Regulation of Postnatal Dentate Gyrus Neurogenesis and its Alteration in Experimental Epilepsy

by

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Abstract

Medial temporal lobe epilepsy (mTLE) is a common, intractable epilepsy of unknown cause. In rodent mTLE, prolonged seizures, termed status epilepticus, stimulate neurogenesis, but many newborn dentate granule cells (DGCs) migrate and integrate aberrantly. We hypothesized that adult-born, rather than pre-existing, neurons contribute to epileptic pathology in the hippocampal dentate gyrus. Using the rat pilocarpine model, which recapitulates many features of human mTLE, we tested our hypothesis by injecting green fluorescent protein-carrying retrovirus to label neural stem cells, or by killing dividing stem cells to suppress neurogenesis pre or post-epileptic injury. We found that an epilepsy-inciting injury differentially influences developing neurons depending upon their ages. Fully mature neurons at the time of injury are resistant, whereas developing neurons of differing maturities contribute to distinct, specific epileptic pathologies.

To address the molecular underpinnings of this phenomenon, we investigated the Reelin pathway. Reelin is secreted into the extracellular matrix, binds its receptors and is internalized, leading to phosphorylation of disabled-1 (Dab1), an adaptor protein. Evidence implicates the Reelin/Dab1 pathway in DGC development, and mice with Reelin, Dab1, or Reelin receptor mutations have abnormalities similar to those in experimental mTLE.
To better understand the role of Dab1 in neurogenesis, we suppressed Dab1 expression using a mouse line with conditional Dab1 knock-in alleles, or by injecting retroviral vector carrying a Dab1 shRNA into the adult rat dentate gyrus. In Dab1-conditional knock-in mice, Dab1 deletion led to aberrant DGC migration, impaired dendritic maturation and axonal disorganization. In rats, retroviral Dab1 shRNA delivery decreased dendritic arborization of DGCs and induced numerous labeled glia in the hilus and DGC layer that were not seen with control shRNA. Aberrant neurogenesis is a proposed cause of seizures and cognitive deficits common in mTLE, and this work supports an important role for Dab1 signaling in both neuronal migration, dendrite formation, and directing DGC progenitors to a neuronal fate.

Taken together, these data suggest that neurons of distinct maturities contribute to specific epileptic abnormalities and that the Reelin/Dab1 pathway may underlie some of these aberrations. Correcting signaling of this pathway after epileptogenic insult may offer a novel strategy to prevent mTLE.
Chapter 1

Introduction

Neurogenesis in the Adult Mammalian Brain

It was previously believed that the CNS did not regenerate, and that,

“... the functional specialization of the brain imposes … proliferation inability and irreversibility of intraprotoplasmic differentiation. It is for this reason that, once the development was ended, the founts of growth and regeneration of axons and dendrites dried up irrevocably. In adult centers, the nerve paths are something fixed, ended and immutable” (Ramón y Cajal, 1913).

We now know, however, that neurogenesis persists into adulthood in two distinct brain regions, the subventricular zone (SVZ) and the dentate gyrus (DG). This is true in all mammals studied to date, including rodents and humans. While the majority of cells are generated early in life, new dentate granule cells (DGCs) are generated at a lower rate throughout adulthood and into senescence both in rat (Schlessinger et al., 1975; Kuhn et al., 1996) and human, where they are generated at least into the 8th decade of life (Eriksson et al., 1998; Kempermann et al., 2004).

Cells born in the SVZ subsequently migrate through the rostral migratory stream (RMS) into the olfactory bulb. In the DG, granule cells are a unique cell
population that are generated continuously throughout life—with thousands of new granule cells produced per day (Cameron and McKay, 2001). In the absence of injury, DGC precursors arise in the subgranular zone, then migrate a short distance to the granule cell layer where they differentiate into dentate granule cells (Figure 1.1A) (Cameron et al., 1993; Kuhn et al., 1996).

In the DG, numerous cell types play a role in neurogenesis. The putative stem cells in the DG, termed Type-1 cells, are radial glia-like progenitors. Type-1 cells express the astrocytic marker glial fibrillary acidic protein (GFAP) and are nestin positive. It is hypothesized that these cells divide asymmetrically to self-renew and give rise to daughter cells, termed type 2 cells. Type 2 cells then differentiate into type 2a or 2b cells. Type-2a progenitor cells are nestin positive, and their morphology suggests they are capable of tangential migration (Kuhn et al., 1996). Type-2b cells are also progenitor cells but, unlike Type-2a cells, they have limited self-renewal capabilities and are lineage determined. Type 2b cells express both nestin and doublecortin (Dcx). Type 3 cells are similar to Type-2b except they only express Dcx.

After progressing through these stages, differentiating DGC precursors exit the cell cycle and enter a post-mitotic stage where they are termed immature granule cells and are actively establishing network connections. The final stage of differentiation is the post-mitotic, mature granule cell that expresses Prox1 (a divergent homeobox gene) and calbindin. These cells are considered to be terminally differentiated granule cells (reviewed in (Kempermann et al., 2004)). Terminally differentiated DGCs project their axons (known as mossy fibers) to CA3 pyramidal cells (Stanfield and
Trice, 1988; Markakis and Gage, 1999) and dendrites to the molecular layer (Figure 1.2).

**The Role of Adult Neurogenesis**

Adult-born neurons make up about 6% of the granule cell layer in adult rats (Cameron and McKay, 2001). New granule cells are thought to integrate into hippocampal circuitry and acquire characteristics of mature DGCs (van Praag et al., 2002; Ge et al., 2006). Bromodeoxyuridine (BrdU) labeling studies indicate that DGCs born during adulthood become integrated into circuits and survive to maturity. These DGCs are very stable and may replace DGCs born during development (Dayer et al., 2003).

Increasing evidence supports a role for adult-born DGCs in learning and memory (reviewed in (Doetsch and Hen, 2005; Ming and Song, 2005a; Deng et al., 2010)). Stimulation of adult DGC neurogenesis with behavioral interventions such as exercise or environmental enrichment is associated with better performance on certain hippocampal learning tasks (Kempermann et al., 1997; van Praag et al., 1999). The degree of adult neurogenesis in some inbred mouse strains also correlates positively with learning ability (Kempermann and Gage, 2002). More direct evidence that neurogenesis supports hippocampal-dependent learning and memory derives from experiments in which depletion of adult-generated neurons impairs specific learning tasks such as associative learning and fear conditioning (Shors et al., 2001; Shors et al., 2002).
Genetic ablation of newly formed neurons in adult mice also leads to both a decrease in the number of DG neurons and an impairment of hippocampal-dependent behaviors in contextual and spatial memory (Imayoshi et al., 2008). In this study, diphtheria toxin fragment A was conditionally expressed in dividing cells, and impairment of behaviors in contextual and spatial memory was observed.

