germany), a Plan-

Neofluar lens (Leica, Bensheim, Germany), a single chip charge coupled device (CCD) color camera (CX9000, Microbrightfield Europe, Magdeburg, Germany), and an AMD Athlon (tm) 64 Processor. An experimenter unaware of the treatment conditions traced the extent of the dentate hilus and of the hippocampal dentate gyrus and performed the counting of cells using the optical fractionator method. Within each contour a step grid was placed. Counting frames were automatically and randomly placed along the grid. The thickness of the counting frame was identical with the thickness of the section (minus guard zones from the top and bottom of the section). Only immunolabeled cells which appeared within the counting frame and came into focus were counted. There was no significant difference between ipsilateral and contralateral parts of the hippocampus based on the injection area and therefore an average number was taken for further analysis. Images were taken using an Olympus BH-2 microscope and Zeiss Axiocam MRc digital camera. For better comparison between groups, lower magnification images are shown.

Cell counts of fluorescent signals from BrdU-positive cells were performed at 400x magnification in an area encompassing the entire dentate granule cell layer (superior and inferior blades) and extending approximately two cell body widths deep into the hilus in five sections per animal as described recently [28]. The hilus was defined as the inner border of the granule cell layer and two straight lines connecting the tips of the granule cell layer and the proximal end of the CA3c region. Counting of BrdU/NeuN double-labeled cells was performed with the same criteria in five sections. Double-labeling was verified by careful analysis of the confocal z-series of multiple cells per animal. Confocal images were further improved for color correction by using Irfanview 4.28 (http://www.irfanview.com/).

In accordance with a previous study [29] CD11b-positive resting microglia were defined as cells with a relatively small cell body (<7.5 µm in diameter) and long processes. Activated microglia exhibited an increased staining intensity with a larger cell body and retracted processes. Using a combination of morphological criteria and a 7.5 µm diameter cut-off for the cell body, activated microglia were counted in the ipsilateral (injected) hemisphere (section about -1.82 from bregma) with 200x magnification.

Five animals, in which pathohistological and immunohistochemical analyses did not substantiate successful infection, were excluded from immunohistological analysis. Due to poor immunostaining of some brain sections we were not able to analyze data from all remaining animals. Therefore, BrdU-positive cells and

BrdU/NeuN double-labeled cells were analyzed in 7-12 animals per group. Doublecortin and CD11b stainings were analyzed in 6-10 animals per group.

Preparation of microglia and flow cytometry

Brain microglia were isolated 14 and 99 dpi (14 dpi: SJL/J non-infected n = 5, SJL/J infected n = 6, C57BL/6 non-infected n = 5, C57BL/6 infected n = 6; 99 dpi: SJL/J non-infected n = 4, SJL/J infected n = 6, C57BL/6 non-infected n = 6, C57BL/6 infected n = 6). The isolation of murine microglia cells was performed according to a procedure previously described for rat microglia (Sedgwick et al, 1991).

Briefly, mice were deeply anesthetized with chloral hydrate and transcardially perfused with 0.01 M phosphate buffered saline. The brains were removed and kept in Hanks' solution with 2% fetal calf serum. Two to three mouse brains had to be pooled to obtain sufficient material for subsequent flow cytometric analyses. After mincing the brain with a razor blade, the cells were separated with a glass potter (neoLab®, Heidelberg, Germany). Following two washing steps, the samples were filtered through a 60 µm cell strainer (Steriflip®, Millipore™, Billerica, USA). Cells were washed again and then subjected to an initial gradient (1.030 g/ml Percoll (GE healthcare, Uppsala, Sweden) and 5 ml of 1.124 g/ml Percoll). A second gradient consisted of four different densities: 2 ml of 1.124 g/ml Percoll and three dilutions of Percoll in Hanks' solution: 2 ml 1.088 g/ml, 2 ml 1.072 g/ml, and 2 ml 1.030 g/ml. Both gradients were centrifuged at 1.250 x g at 20 °C for 20 minutes. Following centrifugation cells were collected from the surface of the 1.072 and 1.088 g/ml-densities and washed in Hanks' solution. After re-suspending and diluting the cell suspension with Hanks' solution, the cells were used for ex vivo immunophenotypical and functional characterization.

The ability of murine microglia cells to phagocytose heat-killed and lyophilized fluorescein isothiocyanate (FITC) labeled *Staphylococcus aureus* (Bio Particles®, wood strain, without protein A, fluorescein conjugate, Molecular Probes Europe B.V., Leiden, The Netherlands) was measured using a flow cytometrical assay [30]. 100 µl of bacteria solution or phosphate buffered saline (PBS) (negative control) were gently mixed with 100 µl non-stimulated microglia cell suspension (mixture adjusted to a concentration of 10^7 bacteria/ml). The mixture was incubated one hour at 37 °C with gentle re-suspension after 30 minutes. Phagocytic activity was stopped and the adhesion of cells on the tube surface was minimized by cooling the tubes on ice for 15 minutes. The assay was performed in duplicates. The percentage of microglia cells with phagocytic activity and the phagocytosis intensity were measured immediately

by flow cytometry after adding FACSFlow™ (BD Biosciences, Heidelberg, Germany). Phagocytosis and further ex vivo microglia assays were measured using FACSCalibur™ (BD Biosciences, Heidelberg, Germany). Data were then analyzed using Cell-Quest™-Software provided by BD Biosciences, Heidelberg, Germany.

Generation of reactive oxygen species (ROS) was measured by flow cytometry as described [31] using the conversion of non-fluorescent rhodamine 123 (DHR 123, MoBiTec GmbH, Göttingen, Germany) into green-fluorescent rhodamine 123 by membrane-adapted myeloperoxidase. ROS generation was measured with and without triggering with phorbol-myristate-acetate (PMA, Sigma, Deisenhofen, Germany) diluted in dimethyl sulfoxide (DMSO) and phosphate buffered saline. Following pre-incubation at 37 °C and 5% CO_2 for 15 minutes, either 10 µL phosphate buffered saline (untriggered) or 10 µL phorbol-myristate-acetate (triggered) were added to 90 µL microglia cell suspensions (37 °C for 15 minutes). Subsequently 20 µL DHR 123 were added and the cells were incubated for 15 minutes at 37 °C. The samples were measured by flow cytometry. The percentage of ROS-generating microglia cells and the mean ROS generation intensity were determined comparing the negative control with non-stimulated and phorbol-myristate-acetate stimulated microglia. The assay was performed in duplicates.

The expression of marker proteins on the surface of murine microglia cells and pro-inflammatory cytokines was analyzed using monoclonal and polyclonal antibodies. Following a blocking procedure primary antibodies were added to the microglia cell suspensions for 20 minutes. Then microglia cell suspensions were washed twice with CellWASH™ (BD Biosciences, Heidelberg, Germany) before adding secondary antibodies for 30 minutes. The following antibodies were used: anti-CD18 (diluted 1:10 AbD Serotec, Eching, Germany), anti-CD21/35 (undiluted, eBioscience, Frankfurt, Germany) (both fluorescein isothiocyanate conjugated), anti-MHC I (undiluted, AbD Serotec, Eching, Germany), and anti-tumor necrosis factor-α (undiluted, BD Biosciences, Heidelberg, Germany)(conjugated with R-phycoerythrine). Anti-interleukin-1ß (diluted 1:10, antibodies-online GmbH, Aachen, Germany), a non-conjugated polyclonal antibody, was detected using a fluorescence-labeled secondary antibody (goat-anti-rabbit cyanine 3, diluted 1:100; Jackson ImmunoResearch Laboratories, Inc., PA, USA). Labeled cells were measured by flow cytometry using three different channels (FL1, FL2, and FL4). The percentage of positive microglia cells and the mean expression intensity were analyzed with the Cell-Quest™-Software.

Statistics

For non-parametric data (clinical scores) a Mann-Whitney U-test was used. Statistical differences in cell counts were analyzed by two-way analysis of variance followed by unpaired student's t-test. The Pearson correlation coefficient was used to test for a correlation between the number of activated microglia and the number of doublecortin-positive cells. Data are expressed as mean ± SEM (cell counts) or median, minimum, and maximum (clinical scores). A value of $p<0.05$ was considered significant. All tests were used two-sided.

2.4 Results

Clinical and histopathological findings

In accordance with previous studies [24, 32] clinical signs started to occur in TMEV infected SJL/J mice at 42 dpi (Fig. 1B). Infected SJL/J mice exhibited progressively increasing clinical scores that exceeded those from non-infected SJL/J mice and from infected C57BL/6 mice (Fig. 1B).

Clinical signs in SJL/J infected animals comprised ataxia, reduction of righting reflex, and spastic paresis of the hind limbs. In a subgroup of SJL/J infected animals activity and locomotion was reduced and the general appearance proved to be impaired. By the end of the experiment, clinical signs were detectable in all infected SJL/J mice (total clinical score at day 98: min = 1, median = 3, max = 8) while infected C57BL/6 mice and the non-infected groups did not show any clinical signs. As the data from the early phase demonstrate, BrdU administrations did not influence the state or the performance of the animals.

As the cerebra were used for the immunohistological investigation of neurogenesis and microglia activation, TMEV infection was verified using brainstem tissue. Pathohistological evaluation revealed lymphohistiocytic, perivascularly accentuated encephalitis in the brainstem in TMEV infected animals of both mouse strains at 14 dpi, which appeared to be more intense in SJL/J mice. At 99 dpi, no perivascular or parenchymal infiltration of mononuclear inflammatory cells was observed in the majority of C57BL/6 mice by light microscopy of hematoxylin and eosin stained sections (Fig. 1C). At the late time point SJL/J mice exhibited enhanced inflammatory changes (Fig. 1D-F), which were frequently accompanied by neuronal cell loss and gliosis (Fig1. D) as well as demyelination demonstrated by loss of Luxol fast blue staining. Neither inflammatory cell infiltrates nor demyelination was detected in non-infected mice of both strains. TMEV antigen was immunohistochemically detected as an intense dark brown signal in the cytoplasm of resident cells and macrophages in the brainstem of 8 out of 9 SJL/J mice and of 5 out of 9 C57BL/6 mice at 14 dpi. At the late time point similar immunoreactivity for TMEV antigen was detected in the brainstem of 8 out of 9 SJL/J mice and (as expected due to effective virus elimination) in none of the C57BL/6 mice (Fig. 1G-I). With the exception of infected C57BL/6 mice at 99 dpi, only animals showing histopathological changes characteristic for TMEV infection and/or exhibiting TMEV antigen expression were used for further analyses.

Impact of TMEV infection on hippocampal cell proliferation and neurogenesis

BrdU-labeled cells were detected in the subgranular proliferation zone and the granule cell layer in all groups (Fig. 2A-H). It is important to note that BrdU administration was performed in the early phase (day 7 to day 11 following infection) in both groups of mice (killed 14 or 99 dpi). Therefore, data from both groups give information about the cell proliferation rate in the acute phase of the infection and data from the late time point provides additional information about the fate and survival rate of cells that have been generated during the acute phase.

In response to virus infection the number of BrdU-labeled cells increased in the early phase of infection (Fig. 3A). The increase in cell proliferation proved to be more pronounced in infected SJL/J mice reaching a significant difference to mice without virus encephalitis (infected: 407.3 59.14, non-infected: 214.6 ± 19.90; p=0.01) . In contrast no significant impact of infection was observed in C57BL/6 mice (infected: 500.0 ± 59.25, non-infected: 384.3 ± 47.11; p=0.15).

Considering data from the late phase, infected mice of the two strains exhibited a contrarious development in the number of BrdU-labeled cells (Fig. 3B). In SJL/J mice the number of BrdU-labeled cells was lowered by 36% as compared to the respective control group (infected: 95.29 ± 12.91, non-infected: 148.6 ± 16.60; p=0.03). In contrast, the number of BrdU-labeled cells in infected C57BL/6 exceeded that in respective control mice by 49% (infected: 114.4 ± 12.07, non-infected: 76.57 ± 6.68; p=0.02).

Pronounced strain differences were also observed in non-infected C57BL/6 mice reaching higher levels of BrdU-labeling than those in non-infected SJL/J mice (p=0.01) (Fig. 3A). Interestingly, the number of BrdU-labeled cells detectable at a late time point reached higher levels in SJL/J as compared to C57BL/6 mice (Fig. 3B). This might in part be due to differences in the survival rate of newborn cells with numbers in SJL/J mice exceeding those in C57BL/6 mice.

The ratio between BrdU-labeled cells detected at an early time point and those still detectable at a late time point further illustrates the differences (Fig. 3C). The ratio of BrdU-labeled cells 99 dpi: 14 dpi amounted to 0.69 in non-infected SJL/J mice and 0.20 in non-infected C57BL/6 mice. The infection decreased the ratio in SJL/J mice to 0.23, whereas the ratio was slightly increased in C57BL/6 mice to 0.23 as compared to controls.

As expected the number of BrdU/NeuN double-labeled newborn neurons was very low when brains were sampled at day 14, i.e. three days following the last BrdU

injection. Therefore, we did not quantitatively compare respective cell counts for the early time point. An analysis of the number of cells that were labeled with BrdU during the 2nd week following infection and that were NeuN-positive (Fig. 3I) when brains were sampled 99 dpi did not reveal significant differences between infected and non-infected mice. However, the comparison of BrdU/NeuN cell counts in non-infected mice indicated strain differences with a higher number reached in non-infected SJL/J mice (p=0.03). (Fig. 3D)

Impact of the TMEV infection on hippocampal neuronal progenitor cells

In the adult rodent brain doublecortin expression levels reflect the rate of neurogenesis in a specific manner [33]. Using an immunohistological approach Rao and Shetty [34] demonstrated the efficacy of doublecortin as a marker for unique labeling of neuronal precursors and of newly generated granule cells during their early phase of synaptic integration.

Analysis of doublecortin immunohistochemistry 14 as well as 99 dpi or vehicle injection rendered information about the long-term development of ongoing neurogenesis based on identification of neuronal progenitor cells and early postmitotic neurons. In all groups numerous cells were labeled for doublecortin in the subgranular zone and the granule cell layer with dendrites extending into the molecular cell layer (Fig. 4A-I). In the acute phase, opposite tendencies for a decrease of neuronal progenitor cells in SJL/J (non-infected: 23086 ± 1986, infected: 19219 ± 1934; p=0.19) vs. an increase in C57BL/6 (non-infected: 26151 ± 2618, infected: 30240 ± 2347; p=0.28) were observed. However, virus encephalitis did not significantly affect the number of doublecortin-labeled cells in both mouse strains (Fig. 4J). In the chronic phase, infected SJL/J mice exhibited a 37% reduction in the number of neuronal progenitor cells as compared to non-infected control animals (infected: 6926 ± 876, non-infected: 10923 ± 580; p=0.001) (Fig. 4K). Respective analyses in C57BL/6 mice did not reveal any significant difference to non-infected control mice (infected: 14649 ± 594, non-infected: 12711 ± 1249; p=0.15) (Fig. 4K).

Strain differences were evident in both non-infected and infected animals. As already mentioned, the number of doublecortin-labeled cells tended to decline in SJL/J mice and to increase in C57BL/6 mice as a consequence of acute infection. Thus, the number of doublecortin-labeled cells in infected C57BL/6 mice significantly exceeded that in infected SJL/J mice (p=0.003) (Fig. 4J). In the chronic phase a comparable difference was observed with more neuronal progenitor cells detected in the granule cell layer of C57BL/6 mice as compared to SJL/J mice (p=0.003) (Fig. 4K). In non-

infected mice aged 134 days (99 dpi) C57/BL6 mice exhibited a higher number of neuronal progenitor cells (p=0.003) (Fig. 4K). Visual inspection of representative sections from all groups did not reveal any differences regarding the localization and morphology of doublecortin-labeled cells.

Impact of the TMEV infection on microglial cells

In non-infected mice of both strains CD11b-labeled cells were detected throughout the hippocampus. In these groups the vast majority of CD11b-labeled cells exhibited a morphology characteristic for resting microglia with small cell bodies, long processes and relatively faint immunoreactivity (Fig. 5A). Fourteen days following infection, most CD11b-positive cells displayed clustering of early activated microglial cells with partial retraction and hypertrophy of processes and rounded cell bodies in SJL/J mice (Fig. 5B). The cells exhibited a higher staining intensity as compared to resting microglia. Cells with a very strong immunoreactivity were evident in CA1 and CA2 regions as well as the molecular layer adjacent to these pyramidal cell layers. Quantitative analysis of activated microglia demonstrated that the number in infected SJL/J mice significantly exceeds that in non-infected controls (infected: 43.71 ± 12.53, non-infected: 123.1 ± 25.51; p=0.02).