Some adult rodent studies also suggest that DGC neurogenesis is necessary for the positive actions of antidepressants (Santarelli et al., 2003; Li et al., 2008). All these findings have been disputed, however, as some studies have found that neurogenesis is not required for the effects of environmental enrichment or for antidepressant effects (Meshi et al., 2006; Holick et al., 2008; Singer et al., 2009).

**Epilepsy and Neurogenesis**

Many stimuli can alter adult neurogenesis, as demonstrated in numerous animal models of human conditions and diseases. One of these is epilepsy, a disorder characterized by spontaneous recurrent seizures. Mesial temporal lobe epilepsy (mTLE) is a common, chronic, debilitating syndrome that disrupts adult neurogenesis. Approximately 50 million people suffer from epilepsy, and of that 40% have mTLE, making it the most common form of adult epilepsy (Engel Jr J et al., 1997). Long-term morbidity includes an increased incidence of depression (Helmstaedter et al., 2004; Mazza et al., 2004) and dysfunction with both learning and memory (Helmstaedter et al., 2003; Elger et al., 2004) that may progress despite adequate seizure control (Blume, 2006). Furthermore, 35% of those with mTLE have chronic seizures that are
pharmacoresistant (Engel, 2001), making the study of this disorder and the development of new therapeutics critical.

Humans with mTLE often have an initial “precipitating” event, followed by a latent period and subsequent development of epilepsy later in life. This knowledge has led to the development of the most common animal models. In these models, prolonged convulsive seizures, termed status epilepticus (SE), are induced by either electrical stimulation or a chemoconvulsant, causing injury as the initial precipitating event. After a seizure-free latent period, spontaneous seizures develop and persist for the lifetime of the animal (Mello et al., 1993; Aridaa et al., 1999).

After an epileptogenic insult such as SE, damage to the hilus of the DG, known as endfolium sclerosis, is the most commonly observed lesion in the brain of patients with mTLE (Margerison and Corsellis, 1966). In animal models of mTLE, hippocampal pathways show structural plasticity that mirror the changes seen in humans with mTLE, recapitulating much of the human pathology (reviewed in (Buckmaster, 2004)). SE provoked by kainic acid or pilocarpine, both common animal models, mimic the human mTLE phenotype, as about half the neurons in the dentate hilus die (Buckmaster and Dudek, 1997; Okazaki et al., 1999).

We study mTLE using the adult rat pilocarpine model. In this model, the chemoconvulsant pilocarpine, a cholinergic muscarinic receptor agonist, induces SE. After 90 minutes of SE, we terminate seizures with diazepam. Rats then enter a several day to several week latent period followed by spontaneous seizures for the remainder of the rat’s life (Mello et al., 1993; Aridaa et al., 1999). After initiation of SE with pilocarpine, rodents display many abnormalities that mimic human mTLE,
including the sprouting of mossy fibers (granule cell axons), abnormal dendrite formation, and aberrant migration, making it an appropriate model for study (Figure 1.1B) (Mello et al., 1993).

In the rat pilocarpine model of epilepsy, the rostral SVZ exhibits an increase in neurogenesis following prolonged seizure activity (Parent et al., 2002). Neuroblasts generated in the SVZ migrate more rapidly to the olfactory bulb, and some exit the migratory stream prematurely (Parent et al., 2002). As assessed by Ki-67 expression or short-pulse BrdU labeling, the DG also responds to SE by increasing neurogenesis in the subgranular zone after pilocarpine or kainic acid treatment (Parent et al., 1997; Jessberger et al., 2005). Dentate gyrus cell proliferation increases 5-10 fold after a latent period of several days (Parent et al., 1997; Gray and Sundstrom, 1998). In the dentate, this early proliferative response seems to be mediated by radial glial-like neural progenitor cells (Huttmann et al., 2003).

The increase in neurogenesis in the DG persists for several weeks (Parent et al., 1997) and may be due to a surge in the number of neural precursors, or may be due to an enhanced survival of newly generated cells. Interestingly, even single seizure-like discharges induce an initial surge in cell proliferation in DGC progenitors (Bengzon et al., 1997).

Approximately 3-4 weeks after the initial SE, proliferation rates return to baseline levels (Parent et al., 1997). In fact, chronic epilepsy after kainic acid-induced SE is associated with a decrease in neurogenesis, with neurogenesis levels substantially below baseline by 5 months after SE (Hattiangady et al., 2004). These findings are also observed in humans, as the DG of children who have had frequent
seizures has decreased numbers of immature neurons (Mathern et al., 2002). Adults with chronic seizures also have decreased neurogenesis, as evidenced by a decrease in doublecortin (Dcx) mRNA in the hippocampus as well as the absence of Ki-67-immunolabeled proliferating cells (Fahrner et al., 2007). Potential reasons for this decrease include exhaustion of the progenitor pool, loss of needed growth/trophic factors or altered cellular interactions (reviewed in (Hattiangady and Shetty, 2008)).

Seizure-induced injury appears to influence both pre-existing and adult-born DGCs in the epileptogenic hippocampal formation (Bengzon et al., 1997; Parent et al., 1999). In rodent models of SE, between 75-90% of cells newly generated after seizure express mature DGC markers within 4 weeks (Parent et al., 1997; Jessberger et al., 2005), and SE appears to accelerate the maturation and integration of adult-born DGCs (Overstreet-Wadiche et al., 2006).

**Morphological Abnormalities in mTLE**

Hippocampi of humans with mTLE and animal models of epilepsy are associated with numerous cellular abnormalities including astrogliosis in the hippocampus, granule cell layer dispersion, mossy fiber sprouting (MFS), DGCs with persistant hilar basal dendrites (HBDs), and ectopic migration of newborn DCGs. Several of these abnormalities and their potential functional significance will be described in the following sections.
Mossy Fiber Sprouting

Mossy fibers are the axons of the hippocampal DGCs. The mossy fiber pathway normally projects to the pyramidal cells of hippocampal area CA3 as well as to mossy cells and interneurons in the dentate hilus. In the non-epileptic brain, mossy fibers are thought to make few, if any, recurrent synapses onto neighboring granule cells. A common feature of human and experimental mTLE, however, is the development of numerous mossy fiber-granule cell synapses that putatively lead to recurrent excitation (Tauck and Nadler, 1985; Sutula et al., 1988; Okazaki et al., 1995).

In the epileptic human DG, Timm staining (used to visualize the zinc present in mossy fiber axons), dynorphin immunoreactivity, and biocytin fills reveal that mossy fibers sprout into the dentate inner molecular layer (de Lanerolle et al., 1989; Babb et al., 1991; Franck et al., 1995; Okazaki et al., 1995). In the pilocarpine model of mTLE, Timm staining also reveals significant amounts of MFS in the rat DG (Mello et al., 1993). Electron microscopy studies demonstrate that mossy fibers synapse onto DGCs, creating recurrent excitatory connections (Okazaki et al., 1995).

Seizure induced mossy fiber reorganization is thought to be progressive in nature, beginning 1-2 weeks after SE with a peak around 100 days post-SE (Cavalheiro et al., 1991; Mello et al., 1993). Based upon this timecourse, one hypothesis is that SE-induced MFS arises from newborn DGCs generated in increased numbers after prolonged seizures (Parent et al, 1997). When neurogenesis was inhibited via X-irradiation around the time of SE, however, MFS was not inhibited (Parent et al, 1999). The development of aberrant mossy fiber remodeling in the
absence of neurogenesis suggests that mature DGCs contribute substantially to seizure-induced network reorganization (Parent et al., 1999; Jessberger et al., 2007). Retroviral labeling studies from other labs also indicate that adult-generated neurons born 4 weeks prior to seizure induction contribute to MFS (Jessberger et al., 2007). Nonetheless, outstanding questions remain regarding MFS in epilepsy. One central question involves the ages of granule cells that contribute to MFS. For example, do only mature DGCs sprout? Or do DGCs still developing at the time of insult lead to the majority of MFS?

**Hilar Ectopic Granule Cells**

The vast majority of newborn neurons born in the subgranular zone normally migrate into the granule cell layer. After SE, many DGCs migrate aberrantly either into the dentate hilus or through the DGC layer into the molecular layer (Parent et al., 1997; Scharfman et al., 2000; Dashtipour et al., 2001; Hattiangady et al., 2004; Shapiro and Ribak, 2005; Parent et al., 2006b). These ectopic cells are found in rodent models of epilepsy (Parent et al., 1997; Scharfman et al., 2000; Dashtipour et al., 2001), and similar ectopically located granule-like neurons are found in the human epileptic hippocampus (Houser et al., 1990; Parent et al., 2006b).

Hilar ectopic granule cells result from abnormal neuroblast chain migration, as the migratory behavior of the progenitor cells appears altered in the post-seizure environment (Parent et al., 2006b; Parent et al., 2006a). Some propose a critical period after the birth of adult-generated neurons during which they are vulnerable to migrating ectopically (Walter et al., 2007).
Although some cells migrate aberrantly after an epileptogenic insult, those cells remain programmed to differentiate into neurons, and they appear to both survive and integrate. Remarkably, ectopically located granule cells develop intrinsic properties and axonal projections that are similar to granule cells that are located normally, although dendritic structure and synaptic structure and function appear to differ (Scharfman et al., 2007). Furthermore, granule cells born after seizures can migrate not only into the hilus, but also as far as the CA3 cell layer, where they become integrated abnormally into the CA3 network yet retain granule cell intrinsic properties.

**Hilar Basal Dendrites**

Under normal conditions, the dendrites of most rodent DGCs arise from the apical pole of the cell body and extend into the molecular layer, while the axons extend from the hilar (basal) pole and terminate in the hilus and stratum lucidum of CA3 (Amaral, 1978). During early postnatal ages, HBDs transiently appear on normal rodent granule cells (Seress and Pokorny, 1981), but they do not persist on mature DGCs (Spigelman et al., 1998; Buckmaster and Dudek, 1999; Ribak et al., 2000). In rats experiencing SE, however, a large portion of newborn DGCs exhibit HBDs that persist for many months (Scharfman et al., 2000; Dashtipour et al., 2001; Shapiro and Ribak, 2006). The persistence of HBDs, normally a feature of only immature rodent DGCs and increased in human mTLE, may be one mechanism underlying the hyperexcitability of DGCs in mTLE.
Functional Implications of Seizure Induced Dentate Gyrus Plasticity

A large body of evidence supports the hypothesis that cellular abnormalities such as MFS, ectopic DGCs and HBDs contribute to epileptogenesis in experimental mTLE.

Mossy Fiber Sprouting

Seizures in mTLE are proposed by some investigators to result from positive feedback through aberrant excitatory recurrent axon collaterals between granule cells (Tauck and Nadler, 1985). Synaptic contacts between the mossy fibers and the apical dendrites of granule cells have been demonstrated at the electron microscopic level in several epilepsy models (Frotscher and Zimmer, 1983; Represa et al., 1993). Mossy fiber sprouting has also been found to induce a shift in the nature of glutamatergic transmission in granule cells, potentially contributing to the physiopathology of the dentate gyrus in epileptic animals (Epsztein et al., 2005).

Analysis of biocytin tracing data suggests that SE provokes the formation of a novel recurrent excitatory circuit in the dentate gyrus via recurrent mossy fiber projections (Okazaki et al., 1995). Sprouted mossy fibers form an average of over 500 new synapses per granule cell, but less than 25 of the new synapses are onto gamma-aminobutyric acid (GABA)-ergic interneurons, suggesting that almost all of the synapses formed by mossy fibers in the granule cell and molecular layers are with other granule cells and thus lead to recurrent excitation (Buckmaster et al., 2002). Evidence also suggests that normal GABAergic inhibition is diminished by mossy fiber terminals, further contributing to tissue excitability (Buhl et al., 1996; Wuarin
and Dudek, 1996). Some data also demonstrate that the intensity of MFS positively correlates with frequency of spontaneous recurrent seizures (reviewed in (Sutula and Dudek, 2007)).