In infected C57BL/6 mice we also detected CD11b-labeled cells in the hippocampus, which exhibited a morphology characteristic for activated microglia. However, the difference to non-infected controls did not reach significance (p=0.07) (Fig. 5C).

In the chronic phase, a significantly increased number of activated microglia cells was evident in infected C57BL/6 mice (53.00 ± 4.74) as compared to non-infected C57BL/6 (25.40 ± 6.07; p=0.01) as well as infected SJL/J mice (33.00 6.17; p=0.03) (Fig. 5D).

Several studies have suggested a link between microglia activation and disease-associated effects on the neuronal progenitor cell population. However, the number of activated microglia cells did not correlate with the number of doublecortin-labeled progenitor cells in the TMEV model (r = -0.0055 in SJL/J mice and r = -0.0716 in C57BL/6 mice at early time point; r = -0.2294 in SJL/J and r = 0.1818 in C57BL/6 mice at late time point).

Additional information about the functional state of microglial cells was obtained based on flow cytometric analyses. The percentage of CD18-positive cells indicated a high purity of the cell preparation ranging from 81-99% (Tab. 1). Note that a contamination of the microglia cell population with blood-derived cells was prevented

by a perfusion of the brain immediately following death [35]. Other cell types were excluded by density gradient centrifugation. A low percentage of cells expressing the lymphocyte marker CD21/35 indicated a limited contamination with B cells (mean 5.5%).

Three additional antibodies were used to characterize microglia cells (data shown in Table 1) (Fig. 5E). At the late time point infected mice of both strains exhibited a higher expression intensity of MHC I as compared to non-infected controls as well as infected mice in the acute phase. This up-regulation of the surface marker MHC I is indicative for an activated state of microglial cells. The highest expression intensity of MHC I was evident in infected C57BL/6 mice 99 dpi (Tab. 1). The expression of interleukin-1ß showed high variance in the samples of infected mice.

The mean expression of tumor necrosis factor (TNF) was reduced in infected mice of both strains at both time points as compared to respective non-infected control animals.

Phagocytic activity was demonstrated in microglia of all mice. The percentage of microglia cells exhibiting phagocytic activity following exposure to opsonized bacteria ranged from 57-78%. The mean phagocytic activity in infected mice reached higher levels at the late time point in comparison to non-infected controls (Tab. 1).

High levels of ROS generation were observed with the phosphate buffered saline testing condition with maximum levels observed in infected SJL/J mice 99 dpi (Tab. 1). These data indicate that enhanced oxidative stress correlates with the chronic progressive disease course in SJL/J mice. No remarkable further stimulation of ROS generation could be observed in response to phorbol-myristate-acetate (data not shown).

Regarding all data from the early time point it needs to be considered that an impact of the intracerebral injection of vehicle might have affected the data.

2.5 Discussion

It is well accepted that brain inflammation can affect adult hippocampal neurogenesis in a complex manner [13]. However, so far respective data on the impact of CNS virus infection are limited [20-22]. In the present study, we addressed the question whether BeAn TMEV infection affects hippocampal cell proliferation and neurogenesis in SJL/J and C57BL/6 mice and whether respective effects depend on the disease course.

In line with our hypothesis, investigations in the TMEV mouse model revealed that the acute and long-term impact of a CNS virus infection on fate of hippocampal cell proliferation and on the neuronal progenitor population differs in mouse strains with a contrasting course of the infection. In susceptible SJL/J mice infection with the BeAn strain of TMEV induces a biphasic disease with an early acute polioencephalitis and a late chronic demyelinating leukoencephalomyelitis with virus persistence [3, 24, 36-37]. In contrast, related to differences in the immune response TMEV-infected C57BL/6 mice develop an early acute disease but efficaciously clear the virus within up to three weeks [3, 36-37]. Clinical data, pathohistological data and detection of TMEV antigen confirmed that both mouse strains exhibited respective differences in the present study.

Regarding an influence of acute TMEV-induced polioencephalitis on the hippocampus it has already been described that viral antigen positive cells can be detected in the hippocampus associated with hippocampal neuropathology. In SJL/J mice a thorough analysis of the brain pathology revealed neuropathology in the hippocampus following infection with the low neurovirulent TMEV strains BeAn and DA [5]. In another study the highly neurovirulent GDVII strain of TMEV caused pronounced neuronal cell damage and loss in SJL/J mice, which proved to exceed that observed with the low virulent DA strain resulting in smaller clusters of apoptotic neurons [38-39]. C57BL/6 mice exhibited apparent neurodegeneration in CA1, CA2 and CA3 following infection with the DA strain with pronounced interindividual differences [40-42]. In C57BL/6 mice with infection-associated seizures hippocampal neurodegeneration proved to be more pronounced as compared to mice which did not develop seizures [40]. In addition to neuronal cell loss, perivascular cuffing with infiltrating macrophages, T cell infiltration, microglia activation and astrogliosis characterized the hippocampal pathology described in C57BL/6 mice with DA virus infection [39, 41, 43].

Our study revealed that depending on the disease course the infection can exert significant effects on hippocampal cell proliferation and generation of neuronal progenitor cells. During the early phase the infection was associated with a significant increase in hippocampal cell proliferation in SJL/J mice, whereas the difference to non-infected animals failed to reach significance in C57BL/6 mice. These data demonstrate that TMEV infection exhibits more pronounced effects on hippocampal cell proliferation in animals which are more susceptible to develop a chronic disease course. Analysis of newborn cells that were generated in the 2nd week following infection and that expressed the neuronal marker NeuN 99 dpi, did not confirm any significant impact of acute infection on hippocampal neurogenesis. Thus, the alterations in BrdU positive cells during the acute phase of the infection might be dominated by changes in the generation of new glia cells. In addition, it needs to be considered that macrophages might penetrate into the brain during the acute infection and might contribute to an increased number of BrdU-labeled cells in this early phase.

Analyses of doublecortin-positive cells reflecting the number of neuronal progenitor cells and early postmitotic neurons [33] revealed a contrarious impact of infection in both strains resulting in lower numbers of doublecortin-positive cells in infected SJL/J mice as compared to C57BL/6 mice. It is notable that these effects were observed as a consequence of an infection with the BeAn strain which is known to induce a rather attenuated grey matter disease as compared to infections with the DA strain of TMEV [3]. Moreover, it needs to be emphasized that the acute phase of TMEV infection is not characterized by any clinical symptoms [3]. Thus, our data demonstrate that even subclinical polioencephalitis can be associated with relevant effects on hippocampal cell proliferation and the fate of newborn cells. The data from SJL/J mice are in line with recent data describing a reduction in doublecortin-positive cells in the late phase of infection with the BeAn TMEV strain [44].

It has been described that the polioencephalitis is limited to the first weeks following TMEV infection regardless of the mouse strain [3]. Thus, it is of particular interest, that despite the limitation of the encephalitis to a short acute phase, long-term consequences on both the long-term survival of newborn cells as well as the neuronal progenitor cell population were evident more than three months following infection with a significantly reduced number of neuronal progenitor cells in susceptible SJL/J mice at this time point. The long-term survival of proliferating cells labeled by BrdU incorporation during the acute phase of the infection proved to be compromised in SJL/J mice, whereas it was rather promoted in C57BL/6 mice. These data raise the

question which cellular and molecular disease-associated alterations contributed to these effects. Brain inflammation is a likely candidate as it is known to influence cell proliferation in the subgranular zone as well as neuronal differentiation and survival of new cells in a complex manner [13]. In particular, microglia activation as an indicator of inflammation can be pro- or antineurogenic depending on the balance between secreted molecules with pro- and anti-inflammatory action [13]. Earlier studies have already described evidence for microglia activation in response to TMEV infection with the DA strain in C57BL/6 and SJL/J mice [38, 41]. In the present study, the immunohistochemical analyses of microglial cells in the acute phase of TMEV infection with the BeAn strain indicated a pronounced activation of microglial cells in the hippocampus of SJL/J mice, whereas activation of microglia in C57BL/6 mice did not reach comparable levels. In contrast, the number of activated microglia in the hippocampus of C57BL/6 mice exceeded that in SJL/J mice in the chronic phase. Alterations in the functional state of microglial cells might have contributed to the effects of TMEV infection on neuronal progenitor cells. A respective impact of microglial activation in viral infections would be in accordance with the fact that activation of microglial cells can affect survival of neural progenitor cells and can prevent neuronal differentiation [45-47]. However, the failure to detect a correlation between the number of activated microglia and the number of doublecortin-labeled cells argues against a critical role of microglia for the impact of TMEV infection on neuronal progenitor cells.

In this context it needs to be considered that the functional state of microglial cells including the generation of cytokine products might be critical in determining the effects on neuronal progenitor cells. Flow cytometric analyses of the functional state of microglia cells in a separate set of animals demonstrated complex alterations with activation in response to infection. Inflammation-induced cytokine products including TNF and interleukin-1 have been reported to play a pivotal role in suppressing neurogenesis [46-49]. An impact of Theiler's virus infection on brain TNF has been described in earlier studies [3, 36, 41, 50]. Chang and colleagues [36] reported a biphasic course of TNF in susceptible SJL/J mice with peaks at day 8 and day 28 and a monophasic course in C57BL/6 mice with a peak at day 21. At the time points investigated in the present study, we observed a down-regulation of TNF expression in the acute and chronic phase in both mouse strains. In comparison with previous studies it needs to be considered that different cells can contribute to cytokine production and that we focused on the activity status of isolated microglia cells.

Considering that Iosif et al [48] described that mice lacking the TNF receptor 1 exhibit an increased proliferation in the subgranular zone, the reduction in TNF levels might have contributed to respective alterations in response to TMEV infection.

Analysis of brain interleukin levels in earlier studies revealed a complex course with fluctuating levels in SJL/J mice whereas a rather constant decline was described in C57BL/6 mice [36]. In our study interleukin-1 production by isolated microglia cells showed a high variance in samples from infected animals. According to the literature interleukin-1 seems to play a dichotomous role in regulating neurogenesis [49, 51]. Thus, considering the up-to-date knowledge on the interaction between microglial-derived mediators and the different steps in hippocampal neurogenesis [13], it is likely that the complex alterations in the functional state of microglial cells, which were determined in the different phases of TMEV infection, critically contributed to the cellular alterations in the hippocampus of infected mice. In this context reactive oxygen species need to be considered as another modulator of hippocampal neurogenesis [52].

Differences in the extent of alterations in the microglial cell functional state between mouse strains, which were evident from the immunhistological findings, were indicated by flow cytometric data and have been described in earlier studies [3], might also have contributed to the varying impact of TMEV infection on hippocampal cell proliferation and neuronal progenitor cells in SJL/J and C57BL/6 mice. It has been proposed that T-cells by interacting with resident microglia can promote progenitor proliferation and possibly also neuronal survival and differentiation [13, 53-54]. Thus, varying T-cell responses in SJL/J and C57BL/6 mice [3] might be another factor accounting for the differences observed between strains in the present study.

The fact that an acute polioencephalitis can cause chronic changes in the population of neuronal progenitor cells suggests that respective alterations might contribute to detrimental long-term consequences of virus encephalitis. In particular, based on the functional link between disease-associated modulation of neurogenesis and learning and memory [19], the impact of virus infection on hippocampal neurogenesis might contribute to cognitive impairment. Moreover, alterations in neurogenesis have been suggested as a contributing factor to epileptogenesis [15-16]. Symptomatic epilepsy can be a consequence of various virus infections [55]. With regard to TMEV infection, the development of acute and chronic seizures has so far only been characterized following infection with the DA strain of TMEV [42-43]. During the intense handling procedures no motor seizure activity was observed in the

present study and a series of recent studies with the same experimental conditions [24, 32, 37]. Moreover, continuous video/EEG recordings in a parallel study with BeAN infected SJL/J and C57BL/6 mice did not indicate any motor or electrographic seizure activity [unpublished data; manuscript in preparation].

Strain differences were not only evident following TMEV infection, but also in the non-infected condition. These data are in accordance with previous reports describing pronounced differences in Ki67 and doublecortin expression between mouse strains [56-57]. Our data additionally indicate that the age-dependent decline described for hippocampal cell proliferation as well as neurogenesis [58-59] reaches different levels in both mouse strains.

In conclusion, TMEV infection differentially affects the neuronal progenitor cell population depending on disease susceptibility of the mouse strain. The cellular alterations are paralleled by complex changes in the functional state of microglial cells, which might affect cell proliferation, neuronal differentiation and survival in the granule cell layer. It is of particular interest, that a sub-clinical polioencephalitis can cause long-term effects in the neuronal progenitor cell population. Future studies are necessary to further investigate whether respective alterations affect the long-term balance of hippocampal neurogenesis and contribute to chronic functional consequences of virus encephalitis. Respective studies will have to include protocols with different time schemes for BrdU administration.

2.6 Acknowledgments

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2.7 Table

Table 1: Impact of TMEV infection on the functional state of microglial cells

	Fluorescence intensity (mean ± SEM); early time point			
Surface antigen or cytokine	**SJL/J non-infected**	**SJL/J infected**	**C57BL/6 non-infected**	**C57BL/6 Infected**
CD18	62.08 ± 14.23	170.92 ± 18.15	93.72 ± 37.56	188.18 ± 39.81
MHC I	659.99 ± 303.91	217.73 ± 53.77	382.33 ± 29.22	287.60 ± 151.84
IL-1ß	44.11*	292.88 ± 230.43	51.73*	415.32 ± 372.89
TNF-α	509.82*	219.97 ± 59.22	432.30*	184.94 ± 37.66
phagocytosis	53.94 ± 7.06	64.30 ± 3.67	56.62 ± 7.79	66.56 ± 8.58
ROS generation	492.70 ± 42.11	259.81 ± 124.80	404.91 ± 8.51	270.74 ± 150.31
	Fluorescence intensity (mean ± SEM); late time point			
Surface antigen or cytokine	**SJL/J non-infected**	**SJL/J infected**	**C57BL/6 non-infected**	**C57BL/6 Infected**
CD18	150.46*	141.73*	96.00*	181.93*
MHC I	146.84 ± 26.18	868.01 ± 493.45	66.75 ± 48.16	2120.71 ± 657.75
IL-1ß	165.99 ± 62.25	63.33 ± 27.50	57.15 ± 44.96	44.01 ± 6.15
TNF-α	219.49*	80.37 ± 12.57	354.06*	95.54*
phagocytosis	22.84 ± 1.61	51.12 ± 20.57	20.56 ± 13.11	95.75 ± 32.49
ROS generation	532.60 ± 175	847.78 ± 158.74	351.70 ± 127.86	489.22 ± 61.69

Fluorescence intensity at early time point (14 dpi) SJL/J non-infected n = 5, SJL/J infected n = 6, C57BL/6 non-infected n = 5, C57BL/6 infected n = 6
Fluorescence intensity at late time point (99 dpi) SJL/J non-infected n = 4, SJL/J infected n = 6, C57BL/6 non-infected n = 6, C57BL/6 infected n = 6
Please consider that samples had to be pooled from 2-3 animals. Data are mean ± SEM.
*= data from one pooled sample only for technical reasons