However, further levels of complexity likely underlie epilepsy pathogenesis, as some studies conclude the density of mossy fiber sprouting is not associated with the total number of lifetime seizures or the seizure frequency in experimental or human TLE (Pitkanen et al., 2000; Frotscher et al., 2006; Rao et al., 2006a). Although the aberrantly sprouted mossy fibers clearly form at least some recurrent excitatory synapses with other granule cells, some report that anatomical analyses of epileptic rat hippocampi demonstrate aberrant granule cell axons densely innervating inhibitory neurons (Kotti et al., 1997), and induction of a glutamic acid decarboxylase (GAD)67 isoform, a GABA-producing enzyme, has been observed in DGCs after SE (Williamson and Spencer, 1995). These data suggest that mechanisms other than MFS might account for enhanced hippocampal hyperexcitability.

Taken together, these findings suggest a large degree of potential complexity to mossy fiber reorganization induced changes in the epileptic hippocampus than was previously appreciated. Given the conflicting data on the topic, it is clear that much remains to be studied about the functional implications of MFS.

**Hilar Basal Dendrites**

Basal dendrites are not normally found on mature granule cells in rats (Seress and Pokorny, 1981), but prolonged seizures induce an increased percentage of granule cells with basal dendrites located at the hilar border and extending into the hilus.
(Ribak et al., 2000; Dashtipour et al., 2002). Similarly, the number of granule cells with HBDs in the epileptic human DG is almost twice that found in the normal human DG (von Campe et al., 1997).

HBDs arising after SE develop mature synapses that show ultrastructural evidence of excess excitatory, including mossy fiber, input (Ribak et al., 2000; Jessberger et al., 2007; Walter et al., 2007). This may implicate DGCs as part of a recurrent excitatory circuit. Interestingly, data from computer models predict that even a small number of highly connected granule cells, such as those with HBDs forming multiple connections, could create an epileptogenic hippocampal network (Morgan and Soltesz, 2008a).

**Hilar Ectopic Granule Cells**

In many ways, hilar ectopic DGCs are similar to newborn granule cells that migrate into the cell body layer of the DG. These aberrantly located cells have intrinsic neuronal properties that are quite similar to properly located, mature granule cells. However, ectopic DGCs are activated by spontaneous seizures as they express the immediate early gene c-fos, which is induced by excessive neuronal activity during seizures (Scharfman et al., 2002). Hilar ectopic cells can also functionally integrate into limbic circuits involved in recurrent seizure generation and burst abnormally, in synchrony with rhythmic bursts of area CA3 pyramidal cells that survive after SE (Scharfman et al., 2000). Furthermore, hilar ectopic granule cells from epileptic rats can be postsynaptic to mossy fibers and have less inhibitory input than granule cells.
contained within the granule cell layer, making them more hyperexcitable than granule cells in the layer (Dashtipour et al., 2001).

Consistent with the idea that aberrant neurogenesis leads to hyperexcitability, ablating neurogenesis after SE can attenuate subsequent epileptogenesis. In one study, the investigators suppressed seizure-induced cell proliferation by infusing the antimitotic agent cytosine-b-D-arabinofuranoside (Ara-C) after pilocarpine-induced SE and found that rats had decreased frequency and severity of spontaneous recurrent seizures compared to those receiving vehicle infusions (Jung et al., 2004). One caveat, however, is that the Ara-C was infused into the lateral ventricle and was thus not specific for the DG.

The idea that seizure-induced neurogenesis is pro-epileptogenic is not without controversy, however, as Jakubs et al. describes that DGCs produced in adult rats under seizure conditions show reduced excitability. Although these cells matured into functional granule cells, they received increased inhibitory and reduced excitatory synaptic input compared to adult-born DGCs in exercising rats (Jakubs et al., 2006). Thus the functional implications of seizure-induced abnormalities and the contribution of adult-born DGCs to intact or epileptic hippocampal network function remain unclear.

**Co-morbidities Associated with Epilepsy**

The co-morbidities associated with epilepsy include cognitive, behavioral and emotional difficulties. Those with epilepsy are at increased risk for depression, anxiety, sleep disturbances and cognitive impairment (reviewed in (Jacobs et al.,
Cognitive impairments in mTLE include problems with memory, verbal fluency, and executive function (Elger et al., 2004; Helmstaedter and Kockelmann, 2006). Approximately 70% of patients with mTLE experience memory dysfunction, which represents the most common cognitive impairment in this group.

Although the co-morbid conditions associated with seizures have often been viewed as purely side effects of seizures, several potential sources of co-morbidity in epilepsy likely exist. Seizures themselves could cause the co-morbidities, as patients with long-standing epilepsy often, though not always, have improved cognition after their seizures are successfully controlled with medication or surgery (Helmstaedter et al., 2003). Alternatively, the medications used to treat epilepsy, anti-epileptic drugs (AEDs), could cause cognitive and psychiatric problems, as patients on both the older and newer groups of AEDs have a higher incidence of depression and cognitive impairments (Meador, 2006). However, cognitive impairment progresses in some patients despite adequate seizure control and changes in their AEDs. A proposed mechanism for this involves altered neurogenesis—both aberrant neurogenesis as well as the decrease in neurogenesis seen in chronic epilepsy.

Significant progress has been made in characterizing the abnormalities observed in mTLE. However, further understanding of the mechanisms underlying seizure-induced neurogenesis is clearly of paramount importance, as it represents a promising target for future treatments of both mTLE and its comorbidities. One potential pathway underlying alterations in seizure-induced abnormalities is the Reelin pathway, a pathway crucial for normal development that is recapitulated in adulthood.
Overview of the Reelin Signaling Pathway

In the mid-1900s, a severely ataxic mouse with a non-laminated cerebral cortex and an abnormally formed cerebellum was first described. It wasn’t until 1995, however, that the gene underlying these abnormalities, the Reelin gene, was discovered (D'Arcangelo et al., 1995). Since then, much has been learned about the structure and function of the Reelin gene and protein, its downstream effectors, animal and human Reelin deficiencies, and the Reelin pathway in development and adulthood.