2.8 Figures

Figure 1: Clinical and histopathological findings

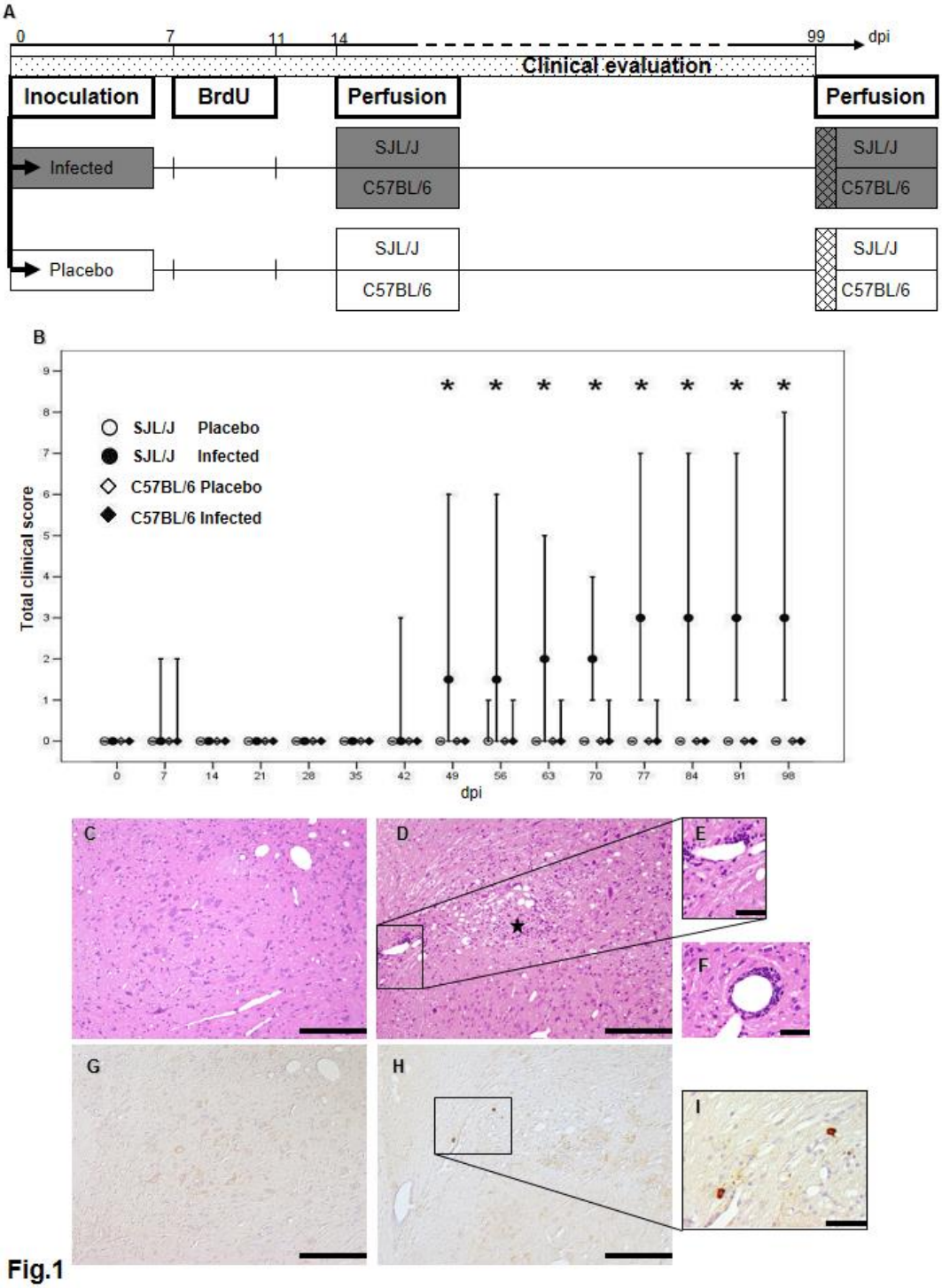

Figure 1: Clinical and histopathological findings

A: Schematic drawing of the experimental design. Each group consisted of 10-14 animals (8 groups). **B:** Clinical course of SJL/J and C57BL/6 mice following TMEV infection in comparison to non-infected control animals. Data are given as median, minimum and maximum of the total clinical score per group and time point. Significantly higher clinical scores of infected SJL/J mice compared to infected C57BL/6 and non-infected SJL/J mice, are indicated by asterisks (Mann-Whitney U-test, $p<0.05$). **C:** Infected C57BL/6 mice do not show obvious inflammatory or degenerative changes in the brainstem 99 dpi. **D:** Brainstems of infected SJL/J mice show multifocal perivascular and parenchymal lympho-histocytic inflammation and gliosis 99 dpi (hematoxylin and eosin staining). An area with neuronal loss and gliosis is presented by an asterisk. **E-F:** Perivascular lympho-

histiocytic inflammation in the brainstem of an infected SJL/J mouse. Infiltrates of lymphocytes and macrophages around empty blood vessels (due to perfusion) are evident in two representative areas from the same animal. **G:** Infected C57BL/6 mice show lack of TMEV immunostaining at the late time point. **H:** Positive immunostaining for TMEV antigen within the lesion of an infected SJL/J mouse. **I:** TMEV-immunopositive cells are shown with higher magnification.
Scale bar: 200 µm in C, D, G, H and 50 µm in E, F, I.

Figure 2: BrdU-labeled cells in the subgranular proliferation zone and the granule cell layer

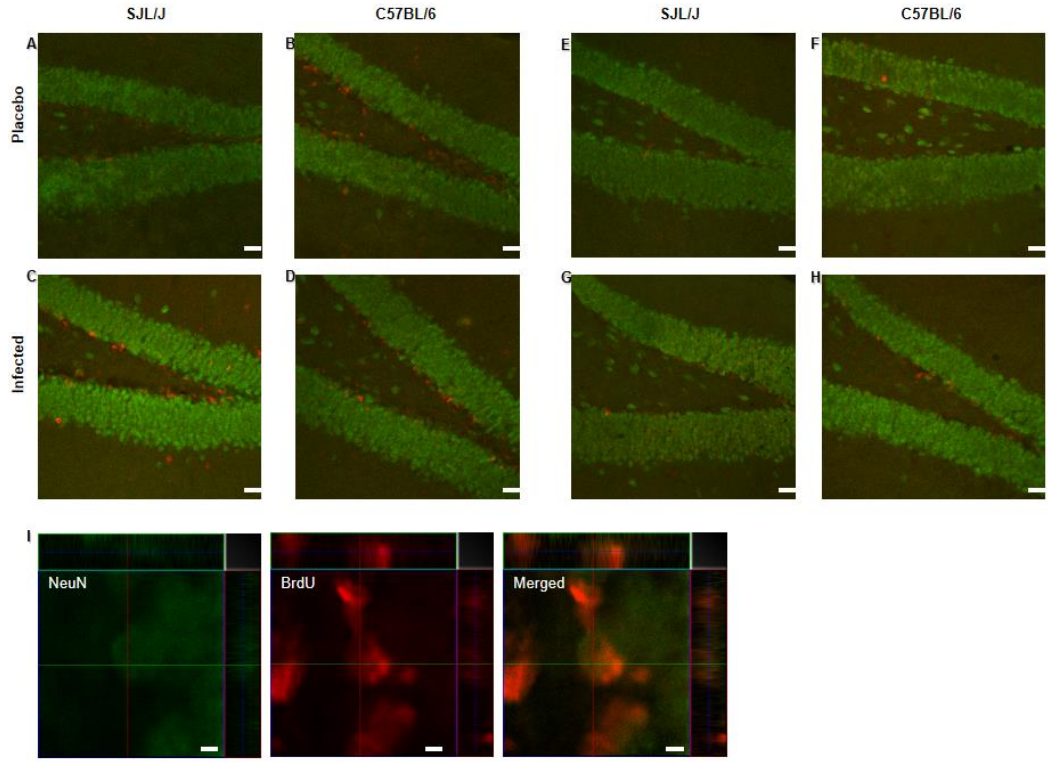

Fig.2

Figure 2: BrdU-labeled cells in the subgranular proliferation zone and the granule cell layer

A-D: BrdU (red) and NeuN (green) immunostaining of proliferating cells in the dentate gyrus shown for representative animals from the different groups (early time point; SJL/J placebo, C57BL/6 placebo, SJL/J infected, C57BL/6 infected). **E-H:** BrdU (red) and NeuN (green) immunostaining in the dentate gyrus shown for representative animals from the different groups (late time point; SJL/J placebo, C57BL/6 placebo, SJL/J infected, C57BL/6 infected). **I:** A BrdU/NeuN double-labeled neuron in the SGZ is visualized by using confocal microscopy (red BrdU, green NeuN).

Scale bar: 20 µm in A-H and 2 µm in I.

Figure 3: Impact of TMEV infection on hippocampal cell proliferation and neurogenesis

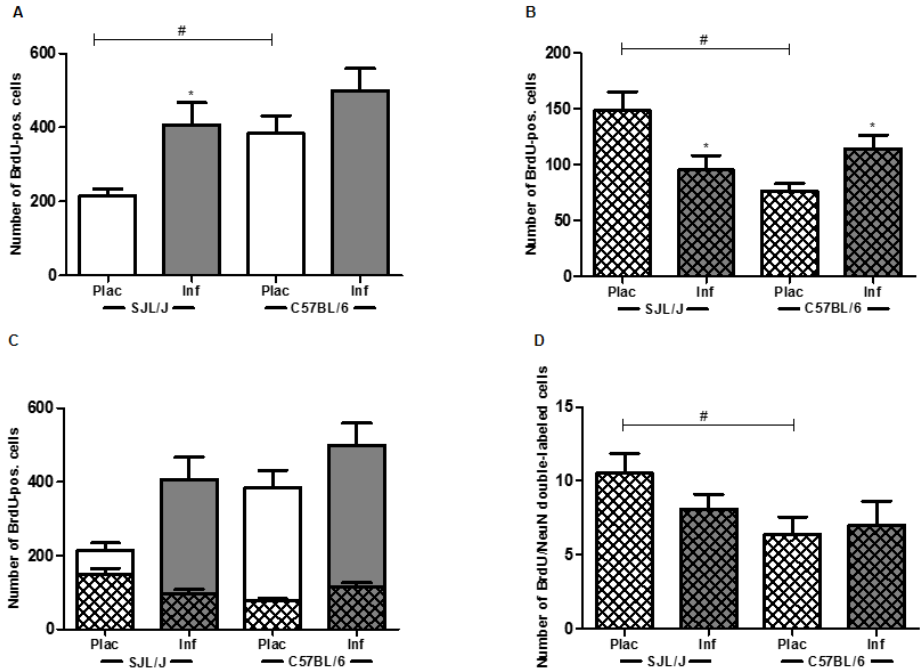

Fig.3

Figure 3: Impact of TMEV infection on hippocampal cell proliferation and neurogenesis

A: Analysis of BrdU-positive cells in the dentate gyrus 14 dpi. **B:** Analysis of BrdU-positive cells in the dentate gyrus 99 dpi. **C:** BrdU-positive cells in the dentate gyrus. The results of cell counts from both time points (14 dpi and 99 dpi) are illustrated allowing a direct comparison. The same column format has been used to indicate the different time points as in Fig 3A and B; i.e. hatched for data from 99 dpi. **D:** Analysis of BrdU/NeuN double-labled cells in the dentate gyrus 99 dpi.

Data are given as mean ± SEM. Significant differences are indicated by asterisk (infected (Inf) vs. placebo (Plac) within one strain) and hash symbol (between strains). (student's t-test, $p<0.05$).

Figure 4: Impact of TMEV infection on hippocampal neuronal progenitor cells

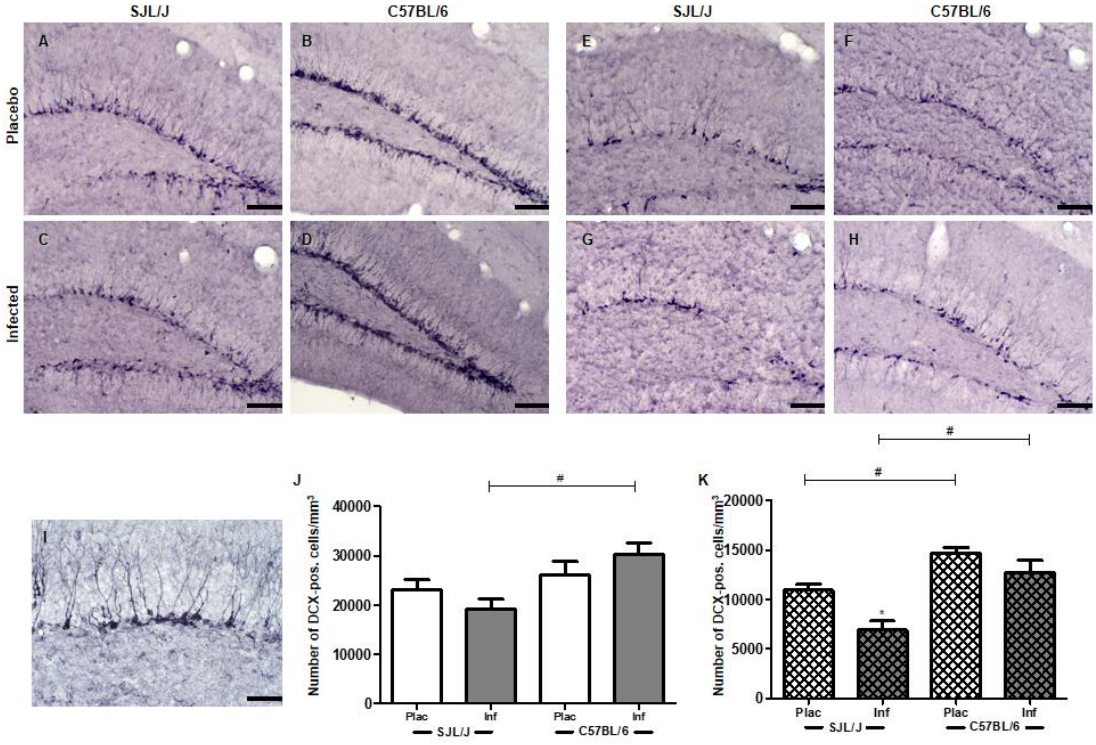

Fig.4

Figure 4: 4 Impact of TMEV infection on hippocampal neuronal progenitor cells

A-D: Doublecortin (DCX) immunostaining of neuronal progenitor cells in the dentate gyrus shown for representative animals from the different groups (early time point; SJL/J placebo, C57BL/6 placebo, SJL/J infected, C57BL/6 infected). **E-H:** Doublecortin immunostaining of neuronal progenitor cells in the dentate gyrus shown for representative animals from the different groups (late time point; SJL/J placebo, C57BL/6 placebo, SJL/J infected, C57BL/6 infected). **I:** Representive doublecortin-labeled neuronal progenitor cells in an infected C57BL/6 mouse. **J:** Analysis of doublecortin-positive cells in the dentate gyrus 14 dpi. **K:** Analysis of doublecortin-positive cells in the dentate gyrus 99 dpi.

Data are given as mean ± SEM. Significant differences are indicated by asterisk (infected (Inf) vs. placebo (Plac) within one strain) and hash symbol (between strains). (student's t-test, $p<0.05$). Scale bar: 20 µm in A-H and 10 µm in I.

Figure 5: Impact of TMEV infection on microglial cells

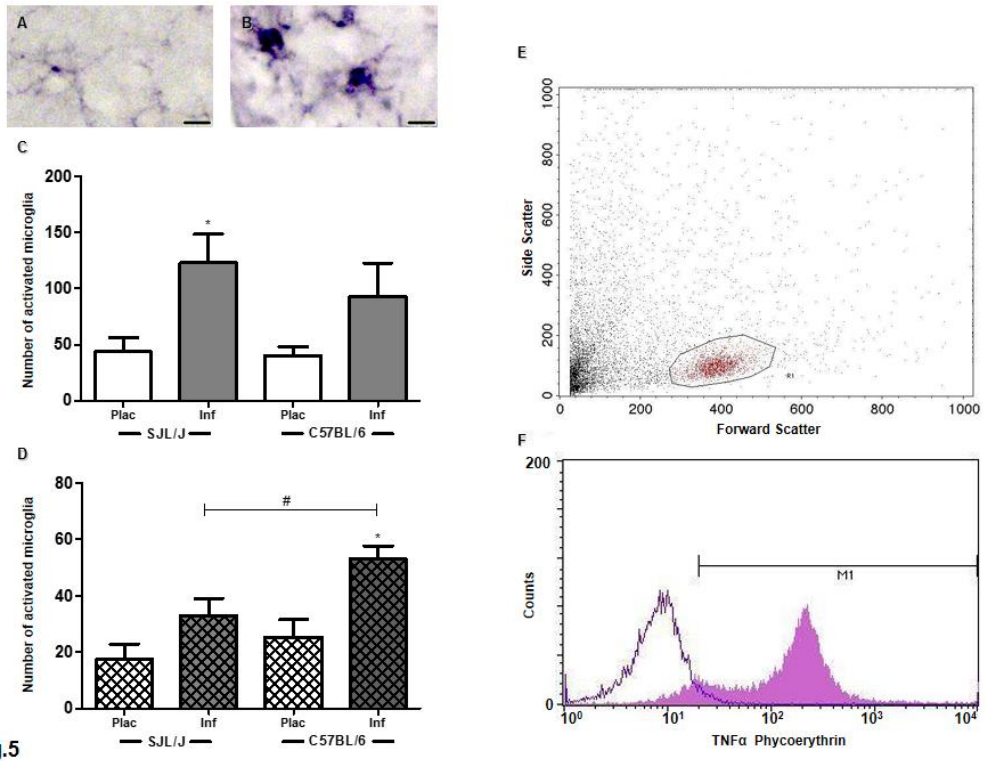

Figure 5: Impact of TMEV infection on microglial cells

A: CD11b immunostained resting microglial cell. Note the small cell body, long processes and relatively faint immunoreactivity. **B:** CD11b immunostained activated microglia cell. Note the relatively large cell body, short retracted processes and intense immunoreactivity. **C:** Analysis of activated microglia cells in the ipsilateral hippocampus of area -1.82 from bregma 14 dpi. **D:** Analysis of activated microglia cells in the ipsilateral hippocampus of area -1.82 from bregma 99 dpi. **E:** TNF expression in microglia cells isolated from infected SJL/J and C57BL/6 mice 14 dpi. The positively stained microglia are displayed under the M1 line with the results for SJL/J depicted as grey area and the results for C57BL/6 as black area. The isotype controls for both strains are shown as grey (SJL/J) and black (C57BL/6) lines, respectively. (x-axis: labeling; y-axis: cell counts).

Significant differences are indicated by asterisk (infected (Inf) vs. placebo (Plac) within one strain) and hash symbol (between strains) (student's t-test, $p<0.05$). Scale bar: 5 µm.

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CHAPTER 3: “Long-term genetic fate mapping of adult generated neurons in a mouse temporal lobe epilepsy model”

3.1 Abstract

In the epileptic brain, seizures can increase hippocampal neurogenesis, while opposingly seizure- associated brain pathology has been shown to detrimentally affect neurogenesis. The long-term impact of recurrent seizures on the number of new neurons as well as their relative contribution to the granule cell layer remains an open question. Therefore we analyzed neuron addition based on genetic fate mapping in a chronic model of epilepsy comparing non-kindled animals and kindled animals having had at least one generalized seizure with and without further seizures. The number of all new granule cells added to the dentate gyrus following the onset of kindling was significantly increased (7.0-8.9fold) in kindled groups. The hyperexcitable kindled state and a prior seizure history proved to be sufficient to cause a pronounced long-term net effect on neuron addition. An ongoing continuous occurrence of seizures did not further increase the number of new granule cells in the long-term. In contrast, a correlation was found between the cumulative duration of seizures and neuron addition following a kindled state.

In addition, the overall number of seizures influenced the relative portion of new cells among all granule cells. Non-kindled animals showed 1.6% of new granule cells among all granular cells by the end of the experiment. This portion reached 5.7% in the animals which experienced either 10 or 22 seizures. A percentage of 8.4% new cells was determined in the group receiving 46 seizures which is a significant increase in comparison to the control group.

In conclusion, permanent genetic fate mapping analysis demonstrated that recurrent seizures result in a lasting change in the makeup of the granule cell layer with alterations in the relative contribution of newborn neurons to the granule cell network. Interestingly, the formation of a hyperexcitable kindled network even without recent seizure activity can result in pronounced long-term alterations in the absolute number of new granule cells. However, seizure density also seems to play a critical role with more frequent seizures resulting in increased fractions of new neurons.

Jafari, M., Soerensen, J., Bogdanović, R. M., Dimou, L., Götz, M., & Potschka, H. (2012). Long-term genetic fate mapping of adult generated neurons in a mouse

temporal lobe epilepsy model. *Neurobiology of disease*, *48*(3), 454–63. doi:10.1016/j.nbd.2012.06.014

The author of this doctoral thesis contributed to this paper by designing and performing the animals experiment, cellular and statistical analysis, and writing the major parts of the manuscript.

3.2 Introduction

Seizures can increase neurogenesis in the subgranular zone of the dentate gyrus, one of the major neurogenic zones of the adult brain (Danzer, 2011; Kokaia, 2011; Parent and Murphy, 2008; Scharfman and McCloskey, 2009). Studies in chronic rodent models of epilepsy including the kindling model and post-status epilepticus models repeatedly demonstrate a considerable expansion of newborn granule cells when seizure activity is induced in naïve animals (Parent et al., 1998; Parent et al., 1999; Scott et al., 1998). In post-status epilepticus models, increased hippocampal neurogenesis characterizes the latent phase; however, in several studies with chemically-induced status epilepticus animals exhibited a reduced neurogenesis in the chronic phase with spontaneous recurrent seizures (Hattiangady et al., 2004; Hattiangady and Shetty, 2010). Evidence exist that hippocampal pathology including cell loss and gliosis can affect the neurogenic niche resulting in decreased survival or neuronal differentiation of progenitor cells (Hattiangady and Shetty, 2010). On the other hand, Cha and colleagues (2004) reported that spontaneous recurrent seizures in the chronic phase can further enhance dentate gyrus neurogenesis.

Respective findings raise the question to what extent the generation of a hyperexcitable network and subsequent recurrent chronic seizures affect the long-term overall balance of hippocampal neurogenesis. Previous studies have been based on administration of DNA base analogues or infection with retroviral vectors (Parent et al., 2002; Taupin, 2007a; Taupin, 2007b). Conclusions from these investigations are limited by the fact that only a small cohort of adult-generated cells is labeled. Moreover, BrdU labeling gives information about only a limited time window. It is well known that the indirect extrapolation of respective data can lead to diverse predictions of overall neuron addition in the dentate gyrus (Imayoshi et al., 2008; Lagace et al., 2007; Ninkovic et al., 2007). Recently, permanent genetic fate mapping was established as a new strategy to monitor adult-generated neurons over long time frames (Mori et al., 2006; Ninkovic et al., 2007). Using this approach, distinct modes of neuron addition have been revealed for the major neurogenic zones of the adult brain (Ninkovic et al., 2007). In the dentate gyrus, adult neurogenesis contributed to only a minor fraction of the entire neuronal network in the granular cell layer.

Considering that in the epileptic brain seizures can increase neurogenesis on one hand and seizure-associated brain pathology can detrimentally affect neurogenesis on the other, we set out to analyze the long-term net impact in a chronic epilepsy model based on permanent genetic fate mapping. We have chosen the kindling model for the

experiments for several reasons. In the kindling model, repetition of electrical stimuli induces seizures with a progressive increase in severity and duration (Loscher and Brandt, 2010). Once an animal exhibits generalized seizures in response to electrical stimulation, the heightened response is permanent, reflecting the generation of a hyperexcitable network in these kindled animals (McIntyre et al., 2002). To our great advantage the frequency of recurrent seizures can be controlled, so that we were able to compare the impact of different seizure history and seizure frequency on the long-term outcome of neurogenesis. In particular, we analyzed neuron addition in the dentate gyrus of kindled animals with and without further recurrent seizures in comparison with non-kindled, electrode- implanted control animals. Moreover, we compared the impact of a different seizure frequency in the chronic phase. The results are of particular interest in view of the ongoing debate about a functional impact of alterations in hippocampal neurogenesis on ictogenesis and epileptogenesis as well as psychiatric and cognitive co-morbidities (Danzer, 2011; Hattiangady and Shetty, 2008; Jessberger et al., 2007; Kokaia, 2011; Pekcec et al., 2008).

3.3 Material and methods

Animals

GLAST::CreERT2 mice (Mori et al., 2006) were crossed to CAG-CAT-eGFP mice (Kawamoto et al., 2000; Nakamura et al., 2006) to monitor newborn neurons using permanent genetic fate mapping. In the GLAST::CreERT2 mouse line the inducible form of Cre (CreERT2) expressed in the locus of the astrocyte-specific glutamate transporter (GLAST) allows for targeting of postnatal and adult neurogenesis at different stages with high efficiency as it originates from astroglial cells (Mori et al., 2006). In the CAG-CAT-eGFP mouse line, expression of enhanced Green Fluorescence Protein (eGFP) is directed upon the Cre-mediated excision of the loxP- flanked CAT gene located between the modified chicken β-actin promoter with the CMVIE enhancer (CAG promoter) and the eGFP gene (Kawamoto et al., 2000). The fusion of Cre to the ligand binding domain of the modified estrogen receptor (ERT2) is restricted to the cytoplasm and translocates only upon tamoxifen stimulation into the nucleus where it can then mediate recombination (Feil et al., 1997) Mori et al., 2006). This allows us to mediate recombination at specific time points to target postnatally generated neurons in the neurogenic zones including newborn neurons of dentate gyrus (Mori et al., 2006). Previous studies demonstrated that following tamoxifen administration the stable expression of the reporter gene allows an efficacious monitoring of the progeny of cells that underwent recombination (Couillard-Despres et al., 2005; Kempermann et al., 2004; Mori et al., 2006; Ninkovic et al., 2007).

Prior to surgery, animals were housed in sibling groups under controlled environmental conditions (21-23°C, 40-50% humidity, 12-h light/dark cycle) with free access to water and standard feed. To minimize the impact of circadian variations each procedure was performed at the same time each day in all experimental groups. Animals were housed separately during the period of experimentation.

Experimental procedures were performed according to the German Animal Welfare Act and were approved by the responsible governmental administration of Upper Bavaria.

Electrode implantation

For implantation of kindling electrodes, 62 GLAST::CreERT2 x CAG-CAT-eGFP male mice aged 10-16 weeks were anesthetized by intraperitoneal injection of 380 mg/kg chloral hydrate (Merck, Germany). A subcutaneous injection of bupivacaine 0.5% (Jenapharm, Germany) was applied for additional local anesthesia before

exposure of the skull surface. The skull surface was exposed, and a bipolar electrode was implanted into the right hemisphere aimed at the amygdala using the following stereotaxic coordinates according to the atlas of Paxinos and Franklin (2004): +1.0 mm caudal, +3.2 mm lateral, +5.3 mm ventral (all respective to bregma). The electrodes consisted of two twisted Teflon-coated stainless steel wires (0.47 mm) separated by 0.55 mm at the tip. One screw, which served as the grounding electrode, was positioned over the left parietal cortex. Bipolar and ground electrodes were connected to plugs, and the electrode assembly and anchor screws were held in place with dental acrylic cement applied to the skull surface. Meloxicam (Boehringer Ingelheim, Germany) was administered as 1 mg/kg subcutaneous injection 30 min prior to the surgery and the administration was repeated 24h later.

Tamoxifen Administration

Tamoxifen (T-5648-5G, Sigma, Germany) was dissolved at 20 mg/ml in corn oil (63156, Fluka, Germany). Animals received intraperitoneal injections of 1 mg twice a day for five consecutive days following the first week of recovery from the surgery (Mori et al., 2006).

Amygdala Kindling

Following a post-operative recovery period of two weeks, the pre-kindling afterdischarge threshold was determined by administering a series of stimulations (1 ms, monophasic square wave pulses, 50 Hz for 1 s) at intervals of 1 min increasing in steps of 20% of the previously applied current. The afterdischarge threshold was defined as the lowest current intensity discharges twice the pre-stimulation amplitude lasting at least 5 s. The next day, the initial kindling phase was started using an individual stimulation current 20% above the previously determined pre-kindling afterdischarge threshold. Stimulation of the amygdala was performed once daily (five times per week) for nine sessions. Following each stimulation, seizure severity, seizure duration and afterdischarge duration were measured. Seizure severity was scored according to Racine (1972): stage 1, immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial clonus; stage 2, head nodding associated with more severe facial clonus; stage 3, clonus of one forelimb; stage 4, bilateral clonus of forelimbs; stage 5, generalized clonic seizures with rearing and falling. Once animals exhibit generalized seizures a stable hyperexcitable network has formed, which is associated with chronically enhanced seizure susceptibility and cellular and molecular alterations mimicking alterations in temporal lobe epilepsy (Goddard et al., 1969). Seizure duration was defined as the time period of limbic and/or motor seizure. Seizure

severity and seizure duration were evaluated by an experimenter unaware of the animal's group membership, and afterdischarge duration was recorded using an EEG. Cumulative afterdischarge duration was calculated as the sum of afterdischarge durations throughout the experiment.

Following the initial kindling phase, animals which exhibited at least one generalized seizure (bilateral forelimb clonus with or without rearing and loss of balance) towards the end of this phase were used for further investigations studying the impact of different seizure frequency during a chronic phase. For this purpose, animals were distributed homogeneously to three groups based on their afterdischarge threshold and the average of seizure severity during the initial kindling phase: one group was connected to the stimulating apparatus but did not receive any further stimulation (kindled/+0); the other two groups were stimulated once (kindled/+1) or three times (kindled/+3) per week for 12 weeks. Mice of the control group were also implanted and were handled the same way as kindled animals but did not receive electrical stimulations during the initial and chronic phase (electrode-implanted non-kindled control group). The experimental design is depicted in Fig. 1A.'

BrdU Treatment

To study cell proliferation in the chronic phase following the initial kindling phase we used the thymidine analog 5'Bromodeoxyuridine (BrdU). The purpose of BrdU application was to study proliferation changes after the formation of a hyperexcitable network. BrdU is incorporated into the DNA during the S phase of the cell cycle and is available for about 15 min following injection and thus labels the proportion of dividing cells that are in the DNA-synthetic phase of the cell cycle during this period (Mandyam et al., 2007). Control and kindled mice received a total of eight intraperitoneal injections of 50 mg/kg BrdU (Sigma, Germany) during a four-week period (Fig. 1A). Each week animals received a BrdU injection following the stimulation session (note that the control and kindled/+0 groups were only connected to the stimulator but did not receive electrical current) and 8h later they received the second administration of BrdU.

PCR genotyping

Mouse tail DNA was used for PCR genotyping before and after the experiments. The following primers were used for GLAST::CreERT2 genotyping:
GLAST F8 (5'-GAGGCACTTGGCTAGGCTCTGAGGA-3'),
GLAST R3 (5'-GAGGAGATCCTGACCGATCAGTTGG-3'),
CER1 (5'-GGTGTACGGTCAGTAAATTGGACAT-3').

Only heterozygous GLAST::CreERT2 mice were used for the experiment to exclude functional defects from lack of GLAST function. So far no defects have been observed in mice with only one functional allele for GLAST (Mori et al, 2006).
For CAG-CAT-eGFP genotyping AG2 (5'-CTGCTAACCATGTTCATGCC-3') and CAT-2 (5'- GGTACATTGAGCAACTGACTG-3') were used.

Tissue preparation

At the end of the experiment (16 weeks after surgery), mice were deeply anesthetized with chloral hydrate (Applichem, Germany) and were transcardially perfused with 0.01 M phosphate buffered saline followed by 4% paraformaldehyde. The brains were removed and transferred into 30% sucrose and stored at 4°C. Five series of 40 μm coronal sections of cerebrum were cut using a Reichert-Jung 1205 freezing microtome. Sections were stored at -80°C in cryoprotecting solution (glycerol and 0.2 M phosphate buffer, pH 7.4, 1:1 in volume).

Fifteen animals were excluded from the experiment for different reasons including lack of behavioral and electrographic seizure induction during initial threshold determination, loss of the electrode during the experiment, general health disturbance and unsuccessful recombination. Six animals did not show generalized seizures during the initial kindling phase and therefore were not included in the final combination of groups.

Seven mice from each group were chosen randomly for counting purposes. The proportion of new granule cells among all granular cells was determined in four animals per group.

BrdU/NeuN Double-Labeling

Free-floating sections were rinsed in 0.05 M Tris-buffered saline (TBS) and incubated in formamid (Sigma, Germany) for 2 hours at 65°C. Then sections were incubated in 2 N HCl for 30 min at 37°C and washed in 0.1 M borate buffer (pH 8.5) for 10 min. Blocking was performed by using donkey serum. Sections were then incubated in an antibody mixture containing rat anti- BrdU (AbD Serotec, UK), 1:30, and mouse anti-NeuN (Chemicon, Germany), 1:500, at 4°C overnight. After washing the sections, carbocyanin-3-labeled donkey anti-rat antibodies (Jackson Immunoresearch Laboratories, USA; 1:1000) and biotinylated donkey anti-mouse antibodies (DAKO, Germany; 1:500) were applied for 60 min. The sections were washed again and incubated in carbocyanin-2-labeled streptavidin (Jackson Immunoresearch Laboratories, USA), 1:2000, for 60 min. Finally, all sections were

washed, mounted onto glass slides, air dried, dehydrated, and cover-slipped with Entellan (Merck, Germany).