Reelin is a large extracellular matrix protein with 8 repeats of 300-350 amino acids and a large N-terminal segment. The central region is crucial for signaling, as Reelin and its central fragment are equally able to rescue the cortical plate abnormalities observed in reeler mice in a slice culture assay (Jossin et al., 2004). The monoclonal antibody CR-50, binds to an epitope in the N-terminal region of Reelin, blocking its function (Ogawa et al., 1995). The N-terminal region has also been shown to regulate dendritic maturation in the postnatal cortex (Chameau et al., 2009). The C-terminus is required for secretion, and mice without it appear similar to mice completely lacking Reelin (de Bergeyck et al., 1997).

The Reelin Pathway

During normal brain development, Reelin is secreted by Cajal-Retzius cells in the embryonic marginal zone. It is required for proper cerebellar and cortical lamination, as mice lacking Reelin lack this proper lamination (reviewed in (Rice and Curran, 2001)). In adult rodent and human, Reelin is expressed by GABAergic
interneurons in the hippocampal formation and cortex (Pesold et al., 1998; Haas et al., 2002; Abraham and Meyer, 2003).

After secretion into the extracellular matrix, Reelin binds to the transmembrane lipoprotein receptors, very low density lipoprotein receptor (VLDL-R) and apolipoprotein-E receptor-2 (ApoER2), present on migrating neurons (D'Arcangelo et al., 1999). ApoER2 exhibits a 6-fold higher affinity for Reelin than the VLDL-R, and alternative splicing of ApoER2 has been shown to control Reelin’s modulation of N-methyl-D-aspartate (NMDA) receptor activity, synaptic transmission, dendritogenesis and long-term potentiation (LTP) (Beffert et al., 2005).

The cytoplasmic domains of the Reelin receptors bind to disabled-1 (Dab1), an adaptor protein (Trommsdorff et al., 1999). Dab1 is a critical member of the Reelin signaling cascade. The Dab1 gene is a 555 aa gene with an N-terminal protein interaction/phosphotyrosine binding domain (PI/PTB). The PTB domain of Dab1 binds to NPxY-containing motifs in several proteins such as member of the low-density lipoprotein (LDL) receptor family (reviewed in (Rice and Curran, 2001)). After Dab-1 is activated by Reelin binding to its receptors, it is then phosphorylated on tyrosine residues (Howell et al., 2000). Yeast 2-hybrid experiments demonstrate that Dab1 interacts with Abl, Src and Fyn (Howell et al., 1997), and the combined deficit of Src and Fyn generates a reeler-like phenotype (Kuo et al., 2005). Dab1 also regulates Reelin binding, as overexpressing Dab1 increases Reelin receptor surface expression to reinforce signaling (Morimura et al., 2005).

There are numerous potential phosphorylation sites essential for Dab1 function. When these sites are mutated in a transgenic mouse, Dab1 is unable to
signal, and the mice display ataxias and abnormal brain morphology similar to reeler and Dab1-deficient mice (Howell et al., 2000). Furthermore, Dab1 is thought to complex with additional signaling molecules including Nckβ, Crk, CrkL, p85 and Lis1 (Assadi et al., 2003; Bock et al., 2003; Pramatarova et al., 2003; Ballif et al., 2004; Huang et al., 2004). Reduction of Crk and CrkL have been shown to decrease Reelin-induced dendritogenesis (Matsuki et al., 2008). Interestingly, Dab1 is also a downstream effector of insulin-like growth factor-I (IGF-1), with evidence suggesting that IGF-1 increases the phosphorylation of Dab1, and reduced Dab1 levels are seen in IGF-1 knockout mice (Hurtado-Chong et al., 2009).

In scramble mutant mice, which have a very similar phenotype to reeler mutant mice, the Dab1 gene on chromosome 4 is disrupted. Northern blot analysis reveals that these mice have a significant decrease in Dab1 mRNA. Mice with a targeted disruption of Dab1 also have identical defects to scramble and reeler mice, suggesting a common pathway (Sheldon et al., 1997).

There are numerous important functions of the Reelin/Dab1 pathway, including proper cell migration, axonal targeting, dendritic maturation, cell survival, and neuronal/glia fate decisions. Although the function of Reelin in the adult is largely unknown, it may be important for cell migration, as chain-migrating SVZ progenitors are directed to disperse from the chains as they enter the olfactory bulb by locally secreted reelin (Hack et al., 2002). In mice lacking either both Reelin receptors or Dab1, chain formation is severely compromised in the rostral migratory stream, and neuroblasts accumulate in the SVZ (Andrade et al., 2007). Thus, Reelin expression in the DG may also play a role in maintaining normal migration of adult-born DGCs.
Reelin also plays an important role in both axonal and dendritic organization. Tracing of DGC mossy fiber axons with Phaseolus vulgaris leukoagglutinin and calbindin immunolabeling reveals an irregular broad projection in reeler mice and ApoER2/VLDLR double knockouts, with a milder phenotype in single knockouts. The authors attribute this to being likely caused by the irregular distribution of granule cell somata related to the migration/lamination defects described above (Drakew et al., 2002). Despite these abnormalities, however, mossy fibers respect the CA3/CA1 border and malpositioned granule cells project normally to CA3 (Drakew et al., 2002).

In terms of dendrites, inactivation of a conditional Dab1 allele reduces process complexity in correctly positioned neurons in the CA1 region of the mouse hippocampus (Matsuki et al., 2008). Dendritic complexity is also severely reduced in mice lacking Reelin both in vivo and in vitro, and Dab1 phosphorylation inhibitors prevent dendrite outgrowth from normal pyramidal neurons (Niu et al., 2004). Deficits in dendritic complexity in reeler cultures are rescued by addition of exogenous Reelin (Niu et al., 2004).

Interestingly, that same group found that in mice heterozygous for the reeler mutation, dendritic complexity was reduced in the absence of ectopic cells, suggesting that cellular ectopias are not necessary for alterations in dendritic complexity (Niu et al., 2004). DGCs in mice with mutations in Reelin, Dab1, or the ApoER2 or VLDLR receptors also display HBDs, a feature characteristic of mTLE as described above (Drakew et al., 2002).

The Reelin signaling pathway also promotes the development of dendritic spines in hippocampal pyramidal neurons (Niu et al., 2008), and the lack of spines in
cultured *reeler* neurons is recovered with incubation with Reelin (Matsuki et al., 2008; Niu et al., 2008). Taken together, these data suggest a role for Reelin in both dendritic development and the formation and maintenance of synapses on dendrites.