BrdU/GFAP Double-Labeling

Pre-treatment was performed as described above. Primary antibodies mixture contained rat anti- BrdU (AbD Serotec, UK; 1:300) and mouse anti-GFAP (Millipore, Germany; 1:500). Cy3- labeled donkey anti-rat antibody (Jackson Immunoresearch Laboratories, USA; 1:1000) and Alexa 647 donkey anti-mouse (Jackson Immunoresearch Laboratories, USA; 1:1000) were used for detection. Cover-slipping was performed as described above.

eGFP/NeuN Double-Labeling

After washing with TBS and blocking with goat serum, all sections were treated with the mixture of primary antibodies chicken anti-eGFP (Aves, USA; 1:500) and biotinylated mouse anti-NeuN (Chemicon, Germany; 1:300) at 4°C overnight for double-labeling of eGFP and neuronal nuclei protein (NeuN). Following washing, DyLight488 goat anti-chicken (Dianova, Germany; 1:200) and Cy3-Sterptavidin (Dianova, Germany; 1:1000) were applied for 60 min. Cover-slipping was performed as described above.

eGFP/Prox1 Double-Labeling

In order to confirm hilar ectopic neurons, additional animals from the kindled/+3 group were used to perform eGFP/Prox1 staining. Primary antibodies mixture consisted of chicken anti-eGFP (Aves, USA; 1:500) and rabbit anti-Prox1 (Covance, US; 1:500). These antibodies were detected using DyLight488 goat anti-chicken (Dianova, Germany; 1:1000) and biotinyliated goat anti- rabbit (Dianova, Germany; 1:1000) and Cy3-Streptavidin (Dianova, Germany; 1:1000). Cover- slipping was performed as described above.

eGFP/DCX Double-Labeling

The primary antibodies mixture consisted of chicken anti-eGFP (Aves, USA; 1:500) and guinea pig anti-DCX (Chemicon, Germany; 1:1000). These antibodies were detected by using DyLight488 goat anti-chicken (Dianova, Germany; 1:250) and Cy3 goat anti-guinea pig (Jackson Immunoresearch Laboratories, USA; 1:500). Cover-slipping was performed as described above.

eGFP/Ki67/GFAP Triple-Labeling

Due to the limit of available tissue, eGFP/Ki67/GFAP were stained in the same sections but analyzed separately (eGFP/Ki67 and eGFP/GFAP). The primary antibodies mixture consisted of chicken anti-eGFP (Aves, USA; 1:500), rabbit anti-

Ki67 (Abcam, UK; 1:500) and mouse anti- GFAP (Millipore, Germany; 1:500). These antibodies were detected using DyLight488 goat anti- chicken (Dianova, Germany; 1:1000), biotinyliated goat anti-rabbit (Dianova, Germany; 1:1000) and Cy3-Streptavidin (Dianova, Germany; 1:1000), and Alexa 647 donkey anti-mouse (Jackson Immunoresearch Laboratories, USA; 1:1000) respectively.

Quantification and image analysis

Counting for double-labeling of cells with neuronal markers was performed in five brain sections in both left and right dentate gyrus. The sections were spaced 200 μm apart. Cell counts of fluorescent signals cells were performed in an area encompassing the entire dentate granule cell layer (upper and lower blades) and extending approximately two cell body widths deep into the hilus. The hilus was defined as the inner border of the granule cell layer and two straight lines connecting the tips of the granule cell layer and the proximal end of the CA3 region. Immunoreactive cells were counted on a computer monitor to improve visualization using a Hitachi HV-C20A (Hitachi, Japan) digital camera connected to a Zeiss LSM 510 microscope (Carl Zeiss, Germany). The signal of the fluorescent labeling was captured with the StereoInvestigator 6.0 (Microbrightfield Europe, Germany). Double-labeling was verified by careful analysis of the confocal z-series of multiple cells in each animal.

Proportions of eGFP-positive cells among NeuN-positive cells were assessed in the aforementioned double-labeling counting areas using an unbiased random systematic sampling method. Three 30 x 30 μm counting frames were applied on the upper and lower blades of the DG on both sides (60 counting boxes per animal). Sampling area was optically sectioned using 3 μm Z-intervals in 9 μm of depth. Cells which were located in the lookup section or were in contact with the exclusion lines were not counted. At least 400 NeuN-positive cells were counted in each animal.

To confirm successful recombination, two animals were selected from each group and DCX- positive cells were counted in two hippocampal regions with a distance of 200 μm. The proportion of eGFP-positive newly generated neurons co-labeled with DCX indicated the efficacy of recombination.

Confocal images were further improved for color correction by using Irfanview 4.28 (http://www.irfanview.com/).

Statistics

Statistical analyses were performed using Prism software (GraphPad; version 5.0). Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test, when appropriate. The Pearson's correlation coefficient was used to test for a correlation

between the kindling data and the number of counted cells. The Student's paired t-test was used to compare counted numbers of left and right hemispheres and no significant difference was found in any of the quantifications. Data are expressed as mean ± SEM. A value of $p<0.05$ was considered significant. All tests were used two-sided.

3.4 Results

Kindling data

Fully kindled animals were distributed homogenously to the different groups for the chronic phase. Statistical comparison of seizure data from the initial kindling phase confirmed that groups did not differ in the initial afterdischarge threshold ($p = 0.40$) and in the mean seizure severity ($p= 0.83$) during the first 10 stimulation sessions.

In the chronic phase, animals which were stimulated once per week exhibited a mean seizure severity of 4.29 ± 0.39, i.e. generalized seizures were observed during the majority of stimulation sessions. Mice which received three stimulations per week exhibited a mean seizure severity of 4.62 ± 0.16 during the chronic phase, i.e. generalized seizures were observed during the majority of stimulation sessions.

In accordance with the project planning and the stimulation protocols (Fig. 1A), the average cumulative seizure duration and afterdischarge duration differed between the groups: kindled/+0 group showed an average cumulative seizure duration of 210.29 ± 16.82 s and an average cumulative afterdischarge duration of 234.00 ± 72.27 s. The respective data for the kindled/+1 group were 646.86 ± 48.25 s and 668.57 ± 52.79 s, and for the kindled/+3 group 1225.00 ± 26.59 s and 1223.14 ± 37.61 s, respectively (Fig. 1B, C).

All the above mentioned data have been determined for the animals that were randomly selected for immunohistological studies ($n = 7$ in all groups).

Hippocampal cell proliferation and neurogenesis during the early chronic phase

Double-labeled eGFP/Ki67 cells (Fig. 3A, B) were quantified in the subgranular proliferation zone of all animals (control: 21.29 ± 3.20, kindled/+0: 23.43 ± 2.36, kindled/+1: 23.43 ± 5.83, kindled/+3: 22.71 ± 3.95) (Fig. 4A). Statistical analysis did not show any significant difference between the groups ($p = 0.98$).

BrdU-labeled cells were detected in the subgranular proliferation zone and the granule cell layer in all groups (control: 21.29 ± 3.76, kindled/+0: 141.70 ± 33.20, kindled/+1: 138.00 ± 29.34, kindled/+3: 159.7 ± 31.79). As mentioned before, BrdU administration was performed following the initial kindling phase during the first four weeks of the chronic phase. During this period the control and kindled/+0 groups had no seizures and 4 or 12 seizures occurred in the kindled/+1 and kindled/+3 groups, respectively. All groups of kindled mice exhibited a significant increase of 6.5 to 7.5fold in the number of BrdU-positive cells, exceeding that in control animals. Interestingly, the degree of increase was comparable in animals not receiving further stimulations and in animals with ongoing repeated seizures (Fig. 2A).

Neuronal differentiation of surviving cells generated during the phase of BrdU administration was assessed based on BrdU/NeuN double-labeling (Fig. 2B, 3C-E). All groups of kindled mice exhibited an increase in the number of newborn neurons during the chronic phase in comparison to the control animals which reached a significant difference in kindled/+3 group (kindled/+0: 3.9fold, kindled/+1: 4.6fold and kindled/+3: 7.7fold). While weekly single additional seizures did not further increase the number of newborn neurons, the elicitation of three seizures per week during the chronic phase further enhanced the rate of hippocampal neurogenesis resulting in a number of BrdU/NeuN double-labeled cells which significantly exceeded that of non-kindled animals (control: 6.57 ± 1.46, kindled/+0: 25.86 ± 7.53, kindled/+1: 30.00 ± 7.59, kindled/+3: 50.71 ± 7.76; p=0.04) (Fig. 2B).

Considering data from all kindled animals, the number of double-labeled cells correlated with the cumulative duration of afterdischarges and seizure duration (Pearson correlation coefficients 0.53 and 0.51; p= 0.01 and 0.02, respectively) (Fig. 2C).

Double-labeling the BrdU-positive cells with anti-glial fibrillary acidic protein (GFAP) proved the presence of astrocytes mostly in the subgranular zone in all groups; however the visual inspection indicated no obvious differences in the number of cells between groups (Fig. 3O, P).

Long-term monitoring of kindling effects on hippocampal neurogenesis

There were eGFP-positive cells present in all of the animals which demonstrated successful recombination in all groups (Fig. 3F-I).

The number of recently born neuronal progenitor cells and early post-mitotic neurons was assessed based on counting of cells expressing both DCX and the reporter eGFP (Fig. 3J, K). One animal of the kindled/+0 group demonstrated to be an outlier due to an extremely high number of eGFP/DCX-labeled cells (335 positive cells). According to the three-sigma rule this animal was not considered in the statistical analysis.

The proportion of eGFP-positive newly generated neurons reached a stable number of 83.19 ± 3.96% of all DCX-labeled cells 15 weeks after the induction of recombination. This number is close to the quantification of β-gal reporter (around 90%) 1.5 and 9 months after induction in the GLAST::CreERT2/R26R mouse line reported by Ninkovic et al. (2007). Therefore our data demonstrate a very efficient labeling of neuronal progenitor cells in the subgranular zone. Analysis of eGFP/DCX-labeled cells did not show any significant difference between the groups (p = 0.06).

Careful attention was paid to the presence of hilar basal dendrites in multiple eGFP-positive cells from all groups. However, our analysis did not reveal a relevant persistence of these formations in our model.

Based on cell counts of eGFP/NeuN-labeled cells (Fig. 3L-N), information was obtained about the generation of newborn neurons during the complete experimental time frame beginning after tamoxifen administration, i.e. consisting of the initial kindling phase and the chronic phase. It is important to note that recombination does not take place in adult neurons, therefore all eGFP/NeuN-labeled cells are newly generated neurons following tamoxifen administration.

In the control group, the kindled/+0 and the kindled/+1 group no double-labeled cells were identified in the hilus. In contrast, the majority of mice from the kindled/+3 group exhibited single newborn neurons located in the hilus expressing both NeuN and the reporter eGFP (Fig. 3T, U). These cells proved to be ectopic granular cells based on a double-labeling of eGFP/Prox1 (Fig. 3V, W). In none of the groups were eGFP/NeuN-labeled cells evident in the molecular layer. In all groups eGFP/NeuN-labeled cells were identified in both blades of the dentate gyrus with most cells being located in the half of the cell layer adjacent to the hilus (Fig. 3L, M). Thus, as previously shown by Ninkovic et al. (Ninkovic et al., 2007) and Lagace et al. (2007), the majority of the new cells did not migrate far into the granule cell layer before becoming integrated.

The number of eGFP/NeuN-labeled cells in the granule cell layer was significantly increased in all kindled groups (control: 17.14 ± 4.03; kindled/+0: 129.1 ± 35.51; kindled/+1: 119.40 ± 33.48; kindled/+3: 152.9 ± 17.98). Interestingly, the number of newborn neurons did not differ between kindled mice without additional seizures and mice with 12 or 36 seizures during the chronic phase (Fig. 4C).

Addressing the question of how alterations in cell proliferation and neurogenesis affect the relative portion of newborn neurons among all granule cells, we used an unbiased random sampling method to count the eGFP/NeuN cells among those NeuN-labeled. The total number of NeuN-positive cells did not differ between groups (control: 439.30 ± 11.11, kindled/+0: 478.8 ± 6.69, kindled/+1: 456.6 ± 12.98, kindled/+3: 444.3 ± 13.76; $p=0.1$).

The percentage of reporter positive cells among the granule cells reached significantly higher levels in the kindled/+3 group as compared to non-kindled controls ($p=0.01$). The same result was confirmed in both upper and lower blades of dentate gyrus ($p=0.03$ and $p=0.01$, respectively) (Fig. 5B, C).

Double-labeling of eGFP positive cells with GFAP marker proved a difference in morphology of astrocytes in kindled groups in compare to the control. Reactive astrocytes were detected in the kindled groups with an increase in length and width of processes (Fig. 3Q-S).

3.5 Discussion

Genetic fate mapping analysis in the mouse amygdala kindling model revealed that recurrent seizures exert an impact on the long-term balance with a net effect on the cellular composition of the granule cell layer.

Analysis of the number of adult-generated reporter-positive neurons during the complete kindling procedure demonstrated a significant increase regardless of the seizure density and the cumulative number of seizures. Interestingly, alterations were also evident in animals which were kindled to generate a hyperexcitable network but did not experience any further seizures. Although the exact functional significance of an increase in adult-generated granule cells remains to be further explored, it is considered rather likely that pronounced alterations in the rate of hippocampal neurogenesis should have functional implications for the course and consequences of epilepsy (Danzer, 2011; Kokaia, 2011; Parent and Murphy, 2008). In view of the present proof of lasting changes in the makeup of the granule cell layer despite lack of recent seizures, these functional implications might even apply to cases with long intervals between seizure events. Previously, Coras and colleagues have shown in ex vivo studies that a longer duration of seizures does not affect neurogenesis in human patients (Coras et al., 2010). Our experiments support this finding for the first time in an animal model.

Earlier studies have already compared the impact of a different number of seizures in the kindling model (Fournier et al., 2010; Parent et al., 1998). Parent and colleagues (1998) described that 4-6 generalized seizures did not affect hippocampal cell proliferation and neurogenesis, whereas increased dentate granule cell neurogenesis was evident in groups experiencing 9-10 or 19-20 generalized kindled seizures. In contrast, genetic fate mapping in the present study revealed that nine kindling stimulations with a mean number of 4.7 ± 1.13 (mean ± SEM) generalized seizures are sufficient to cause lasting changes in the composition of the granule cell layer. The discrepancy to the previous findings underlines the advantages of permanent genetic fate mapping approaches. BrdU is incorporated during a short time span following its injection, so that even repeated BrdU injections will label only a minor fraction of newborn cells (Taupin, 2007a). Therefore, analysis of BrdU-labeled cells can result in an underestimation of hippocampal neurogenesis and the extent of its modulation (Imayoshi et al., 2008; Lagace et al., 2007; Ninkovic et al., 2007).

Whereas analysis of the number of adult-generated neurons introduced during the complete time span following implantation did not reveal any impact of the cumulative

number of seizures or seizure frequency, differences in neurogenesis were evident based on BrdU/NeuN labeling during the first four weeks of the chronic phase, i.e. after the animals acquired a generalized-state. The number of newborn neurons generated during this phase reached a significant difference only in animals with a high seizure density in comparison to the control group. In contrast, hippocampal cell proliferation rates analyzed based on eGFP/Ki67 double-labeling did not differ by the end of the experiment among the groups. In the early chronic phase, cell proliferation proved to be enhanced in a comparable manner regardless of the occurrence of additional seizures and their number.

Looking at previous studies indicates that controversial findings have been obtained regarding the impact of chronic recurrent seizures in both kindling and post-status epilepticus models. Recently, Fournier and colleagues (2010) described that short-term kindling with 30 electrical stimulations increased the number of immature neurons, whereas long-term kindling with 99 electrical stimulations did not. The differences to our results might be related to higher seizure density and the fact that conclusions in their study were merely based on doublecortin analysis. In post-status epilepticus models with chemical induction of status epilepticus a long-term decline in hippocampal neurogenesis has been repeatedly reported (Hattiangady et al., 2004; Hattiangady and Shetty, 2008). Recent findings revealed that decreased neuronal differentiation underlies this reduction in hippocampal neurogenesis (Hattiangady and Shetty, 2010). Thus, chemically- induced status epilepticus seems to result in anti-neurogenic long-term conditions which modulate neuronal fate-choice decision. This effect might be related to the pronounced pathology occurring as a consequence of chemically induced status epilepticus (Loscher and Brandt, 2010). In support of this hypothesis, we did not observe a respective decline in neurogenesis in a model with electrically-induced status epilepticus which is associated with milder pathological alterations (Pekcec et al., 2008; Seeger et al., 2011). Thus, recurrent seizures in the chronic phase might actually be pro-neurogenic. However, the effect can be masked or counteracted by anti- neurogenic conditions related to pathological alterations affecting the neurogenic niche such as inflammatory events.