In addition to its effects on neuronal projections, Dab1 also appears to impact neuronal fate decisions. One group labeled dividing cells in adult *reeler* mutants and their wild-type littermates with bromodeoxyuridine (BrdU) and found fewer BrdU-labeled cells in the adult *reeler* DG when compared to wild-type animals (Zhao et al., 2007). Moreover, whereas there was a dramatic decrease in the number of newly generated granule cells identified by double labeling for BrdU and NeuN, the number of BrdU-labeled, GFAP-positive astrocytes had increased. These results indicate that adult neurogenesis is altered in the *reeler* DG and that adult-born cells preferentially differentiate into astrocytes.

Disruption of Dab1 also appears to increase glial differentiation. Neurospheres from the Dab1\(^{-/-}\) mouse, *yotari*, show increased GFAP expression and less neuronal marker expression, suggesting that Dab1 suppresses astroglial differentiation (Kwon et al., 2009). This is also true *in vivo*, with a decrease in neuronal numbers and increase in astroglia in *yotari* brains. In addition to changing neuronal morphology, evidence suggests that Reelin signaling also alters cell survival. Using P19 cells, Ohkuba et al. demonstrated that Reelin protects cells from apoptosis during retinoic acid-induced neuronal differentiation (Ohkubo et al., 2007). They found that Reelin acted through the Src-family Kinases/PI3 pathway and led to selective phosphorylation of the Bcl-2/Bcl-XL associated death promoter (BAD). Thus Reelin was found to enhance cell survival, and loss of Reelin enhanced apoptotic cell death.
Taken together, these data implicate Reelin as a molecule with complex physiological roles, including influences on cell migration, process development, neuronal survival and fate decisions. Many of these processes are altered in mTLE, suggesting that impaired Reelin signaling may also play a role in epileptogenic plasticity.

**The Reelin Pathway in Epilepsy**

The results of Reelin and Dab1 deletion studies mirror several abnormalities observed in epilepsy, most notably granule cell layer dispersion and ectopic DGC migration. Abnormal DGC layer lamination is a feature of human mTLE (Houser, 1990), and loss of Reelin mRNA expression correlates with the degree of DGC layer dispersion in human mTLE (Haas et al., 2002). Furthermore, increased Reelin promoter methylation, and thus decreased Reelin expression, is associated with granule cell dispersion in human temporal lobe epilepsy (Kobow et al., 2009). Mice with Reelin signaling defects also have abnormal DGC layer dispersion (Drakew et al., 2002), further implicating Reelin as an important player in the similar DG abnormalities seen after SE.

Decreased Reelin expression is also observed in experimental models of epilepsy, where a significant decrease in Reelin mRNA synthesis parallels the development of granule cell layer dispersion in the post-seizure environment (Heinrich et al., 2006), and there is a decrease in Reelin immunoreactivity in the adult rat DG following SE (Gong et al., 2007).
Evidence suggests that Reelin may influence postnatal and adult DGC migration, and Reelin directly modulates DG neuroblast migration in vitro (Gong et al., 2007). Although some conclude the Reelin has a direct effect on migrating neuroblasts (Gong et al., 2007), other suggest the effects of Reelin on DGC migration are secondary to changes in the radial glia scaffold (Fahrner et al., 2007). In vivo, antibody blockade of Reelin function in adult mice induces granule cell dispersion, and, granule cell dispersion can be prevented by infusion of exogenous Reelin (Haas and Frotscher, 2009).

**Questions Addressed in this Dissertation**

In this dissertation, the role of adult neurogenesis in epilepsy and the impact of manipulating the Reelin/Dab1 pathway on adult neurogenesis are explored. In the second chapter, the role of cellular age on DGC response to SE is investigated using retroviral (RV) injections into the DG and targeted neuronal progenitor ablation using x-irradiation. Through these experiments, we have found that neuronal age dictates the response of a given DGC to SE.

In the third chapter, the role of the Dab1 adaptor protein, part of the Reelin signaling cascade, is examined. A transgenic mouse line with conditional Dab1 deletion in newly born granule cells and RV-mediated shRNA to Dab1 in adult rats are used for loss of function experiments. With these studies, we show that several of the morphological abnormalities induced by SE are recapitulated when Dab1 expression is knocked down in the neonatal or adult rodent DG.
Figure 1.1: The epileptic hippocampus displays several characteristic abnormalities

Top- Normal dentate gyrus
OML=outer molecular layer
MML= middle molecular layer
IML= inner molecular layer
GCL= granule cell layer
SGZ= subgranular cell layer.

Bottom- Dentate gyrus after seizure
1=mossy fiber sprouting
2=hilar basal dendrites
3=ectopic granule cells
Figure 1.2: Schematic representation of the generation of new granular neurons in the dentate gyrus of the hippocampus

DG = dentate gyrus region
ML = molecular cell layer
G = granular cell layer

Modified from (Ming and Song, 2005b)
Reelin is an extracellular protein that binds to lipoprotein receptors, Vldlr and ApoER2, expressed on neurons. Dab1 is an adapter protein that associates with an NPxY motif (star) in the cytoplasmic domain of these receptors. Reelin binding to lipoprotein receptors activates a tyrosine kinase, resulting in phosphorylation of Dab1. This effect is inhibited by ApoE. Reelin is internalized following binding to lipoprotein receptors, and Dab1 is degraded. The Reelin signaling pathway activates a complex array of biological responses, including cell positioning, cytoskeleton rearrangements and altered gene expression. Mutations in Cdk5, p35, and integrins elicit similar responses that may be mediated through interactions with the Reelin pathway.