As emphasized by Meltzer and colleagues (2005), the integration of new neurons into adult neural circuits can promote or impair circuit function depending on whether homeostatic mechanisms are in place to regulate the resulting changes in neural activity. In line with this statement, there is concern about detrimental long-term consequences of seizure- and epilepsy- associated alterations in the generation of new

neurons (Danzer, 2011; Kokaia, 2011; Parent and Murphy, 2008). So far the long-term net effect of seizure-associated alterations has remained an open question. Permanent genetic fate mapping now rendered information about the impact of recurrent seizures on the relative contribution of newborn neurons to the dentate gyrus. Whereas the initial kindling phase and low intensity stimulation of one seizure per week did not significantly affect the relative portion of new neurons among all dentate granule cells, additional recurrent seizures resulted in an expansion of the fraction of new granule cells. Thus, repeated seizure activity seems to disturb the homeostatic mechanisms of granule cell turnover rates with a shift towards integration of new cells vs. cell loss. A disturbance of homeostasis in the granule cell layer via an increased percentage of new cells might result in different functional consequences. Each new excitatory neuron will probably add excitatory drive affecting dentate gyrus outputs (Meltzer et al., 2005). As Meltzer and colleagues discussed (2005), memory recall might be impaired related to erroneous recruitment of output cells if a given memory depends on activation of a precise pool of hippocampal neurons. In line with this hypothesis, data from animals indicate that recall of older memories benefits from a reduction in neurogenesis (Feng et al., 2001). Moreover, we already reported that partial normalization of hippocampal neurogenesis in a rodent epilepsy model improves spatial learning (Pekcec et al., 2008). Thus, the excessive addition of new neurons seems to disturb hippocampal neuronal circuits involved in information processing and might hamper subsequent integration of further newborn neurons. This may impact memory formation that is discussed to involve the integration of new neurons into hippocampal networks (Wiskott et al., 2006). Moreover, studies on human patients with temporal lobe epilepsy suggest that neuronal density in the internal limb of the dentate gyrus (anatomically comparable to the lower blade of the dentate gyrus in rodents) is an important predictor for the patient's capability to store and recall declarative memory (Pauli et al., 2006). Therefore, alterations in the proportion of new granule cells might contribute to epilepsy-associated cognitive deficits.

Regarding a putative role of adult-generated neurons in ictogenesis and epileptogenesis, controversial findings have been reported, thereby hindering the development of clear-cut conclusions. In general, the addition of excitatory cells suggests an increase in excitability. However, it has been reported that newborn granule cells that integrate into the granule cell layer of epileptic animals are less excitable and have fewer dendritic spines than cells from control animals (Jakubs et al., 2006; Murphy et al., 2011; Wood et al., 2011). Thus, variations in morphological

development of adult-generated granule cells might help to maintain homeostasis and might prevent an excessive increase in dentate gyrus excitability in the epileptic brain (Danzer, 2011). In support of this concept, various studies indicated that modulating neurogenesis in different epilepsy models has no relevant and consistent effects on seizure development (Danzer, 2011; Kokaia, 2011; Pekcec et al., 2008; Pekcec et al., 2011; Pekcec et al., 2007). Considering these data, an increase in the fraction of newborn cells might not affect ictogenesis and epileptogenesis.

In contrast, ectopic granule cells in the hilus exhibit bursting behavior and a higher ratio of excitatory inputs to inhibitory inputs (Scharfman et al., 2000; Zhan et al., 2010). The fact that reporter-positive ectopic neurons in the hilus only occurred in kindled animals with high seizure frequency suggests that these cells are not critical for kindling development. However, considering that these can develop with a glutamatergic granule cell phenotype (Scharfman and McCloskey, 2009) they might contribute to disease severity in individuals with a high seizure density.

Several studies suggest that astrocytes play a key role in epilepsy development through various mechanisms (Arisi et al., 2011; Seifert et al., 2010; Tian et al., 2005). Kindling-induced seizures have been shown to result in prominent cytoskeletal changes in astrocytes which take place early in the development of kindling and persist for long periods of time (Khurgel and Ivy, 1996). On the other hand, it has been shown that altered neurogenesis in epileptic animals changes the one- to-one relationship between astrocytes and newly born granule cells (Shapiro and Ribak, 2005), which may contribute to a hyperexcitable condition by making the newborn neurons more susceptible to aberrant synaptic targeting (Arisi et al., 2011). Reactive astrocytosis involves an increase in the size of the soma as well as in the length and width of astrocytic processes which are indicative of hypertrophy and is occasionally accompanied by proliferation of these cells (Khurgel and Ivy, 1996; Rogawski, 2005). Hattiangady & Shetty (2010) showed that in a chronic kainic acid-induced status epilepticus in rats, differentiation of newly born cells into S-100ß positive astrocytes increased in epileptic animals in comparison to the controls. On the other hand, in a kindling model of aged rats, Arisi et al. (Arisi et al., 2011) reported an increased volume and activated morphology of astrocytes in the kindled group but no quantitative difference of GFAP positive cells in the hilus and CA1. As Hawrylak et al. (1993) point out, this might be due to the fact that it is unlikely to see astrocytic proliferation in the kindling model in a region distant from the implantation site (Hawrylak et al., 1993). Our results from double- labeling the proliferative cells in the

chronic phase with the astrocytic marker GFAP, proved the presence of astrocytes in all of the animals especially in the subgranular area. However, we did not observe any major difference in the number of these cells between the groups through our visual observation. Using an eGFP/GFAP staining, we confirmed astrocytic identity of cells in the subgranular zone similar to the findings of Mori et al. (2006) in GLAST::CreERT2 – Cx43::LacZ mice. We could see hypertrophic astrocytes in all of the kindled groups but not in the controls. Therefore, our model suggests that seizures result in structural changes of astrocytes that are detectable long after the termination of these insults.

Regarding the homogeneity in the number of BrdU-labeled cells among kindled animals and the differences between BrdU/NeuN-labeled cells, it needs to be considered that the formation of other types of glial cells, i.e. microglia and oligodendrocytes can also be enhanced in epilepsy models (Hattiangady and Shetty, 2010; Vezzani et al., 2011). Hattiangady and Shetty (Hattiangady and Shetty, 2010) reported that NG2+ oligodendrocyte progenitors and S100beta- positive astrocytes increased in a chronic rodent model of temporal lobe epilepsy. In addition, microglia cell proliferation as well as migration of bone marrow cells into the brain can occur in the chronic epileptic brain (Longo et al., 2010; Vezzani et al., 2011), and might, thus, have contributed to the number of BrdU labeled cells. In this context, it is of particular interest that Hattiangady and Shetty (2010) have concluded that diminished dentate granule cell neurogenesis in chronic temporal lobe epilepsy is linked to a dramatic decline in the neuronal fate-choice decision of newly generated cells with newly born cells mainly turning into glia. The fact that we did not observe any major alterations in the number of astrocytes might be related to the use of another astrocytic marker or to differences between the animal models used. Future investigations will be necessary to determine to which extent the other cell types mentioned above have contributed to the overall number of new cells in the chronic kindling paradigm used in the present study.

3.6 Conclusion

Genetic fate mapping analysis demonstrated that recurrent seizures result in a lasting change in the makeup of the granule cell layer with alterations in the relative contribution of newborn neurons to the granule cell network. Interestingly, the formation of a hyperexcitable network and a prior seizure history without recent seizure activity can result in pronounced long-term alterations regarding the absolute number of new granule cells. However, seizure density also seems to play a critical role with more frequent seizures resulting in increased portions of new neurons.

In futures studies it is of interest to use the genetic fate mapping approach to further study electrophysiological properties of newly generated and integrated neurons in the kindling model as well as functional consequences with respect to memory impairment in a hippocampal kindling paradigm.

3.7 Acknowledgments

This research was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG FOR 1103 PO- 681/5-1; SFB 870). The authors thank Andreas Blutke for his valuable comments regarding the counting methods. We would like to thank Carmen Mayer, Heidrun Zankl, Marion Fisch and Angela Vicidomini for their technical assistance and Anton Pekcec for his contributions to the initial planning phase. In addition we thank Hannes Wendt for help during electrode implantations.

3.8 Figures

Figure 1: Kindling data

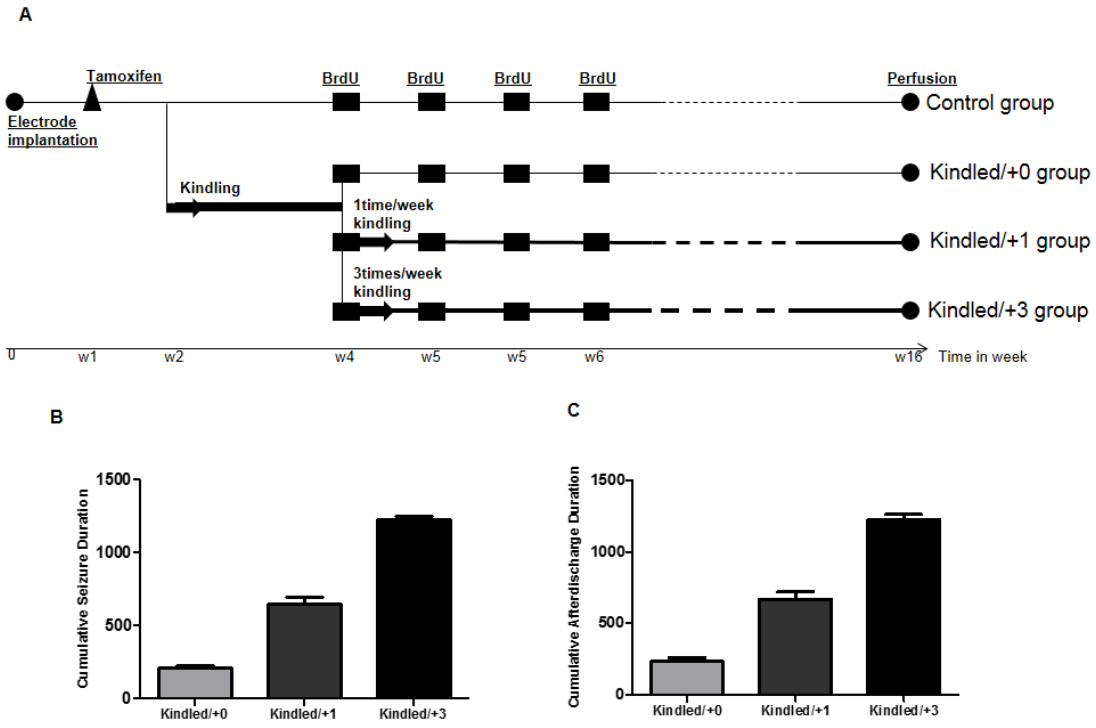

Fig. 1

Figure 1: Kindling data

A: Experimental design including project planning and stimulation protocols. B: Cumulative seizure duration reflects the difference of stimulation protocols between groups. C: Cumulative afterdischarge duration reflects the difference of stimulation protocols between groups.

Data are given as mean ± SEM. Significant differences are found between all of the groups. (One-way ANOVA test, $p<0.05$).

Figure 2: Hippocampal cell proliferation and neurogenesis during the early chronic phase

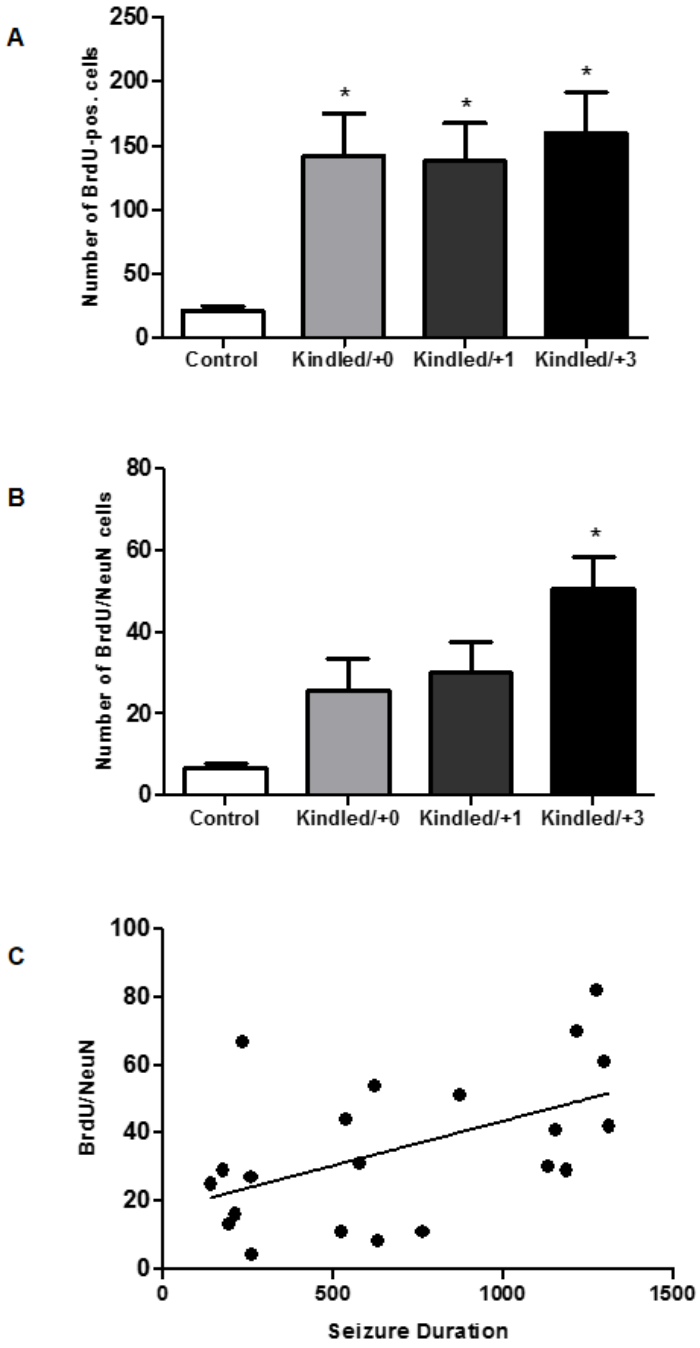

Fig. 2

Figure 2: Hippocampal cell proliferation and neurogenesis during the early chronic phase

A: Analysis of BrdU-positive cells in the dentate gyrus showed a 6.5 to 7fold increase in all kindled groups. B: Neuronal differentiation was assessed based on analysis of BrdU/ NeuN double-labeled cells in the dentate gyrus. The previous kindling procedure resulted in an induction of hippocampal neurogenesis which was enhanced with elicitation of three seizures per week, but not with single additional seizures. C: The number of BrdU/NeuN-positive cells showed positive correlation with cumulative seizure duration.