Modified from (Rice and Curran, 2001)
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Chapter 2

The Developmental Stage of Dentate Granule Cells Dictates Their Contribution to Seizure-Induced Plasticity

Abstract

Dentate granule cell (DGC) neurogenesis persists throughout life in the hippocampal dentate gyrus. In rodent temporal lobe epilepsy models, status epilepticus (SE) stimulates neurogenesis, but many newborn DGCs integrate aberrantly and are hyperexcitable while others may integrate normally and restore inhibition. The overall influence of altered neurogenesis on epileptogenesis is therefore unclear. To better understand the role DGC neurogenesis plays in seizure-induced plasticity, we injected retroviral (RV) reporters to label dividing DGC progenitors at specific times before or after SE, or used X-irradiation to suppress neurogenesis. RV injections 7 weeks before SE to mark DGCs that had matured by the time of SE labeled cells with normal placement and morphology 4 weeks after SE. RV injections 2 or 4 weeks before seizure induction to label cells still developing during SE revealed normally located DGCs exhibiting hilar basal dendrites and mossy fiber sprouting (MFS) when observed 4 weeks after SE. Cells labeled by injecting RV after SE displayed hilar basal dendrites and ectopic migration, but not sprouting, at 28 days after SE; when examined 10 weeks after SE, however, these cells showed robust MFS. Eliminating
cohorts of newborn DGCs by focal brain irradiation at specific times before or after SE decreased MFS or hilar ectopic DGCs, supporting the RV labeling results. These findings indicate that developing DGCs exhibit maturation-dependent vulnerability to SE and suggest that abnormal DGC plasticity derives exclusively from aberrantly developing DGCs. Treatments that restore normal DGC development after epileptogenic insults may therefore ameliorate epileptogenic network dysfunction and associated morbidities.

**Introduction**

Medial temporal lobe epilepsy (mTLE) is often associated with pharmacoresistant seizures and co-morbidities of cognitive dysfunction and depression (Mendez et al., 1986; Helmstaedter et al., 2003; Elger et al., 2004). In addition to neuronal loss and gliosis, human and experimental mTLE pathology involves prominent dentate granule cell (DGC) abnormalities, including mossy fiber sprouting (MFS), DGCs with abnormal dendrites, and dispersed or ectopically located DGCs (Tauck and Nadler, 1985; de Lanerolle et al., 1989; Houser et al., 1990; Mello et al., 1993; Parent et al., 1997; Spigelman et al., 1998; Buckmaster and Dudek, 1999; Ribak et al., 2000; Scharfman et al., 2000; Dashtipour et al., 2001). DGC abnormalities are associated with network hyperconnectivity that may impact mTLE pathogenesis. Timm staining, ultrastructural studies and electrophysiological evidence from human or experimental mTLE, for example, suggest that mossy fibers form recurrent excitatory synapses onto neighboring DGCs [reviewed in (Sutula and Dudek, 2007)].
In terms of abnormal dendrites, hilar basal dendrites are normally present only transiently on developing rodent DGCs but persist chronically after chemoconvulsant-induced status epilepticus (SE). Hilar basal dendrites in epileptic rats develop mature synapses with ultrastructural evidence of excess excitatory input (Ribak et al., 2000). Hilar ectopic DGCs also arise after SE (Parent et al., 1997). These cells aberrantly integrate into the local circuitry, receive excess excitatory input, exhibit abnormal bursting, and are recruited during spontaneous seizures (Scharfman et al., 2000; Dashtipour et al., 2001; Scharfman et al., 2002).

Although DGC abnormalities in mTLE are proposed to arise from abnormal plasticity of mature granule neurons, mounting evidence suggests that epileptogenic insults influence developing or newborn DGCs. For example, many hilar ectopic or basal dendrite-containing DGCs found after pilocarpine-induced SE in rats are adult-born (Parent et al., 1997; Scharfman et al., 2000; Parent et al., 2006b; Jessberger et al., 2007; Walter et al., 2007). DGCs born after SE, however, have been thought to not contribute to MFS because suppression of neurogenesis after pilocarpine treatment with low-dose irradiation does not inhibit MFS at 4 weeks after SE (Parent et al., 1999). One interpretation of these data is that mature DGCs are responsible for MFS, but other work suggests that developing DGCs generated several weeks before SE can sprout (Jessberger et al., 2007).

We sought to determine the precise developmental stages at which DGCs are vulnerable to aberrant plasticity found in human and experimental mTLE. We labeled dividing DGC progenitors with retroviral (RV) reporters or transiently suppressed neurogenesis before or after SE using low-dose X-irradiation. We found that the
developmental stage of DGCs at the time of SE dictates their contribution to seizure-induced plasticity. DGCs still developing during SE or those generated after SE, but not mature DGCs, differentially contribute to the cell populations that develop MFS, hilar basal dendrites or ectopic migration. These findings indicate that seizure-induced DGC plasticity results from altered development, and that DGCs exhibit maturation-dependent vulnerability to specific SE-induced pathologies. Restoring normal DGC development after epileptogenic insults may therefore ameliorate epileptogenic network dysfunction and associated cognitive and mood-related morbidities.

Materials and Methods

Animals: All animal procedures were performed in accordance with protocols approved by the University Committee on Use and Care of Animals of the University of Michigan. Animals were purchased from Charles River and kept under a constant 12 h light/dark cycle with access to food and water ad libitum.

Pilocarpine-Induced SE: Adult male Sprague-Dawley rats (postnatal day [P] 56) were pretreated with atropine methylbromide (5 mg/kg intraperitoneally [IP]; Sigma, St. Louis, MO), and 15-minutes later were given pilocarpine hydrochloride (340 mg/kg IP; Sigma) to induce SE. If convulsive seizure activity was not initiated within 1 hour of the initial pilocarpine hydrochloride dose, an additional dose of 170 mg/kg was given. Seizures were monitored behaviorally, and then terminated with diazepam (10 mg/kg) after 90 minutes of SE as previously described (Parent et al., 2002; Parent
et al., 2006b). Controls received the same treatments as experimental animals except that they were given saline in place of pilocarpine.

**Production of high titer RV:** Replication-incompetent recombinant RV vectors were pseudotyped by co-transfection of GP2-293 packaging cell line (Clontech, Mountain View, CA) with plasmids containing the RV vector (RV-CAG-GFP-WPRE, gift of S. Jessberger and F. Gage) (Jessberger et al., 2007) and vesicular stomatitis virus (VSV)-G envelope protein (Clontech). Transient transfection was carried out using calcium phosphate precipitation in 10-cm dishes after 5x10^6 cells/dish were plated in 10 ml of DMEM supplemented with 10% FBS the day prior to transfection. The cells were incubated at 37°C for 6 hours, the medium was replaced and then the cells were cultured for 65 h. The supernatant containing RV was harvested and filtered through a 0.45-µm pore size filter (Gelman Sciences, Ann Arbor, MI). The filtered supernatant was then centrifuged in a Sorvall model RC 5C PLUS at 50,000xg at 4°C for 90 minutes. The RV-containing pellet was resuspended in 1X PBS (~0.003X the volume of medium initially harvested), aliquoted, and subsequently stored at -80°C until use. The concentrated RV titer was determined using NIH 3T3 cells and found to be approximately 1-5 x 10^8 CFU/ml.