Data are given as mean ± SEM. Significant differences are indicated by asterisks (kindled groups vs. control group). (One-way ANOVA test, $p<0.05$; Pearson correlation coefficient = 0.51, $p = 0.02$)

Figure 3: 3 Fluorescence micrographs

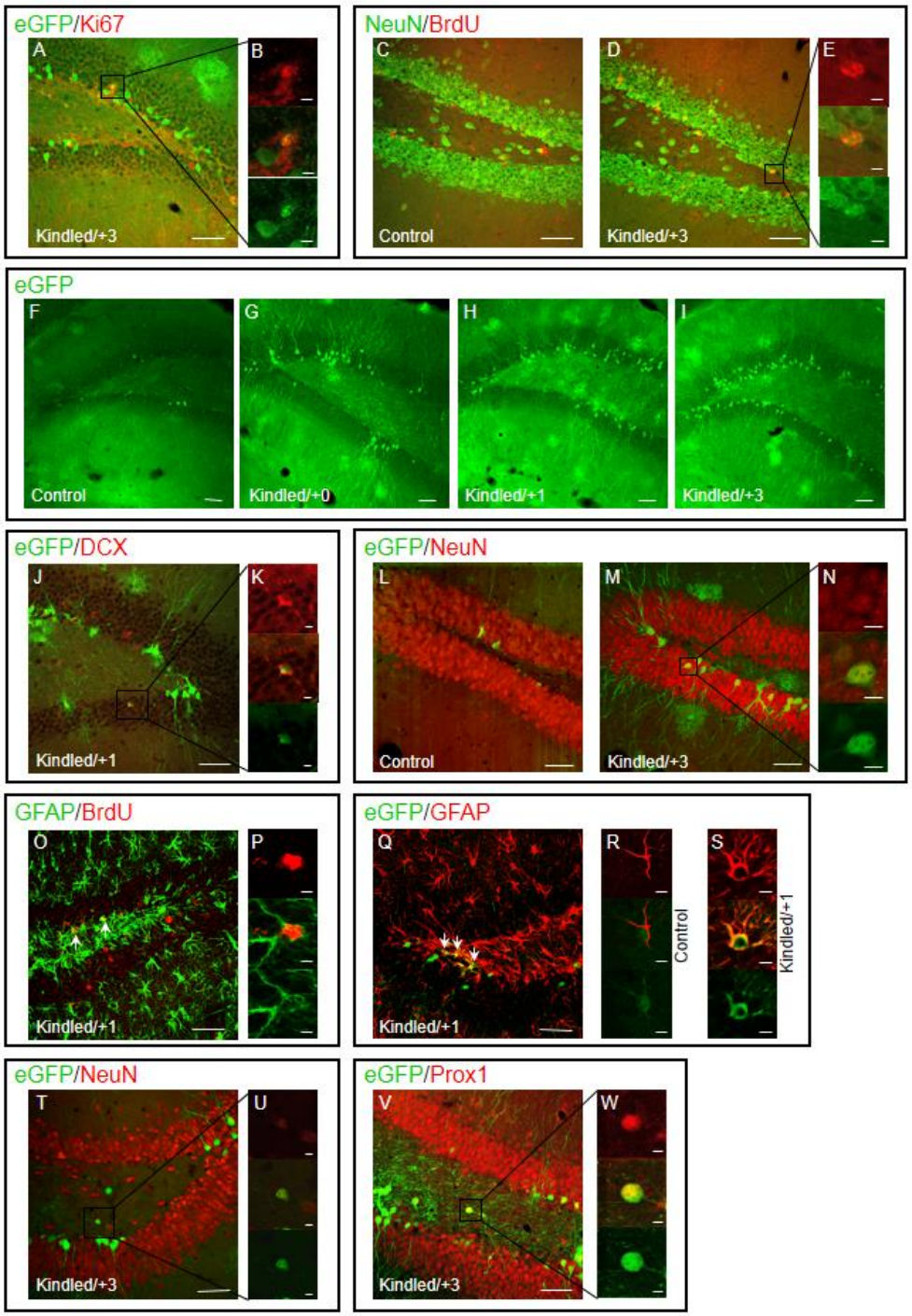

Figure 3: Fluorescence micrographs

A: eGFP (green) and Ki67 (red) immunostaining in kindled/+3 group. B: High magnification of an eGFP/Ki67-labeled cell. C: BrdU (red) and NeuN (green) immunostaining in control group. D: BrdU/NeuN immunostaining in kindled/+3 group. E: High magnification of a double-labeled BrdU/NeuN subgranular neuron. F-I: eGFP (green) immunostaining in all four groups. J: eGFP (green) and DCX (red) immunostaining in kindled/+1 group (no significant difference in double- labeling seen between all four groups). K: High magnification of a double-labeled eGFP/DCX newborn neuron. L: eGFP (green) and NeuN (red) immunostaining in control group M: eGFP/NeuN immunostaining in kindled/+3 group. N: High magnification of a double-labeled eGFP/NeuN neuron. O: GFAP (green) and BrdU (red) immunostaining is shown in an animals from kindled/+1 group. No difference was observed between the groups. Arrows show several double-labeled cells in subgranular zone. P: High magnification of a

double-labeled GFAP/BrdU cell. Q: eGFP (green) and GFAP (red) immunostaining in animal from kindled/+1 group. Arrows show several double-labeled cells in subgranular zone. R: High magnification of a double-labeled eGFP/GFAP astrocyte in control group. S: High magnification of a double-labeled eGFP/GFAP reactivated astrocyte in kindled/+1 group. Hypertrophic astrocytes were observed in all kindled groups. T: Ectopic hilar neurons were found in kindled/+3 group using eGFP/NeuN staining. U: High magnification of an eGFP/NeuN double-labeled ectopic hilar neuron. V: eGFP (red) and Prox1 (green) immunostaining shows ectopic hilar neuron in kindled/+3 group. W: High magnification of an eGFP/Prox1 ectopic hilar neuron.

Scale bar is 5 μm in high magnification micrographs (B, E, K, N, P, R, S, U, W) and 50 μm in the rest of the images.

Figure 4: Long-term monitoring of kindling effects on the hippocampal neurogenesis

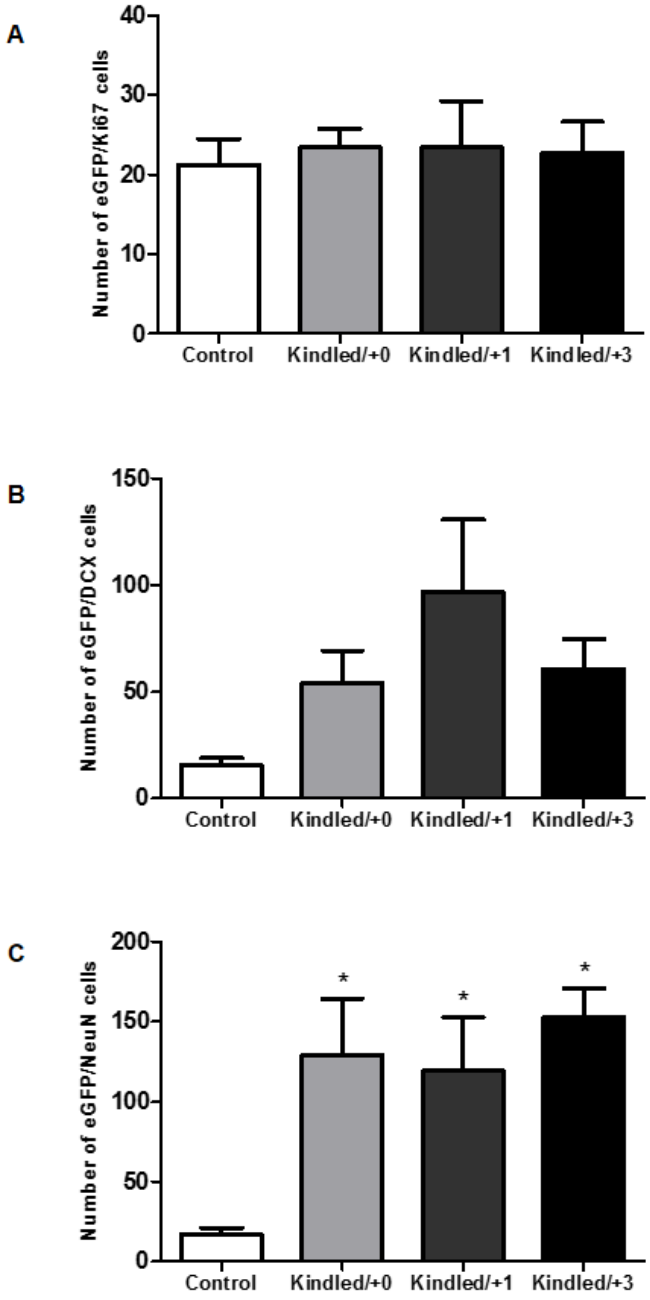

Fig. 4

Figure 4: Long-term monitoring of kindling effects on the hippocampal neurogenesis

A: Analysis of eGFP/Ki67 double-labeled cells in the dentate gyrus showed no difference of proliferation between the groups. B: Analysis of eGFP/DCX double-labeled cells in the dentate gyrus did not reveal any significant difference among different groups. C: Analysis of eGFP/ NeuN double-labeled cells in the dentate gyrus gives information about the generation of newborn neurons during complete experimental time frame.

Data are given as mean ± SEM. Significant differences are indicated by asterisks (kindled groups vs. control group). (One-way ANOVA test, $p<0.05$).

Figure 5: Relative portion of newborn neurons among all granule cells

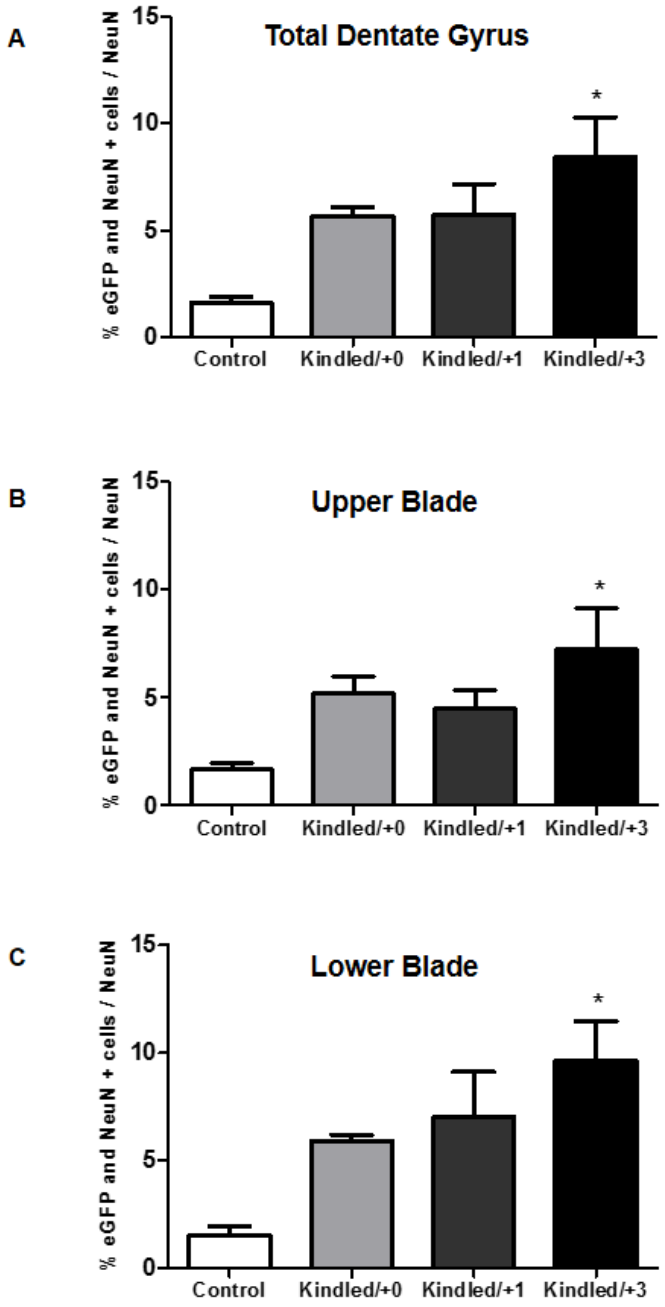

Fig. 5

Figure 5: Relative portion of newborn neurons among all granule cells

A: Proportion of eGFP/NeuN double-labeled cells among all NeuN cells in the counted frames of dentate gyrus was significantly increased in kindled groups with further stimulations. B: Proportion of eGFP/NeuN double-labeled cells among all NeuN cells in the counted frames of the upper blade was higher in the kindled/+3 group. C: Proportion of eGFP/NeuN double-labeled cells among all NeuN cells in the counted frames of the lower blade of the dentate gyrus was higher in the kindled/+3 group.

Data are given as mean ± SEM. Significant differences are indicated by asterisks (kindled groups vs. control group). (One-way ANOVA test, $p<0.05$).

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CHAPTER 4: General Discussion

4.1 Overview of the main findings

We provide evidence that Theiler's virus can show acute and long-term effects on the production of new cells and the neuronal progenitor cell population of the hippocampus depending on the disease course and the strain of the animal. While the hippocampal cell proliferation was enhanced in infected SJL/J mice during the acute phase of the disease, the C57BL/6 infected mice did not show a significant difference to the non-infected controls. The number of neuronal progenitor cells and immature neurons was slightly decreased in the infected SJL/J mice, whereas, the infected C57BL/6 showed a slight increase of these cells. Despite the limitation of the polioencephalitis to the first weeks of infection, long-term consequences of the viral infection were evident in both resistant and susceptible strains. The survival of hippocampal proliferating cells was compromised in the infected SJL/J mice, whereas, the infected C57BL/6 mice showed a promoted survival of the cells. The number of mature neurons during the chronic phase of the disease was not altered in the infected mice. The number of the neuronal progenitor cells and immature neurons was not affected in the C57BL/6 animals that cleared the virus, but this number was reduced in the susceptible SJL/J mice. The number of activated microglia was more pronounced in the infected SJL/J mice during the acute phase, while this number was enhanced in the infected C57BL/6 mice during the chronic phase. We could not confirm a correlation between the number of activated microglia and the immature neurons. However, the functional state of microglia may have contributed to hippocampal changes as complex alterations in response to infection were found in both strains. Moreover, we were able to show strain differences in proliferation and production of new neurons in the non-infected condition.

In a parallel study conducted by Dr. Marion Bankstahl at the Institute for pharmacology, toxicology and pharmacy of the University of Veterinary Medicine Hannover (TiHo), infected and non-infected mice were video monitored for 24h over 14 days of the acute phase of infection. No seizures were observed during the two-hour observation periods. In the chronic phase, animals had cortical electrodes implanted and after a week they were observed with EEG/video monitoring for 24h over 7 days before they were sacrificed. No seizures were observed during this period (the results of the study were kindly provided by Dr. Bankstahl).

In our second experiment, using permanent genetic fate mapping, we show that the total number of granule neurons was increased during a 14-week period following

the start of kindling in comparison to the control animals. Our data demonstrated that a short period of kindling is sufficient for observing this enhancement 12 weeks following the generalized kindled state without any additional stimulation. Receiving 1 or 3 further electrical stimulations per week for 12 weeks did not change the net number of newborn neurons. The majority of newborn neurons were detected in the inner half of both blades of the DG. Ectopic hilar granule neurons were only observed in the animals that were exposed to a higher frequency of electrical stimulation during the 12-week period. Moreover, the proportion of newborn neurons to the total granule neurons showed a higher fraction of these cells in the group with more stimulations. Thus, additional seizures do not increase the absolute number of hippocampal neurons but ectopic granule neurons may modulate hippocampal activity. The analysis of the Ki67 positive cells and reporter-positive immature neurons did not confirm any significant differences between controls and kindled animals at the end of the experiment. Therefore, it seems that following a chronic period of kindling-induced seizures, proliferation and neuronal differentiation are not altered. On the other hand, the data from the first 4 weeks following the kindled state, showed that proliferation transiently increases in animals with a history of stimulation and that the production of new neurons during this time frame gradually increases in correlation with the seizure density. However, this could be the result of an underestimation of neurogenesis based on BrdU analysis in comparison with the fate-mapping method. Structural changes of astrocytes were observed in the kindled animals but we did not observe major differences in their number.

4.2 Acute effects of TMEV on the hippocampal neurogenesis

Early acute infection of mice with the BeAn strain of Theiler's virus results in a mild, attenuated, clinically inapparent polioencephalomyelitis in SJL/J and C57BL/6 strains (Dal Canto et al., 1996; Oleszak et al., 2004; Mecha et al., 2013).

In our study, weekly evaluation of clinical signs confirms that there was no detectable clinical impairment during the acute phase of the disease in the animals. Pathohistological evaluation showed lymphohistiocytic encephalitis, which was more pronounced in the susceptible SJL/J animals. TMEV antigen was detected in the resident cells and macrophages of the brainstem in the majority of infected animals of both strains, in accordance to the reported high levels of viral replication during this phase (Lipton et al., 2005).

Analysis of proliferation based on BrdU-labeling revealed an increase of cells in the SGZ and the granular zone of the DG in the infected animals in comparison with the controls that reached a significant difference in the infected SJL/J mice. Thus, hippocampal cell proliferation was enhanced in animals that were more susceptible to develop a chronic demyelinating disease. Evaluation of neuronal progenitor cells and immature neurons did not confirm an increase of these cells during the acute phase of the disease in the infected SJL/J mice. Also, analysis of the BrdU-positive cells 99 days following the infection did not substantiate changes in the number of newborn neurons. Therefore, the proliferating cells in the acute phase may originate from other cell types including T cells, glia or infiltrating mononuclear cells. Previous studies have shown an increase in the number of these cells during the acute phase of the TMEV infection (Oleszak et al., 2004; Kim et al., 2005; Mecha et al., 2013).

Although analysis of neuronal progenitor cell and immature neurons that expressed DCX did not prove a significant difference between the infected and control animals, a contrarious impact of the infection was observed between the strains resulting in a higher number of these cells in the infected C57BL/6 compared to the SJL/J infected mice.

Results from the acute phase of our study demonstrate that even a subclinical polioencephalitis can be associated with modulation in the hippocampal cell proliferation. We also show that viral infection could induce opposing effects on the neuronal progenitor cells and immature neurons in the two mouse strains resulting in a significant difference in the population of these cells.

4.3 Chronic effects of TMEV on hippocampal neurogenesis

The gray matter disease phase of the TMEV infection heals spontaneously after 2-3 weeks in both resistant and susceptible strains of mice, however, the virus persists in the white matter of spinal cord in susceptible mice resulting in a late chronic demyelinating disease associated with perivascular and parenchymal infiltrates (Oleszak et al., 2004; Lipton et al., 2005).

In our study, infected SJL/J mice showed a progressive clinical impairment starting day 42 following the infection while C57BL/6 mice did not develop significant clinical signs. All of the C57BL/6 infected mice cleared the brainstem from viral antigen at day 99 and no perivascular or parenchymal infiltration of mononuclear cells was observed in the majority of them. In contrast, TMEV antigen was detectable in the majority of the infected SJL/J mice, and the inflammatory changes were enhanced and frequently accompanied by neuronal loss and gliosis in the brainstem.

During the chronic phase, the majority of the BrdU-labeled cells generated in the acute phase were not detectable in the infected SJL/J. In contrast, the long-term survival of the BrdU-labeled proliferating cells was slightly promoted in the resistant C57BL/6 mice compared to the non-infected mice of the same strain.

Analysis of cells that incorporated BrdU and express NeuN in the chronic phase did not confirm any difference in the number of mature neurons between the infected and control animals. This finding supports the idea that the enhanced proliferation during the acute phase of the disease can be due to other cell types including T cells, glia or infiltrating mononuclear cells rather than the neuronal population.

The number of neuronal progenitor cells and immature neurons expressing DCX was detrimentally affected during the chronic phase in the susceptible SJL/J mice. A previous study by Goings and colleagues of alterations of neurogenesis in the SVZ following TMEV infection in SJL/J mice, showed a similar pattern with an unchanged population of DCX positive cells during the period of greatest inflammation and a decrease of these cells during the chronic phase (Goings et al., 2008). Moreover, we did not observe a change in the number of neuronal progenitors in the C57BL/6 animals that cleared the early infection of the virus in comparison to the non-infected C57Bl/6 mice. The number of DCX-positive cells of the infected C57Bl/6 was significantly higher than that of the infected SJL/J mice.

Our study demonstrates that acute subclinical encephalitis without a chronic course is sufficient to induce a long-term modulation of the neuronal population.

Moreover, Theiler's virus proved to have a differential impact on hippocampal neuronal progenitor cells in long-term depending on the strain of the infected mice.

4.4 Neuroinflammation in the TMEV model

Several studies have described a dual role of brain inflammation on hippocampal neurogenesis (Monje et al., 2003; Carpentier and Palmer, 2008; Das and Basu, 2008; Taupin, 2008; Ekdahl et al., 2009; Volobouéva and Giffard, 2011; Yoneyoma et al., 2011; Gonzalez-Perez et al., 2012). Microglia can regulate neurogenesis in, both, positive and negative ways (Gemma et al., 2010). Furthermore, microglia have been suggested as the main reservoir of Theiler's virus replication during the chronic phase (Kim et al., 2005; Tsunoda and Fujinami, 2010). Therefore, we investigated how brain inflammation may have contributed to the complex disease-associated alterations of hippocampal cell proliferation and neuronal progenitor cells in our study.

An expansion of the activated microglia population during the acute phase of TMEV infection has been previously described in the SJL/J and C57BL/6 mice (Jin et al., 2007; Tsunoda and Fujinami, 2010). Acute microglial activation has been shown to exert detrimental effects on survival and differentiation of newly formed neurons in the adult brain (Ekdahl et al., 2009). In our study, analysis of the cells with phenotypical characteristics of activated microglia that expressed CD11b marker showed an increase of these cells during the acute phase in the infected mice reaching a significant difference in the SJL/J infected mice compared to controls. We did not find a strain difference during the acute phase, which confirms the previous findings that similar levels of microglial cells are present in the CNS of susceptible SJL/J mice compared to those of resistant C57Bl/6 mice during the early course of infection (Jin et al., 2007). During the chronic phase, higher numbers of activated microglia were found in the infected mice; however, this number only reached a significant difference in the resistant C57BL/6 mice. We did not find a correlation between the number of activated microglia and the number of immature neurons during acute and chronic phases.

Therefore, we further studied the functional state of microglia to observe how inflammatory factors and cytokines induce effects on hippocampal cell proliferation and population of neuronal progenitors in the TMEV model. Unfortunately, the number of samples was not sufficient for statistical analysis due to technical reasons.

CD18, like CD11b, is one of the integrin subunits of the Mac-1 protein (Lynch, 2009). Microglia cells express this complement receptor to signal for phagocytosis, and microglia-neuron interaction during development (Linnartz and Neumann, 2013). During the acute phase of our study, CD18-labeled microglia cells measured by flow cytometry were increased in the infected mice, similar to our immunohistochemical

finding for CD11b. The ability of phagocytosis was also in line with the CD11b alterations, suggesting that the immune staining for CD11b is a good representative of the microglia function.

Enhanced expression of MHC class proteins has been reported to contribute to the recruitment and activation of inflammatory cells (Chang et al., 2000). Although the data for MHC I fluorescence intensity were not conclusive in the acute phase, infected mice showed an upregulation of MHC I in both stains during the chronic phase.

IL-1β signals are associated with both host protection from viral infections and pathogenesis of inflammatory immune-mediated disease (Kim et al., 2012). The absence of IL-1β permits viral persistence while high levels can contribute to pathogenesis by elevating Th17 response (Kim et al., 2012). Our study on the cytokines shows the presence of high IL-1β level during the acute phase in the infected mice of both strains suggesting its role in promoting the infection during the acute phase. However, we found a high variance in our samples from the infected animals. TNF-α levels were downregulated in the infected animals of both strains during the acute and chronic phase.

ROS generation levels did not confirm a distinct difference between infected and non-infected animals during acute and chronic phase.

In summary, we determined complex alterations in the functional state of the microglial cells during different phases of the TMEV infection. These changes may have contributed to the cellular alterations in the hippocampus of infected mice considering the interaction between microglial-derived mediators and the different stages of hippocampal neurogenesis (Koo et al., 2008; Ekdahl et al., 2009; Seguin et al., 2009; Taupin, 2010). Further studies are necessary to determine the role of specific mediators in TMEV infection. Using transgenic mice that overexpress or lack selected mediators, and/or pharmacological modulation of IL-1 β in the TMEV model may provide a better understanding of the complex role of neuroinflammation on hippocampal neurogenesis.

4.5 Strain differences of neurogenesis in mice

The TMEV study provides evidence that strain differences can determine the rate of production of new cells. Non-infected C57BL/6 mice that were evaluated 14 days following the vehicle inoculation for BrdU labeling showed higher proliferation in comparison with SJL/J mice. Moreover, the ratio of the number of the BrdU positive cells in the chronic phase to that of the acute phase, suggested a higher survival rate of the proliferated cells in the non-infected SJL/J mice. Also, a stereological analysis of neuronal progenitor cells and immature neurons showed a higher number of these cells in the C57BL/6 animals by the end of the experiment. Our data support the previous finding by Kim and colleagues that C57BL/6 mice exhibit more Ki67 and DCX positive cells in the adult hippocampus compared to the SJL/J mice (Kim et al., 2009). In addition, we demonstrated that the number of neuronal progenitor cells does not differ between the strains in young animals; however, an age-dependent decline of neurogenesis reached different levels for these cells resulting in the presence of more DCX-positive cells in the C57BL/6 mice. Analysis of the BrdU-labeled cells that expressed the neuronal marker (NeuN) by the end of the experiment showed a higher number of these cells in the SJL/J mice, however, this could be due to the high survival of the proliferated cells labeled during the acute phase.

Strain differences were also evident following the TMEV infection. Infected C57BL/6 mice showed higher populations of neuronal progenitor cells in both acute and chronic phases of the disease in comparison to the infected SJL/J mice. Furthermore, the number of activated microglia based on the CD11b labeling was higher in infected C57BL/6 mice during the chronic phase compared to the SJL/J infected animals.

4.6 Genetic fate mapping analysis of seizure-associated modulation of neurogenesis

We used genetic fate mapping to investigate the long-term impact of recurrent seizures on the cellular composition of the granular layer of the DG. Double labeling of the eGFP reporter cells with anti-NeuN showed an increase of neurons in the DG area in the kindled animals compared to the control group. Interestingly, the density of seizures did not affect the overall production of neurons. This finding is supported by previous data from human patients that longer history of repeated seizures does not affect neurogenesis (Coras et al., 2010).

On the other hand, our data demonstrate that in the animals that experienced more seizures both lower and upper blades of the DG of hippocampus exhibit a higher fraction of newborn neurons in relation to the total population of granule neurons. Therefore, it seems that repeated seizures disturb the homeostatic mechanisms of neuronal turnover rate. Integration of adult-born neurons into hippocampal network contributes to memory formation, while erroneous recruitment of granular neurons may contribute to impairment of memory recall (Meltzer et al., 2005; Mongiat and Schnider, 2011; Toni and Sultan, 2011; Snyder and Cameron, 2012). Therefore, we discussed that alterations in the proportion of new granule cells in the hippocampal network in animals that experience recurrent seizures, might contribute to epilepsy-associated cognitive deficits. In line with our hypothesis, recently, it was reported that kindling seizures promote the survival of new neurons but decreases the integration of these cells into functional networks important in hippocampal-dependent learning such as fear memory retrieval (Fournier et al., 2012).

Double labeling of eGFP/Ki67 was used to study cell proliferation by the end of the experiment. The results did not confirm any difference between the kindled animals and control and kindled animals. These data are in line with data obtained from human patients with epilepsy, which did not reveal changes of Ki67 positive cells in older patients with chronic disease (Siebzehnrubl and Blumcke, 2008).

Previously it has been reported that 30 stimulations lead to an increase of DCX-labeled cells, whereas 99 stimulations did not affect the number of these cells (Fournier et al., 2010). In our study, analysis of the reporter positive cells that expressed DCX showed no difference among groups, however, a high variance was detected in the animals that were stimulated one time per week during the chronic phase (a total of 22 stimulations).

These findings suggest that by the end of the experiment, proliferation and neuronal fate-choice did not differ between the control and kindled animals.

4.7 Impact of recurrent seizures on proliferation

Once the animals reached the fully kindled state we used BrdU incorporation to study the effects of recurrent seizures on proliferation and neuronal survival in a selected time interval. Nine weeks following the last injection of the BrdU, we found that all of the animals with a history of a kindled state showed an increase in the number of labeled cells. Interestingly, our results show that the initial kindling-associated formation of a hyperexcitable network is sufficient to induce a persisting increase of proliferation without an impact of further subsequent seizures.

An analysis of the expression of the neuronal marker NeuN in BrdU positive cells, revealed a positive correlation between double-labeled cells and the cumulative duration of seizures leading to a significant increase of the newborn neurons in the group that was stimulated more often.

Double-labeling of the BrdU positive cells with anti GFAP protein proved the presence of astrocytes in the SGZ of all of the animals, however, we did not detect any major difference in the number of these cells between the groups by visual inspection. On the other hand, a careful analysis of the double-labeled eGFP/GFAP positive cells revealed hypertrophic structural changes in the astrocytes of the kindled animals that were not visible in the control animals.

The formation of other cell types of glial cells such as microglia and oligodendrocytes, and/or migration of bone marrow cells into the brain might also contribute to the increased proliferation of cells in the kindled mice (Hattiangady and Shetty, 2010; Vezanni et al., 2013).

4.8 Ectopic hilar granule neurons

We were able to identify single new neurons in the hilus in the majority of mice that were stimulated three times per week. The granular nature of these cells was confirmed by Prox1 labeling. Ectopic hilar granule neurons were not observed in the animals from the other groups. This finding is in accordance with a previous study that showed a correlation between the number of ectopic granule neurons and the seizure frequency in status epilepticus model (McCloskey et al., 2006). In a kindling model of rats, it was shown that ectopic neurons are only increased in the ipsilateral hippocampus of the animals that received 99 stimulations, and not in the group that received 30 stimulations (Fournier et al., 2010). The same study suggested that aberrant neurogenesis depends on the number of elicited seizures and the resultant circuitry rearrangement that follows the repeated induction of seizures (Fournier et al., 2010). Ectopic granule cells receive increased excitatory input and exhibit spontaneous bursting in synchronization with CA3 that may contribute to hyperexcitability of the network (Scharfman et al., 2000; Parent and Murphy, 2008; Zhao and Overstreet-Wadiche, 2008; Zhan et al., 2010). Abnormal localized granule cells may lead to disorganization of the DG and cause potential problems for normal for information processing (Scharfman and Gray, 2007).

4.9 Conclusions and remarks

In this doctoral thesis, I studied the disease-induced modulation of adult hippocampal neurogenesis. For this purpose, I focused on two mouse models that are widely used in disease studies: TMEV model of the MS disease and the kindling model of epilepsy. I used the widely used technique of BrdU labeling in combination with various cell markers to identify the newborn neurons at different stages. Also, I took advantage of a genetic-fate mapping method in my second study to accomplish a better understanding of the disease-associated changes of neurogenesis.

Recently, TMEV has been proposed as a viral encephalitis model of epilepsy (Libbey and Fujinami, 2011; Libbey et al., 2011). However, our colleagues at the University of Veterinary medicine Hannover (TiHo) could not confirm any seizures in this model. Our results from the hippocampal neurogenesis study proved the impact of Theiler's virus infection on hippocampal proliferation and neuronal progenitors. We showed that Theiler's virus may exert delayed effects on the hippocampal progenitor population with long-term changes evident 3 months after the infection. These alterations proved to depend on the strain of mice and its susceptibility to the disease.

The experiment reflects the complexity of hippocampal neurogenesis study in a model of a biphasic disease. In the acute phase, the common BrdU method was not helpful to detect newborn neurons because the newborn cells do not mature within the short time frame that is interesting to study the peak of the clinical symptoms. On the other hand, in the chronic phase, although BrdU labeling can distinguish the newborn neurons, the interpretation could be complicated because of the differences in survival of proliferating cells in a disease model. We also demonstrated that the complex alterations of microglial functional state during different phases of the infection in each strain might have affected the generation of neurons.

In the second experiment, genetic fate mapping was used to monitor the newborn neurons in the kindling model of epilepsy in long-term. We showed that the initial kindling-related formation of a hyperexcitable network is sufficient to induce a long-term increase of the production of neurons, and that further subsequent seizures do not enhance this effect. A high seizure density, however, can lead to the generation of ectopic granule neurons in the hilus area that can participate in cognitive deficits. Thus, the genetic fate mapping method was confirmed as an excellent tool for studying chronic changes induced by electrical stimulation. Conditional recombination at desired time points gives the opportunity to study a chronic disease in different stages

for a better understanding of the accumulative effect of the insult on hippocampal neurogenesis. Moreover, the transgenic expression of reporter protein makes this method valuable for further in vivo studies.

In conclusion, different central nervous system diseases can modulate hippocampal neurogenesis at different stages of the process. The nature of the insult (infection vs. electrical stimulation), duration (acute vs. chronic), the mice susceptibility to the insult (SJL/J vs. C57BL/6 mice in the TMEV model), and the method used to identify the newborn neurons (BrdU method vs. genetic fate mapping) are critical in analyzing the disease-induced modulation of hippocampal neurogenesis.

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The author of this doctoral thesis contributed to this paper by participating in the experimental part of the study, performing cellular studies of adult hippocampal neurogenesis, statistical analysis, and writing the major parts of the manuscript.

Jafari, M., Soerensen, J., Bogdanović, R. M., Dimou, L., Götz, M., & Potschka, H. (2012). Long-term genetic fate mapping of adult generated neurons in a mouse temporal lobe epilepsy model. *Neurobiology of disease*, *48*(3), 454–63. doi:10.1016/j.nbd.2012.06.014

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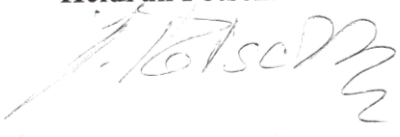

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Jafari M., Haist V., Baumgärtner W., Wagner S., Stein VM., Tipold A., Wendt H., Potschka H. (2012). Impact of Theiler's virus infection on hippocampal neuronal progenitor cells: differential effects in two mouse strains. *Neuropathology and applied neurobiology*, 1–41. doi: 10.1111/j.1365-2990.2012.01256.x

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