**Intrahippocampal RV Injections:** Animals were anesthetized with a ketamine/xylazine mixture and placed on a water-circulating heating blanket. After positioning in a Kopf stereotaxic frame, a midline scalp incision was made, the scalp reflected by hemostats to expose the skull, and bilateral burr holes drilled. RV vector
(2.5 μl of viral stock solution/site except for P7 pups, where 1 μl virus/site was injected) was injected into the left and right dentate gyri over 20 minutes each using a 5 μl Hamilton Syringe, and the micropipette left in place for an additional 2 minutes. Coordinates for injections (in mm from Bregma and mm depth below the skull) were: at P7 - caudal 2.0, lateral 1.5, depth 2.7; at P28 – caudal 3.5, lateral 2.1, depth 3.8; at P42 – caudal 3.8; lateral 2.3, depth 4.2; at P60 - caudal 3.9; lateral 2.3, depth 4.2.

**Brain Irradiation:** Irradiation was done using an IC-320 Specimen Irradiation System (Kimtron Medical, Woodbury, CT) operated at 300 kilovolts and 10 mA. Rats were anesthetized with ketamine and xylazine, placed in sternal recumbency on a treatment table, and irradiated individually. The radiation dose was centered in a 2 cm x 2 cm treatment field that covered the hippocampi, with the remainder of the head, neck and body protected by lead shielding. The corrected dose rate was 1.25 Gy/minute. Dosimetry was carried out using an ionization chamber connected to an electrometer system that is directly traceable to a National Institute of Standards and Technology calibration. Rats received a 6 Gy total irradiation dose given in 2 fractions over 3 days. Sham-irradiation controls were handled, but not irradiated.

**Tissue Processing, Histology and Immunohistochemistry:** Either 4 or 10 weeks after SE, animals were deeply anesthetized and perfused with 4% paraformaldehyde (PFA). The brains were removed, postfixed for 4-6 hours in 4% PFA, cryoprotected in 30% sucrose and frozen. For some animals, a 0.37% sulphide fixative was also used for subsequent Timm staining. Coronal sections (40 μm thick) were cut with a
freezing mictrotome and peroxidase and fluorescence (single and double-label) immunohistochemistry performed on free-floating sections (Parent et al., 1997; Parent et al., 1999). Sections were immunostained with the following antibodies: goat anti-doublecortin (Dcx) (1:1000, Santa Cruz, Santa Cruz, CA), rabbit (Rb) anti-Prox1 (1:10,000- gift of S. Pleasure, UCSF), Rb or mouse (Ms) anti-GFP (1:1000 or 1:250, respectively, Molecular Probes, Carlsbad, CA), Ms anti-BrdU (1:1000, Roche, Indianapolis, IN) or Ms anti-NF-M (1:500, Chemicon, Billerica, MA). MFS was examined by Timm staining. Briefly, coronal sections were mounted onto superfrost plus slides (Fisher, Pittsburgh, PA) and then dehydrated, rehydrated and placed in a staining solution containing gum arabic, citrate, hydroquinone and silver nitrate and incubated at 26°C (Parent et al., 1999). After development of the stain, sections were washed, dehydrated and delipidated in graded ethanol and xylenes, and coverslipped with Permount (Sigma).

**Epifluorescence and Confocal Microscopy:** Single-label images were captured using a Leica DSMIRB epifluorescence microscope attached to a SPOT-RT digital camera. For double-labeling, images were acquired using a Zeiss LSM 510 confocal microscope as 1 μm thick z-series stacks, imported into Adobe Photoshop, and analyzed for co-localization of immunoreactivity.

**Cell quantification after RV reporter injections:** All quantification was performed blind to experimental condition. SE and control groups at all RV reporter injection time points were examined for GFP-immunoreactivity. A total of 4,978 GFP-
expressing granule cells were counted (at least 100 cells from at least 3 sections were counted per animal). Only cells with clearly demarcated, brightly labeled cell bodies were included. Cells in the DGC were only counted if properly oriented (perpendicular to the granule cell layer) in order to unambiguously identify hilar basal dendrites. Each cell was analyzed for the presence of a hilar basal dendrite (using criteria of extension into the hilus and presence of clear spines, as in (Dashtipour et al., 2003), and for ectopic location, defined as greater than or equal to 2 cell body widths outside the granule cell layer (n=3 rats for each control group and n=4-5 for each experimental group). For Timm staining analysis, a minimum of 3 sections, spanning the rostral, mid, and caudal dentate gyrus, were examined by densitometry. Pixel density from ten sampled areas/section were measured in the inner molecular layer using NIH ImageJ, 5 from the lower blade and 5 from the upper blade. The average background level (average of 5 samples/section) was subtracted and an average mean pixel density calculated for each animal (n=4-5 per experimental group, n=6 for each control group). Animals with severely damaged or missing superior blades were excluded from analysis. Statistical comparisons between groups for irradiation and RV studies were made using an ANOVA with post-hoc t-tests. For animals that were processed 10 weeks post-SE, ImageJ was used to determine the percent Timm positive area in the lower blade as previously described (Buckmaster and Dudek, 1997) because many animals at this time point had damaged suprapyramidal blades. To accomplish this, the lower blade granule cell layer and molecular layer were carefully outlined and the percent area stained was calculated in control animals that did not receive irradiation (n=6) and experimental animals that were irradiated post-SE (n=4). A minimum of
three slices were averaged per animal, and a two-tailed student’s t-test was performed to compare the two groups. Data are presented as means ± SEM with the significance level set at p<0.05.

Results

Dentate granule cells born 2-4 weeks before or 4 days after SE form hilar basal dendrites, whereas only DGCs born after SE migrate ectopically

Evidence suggests that both mature and immature or newborn DGCs contribute to seizure-induced plasticity in adult rodents (Parent et al., 1999; Overstreet-Wadiche et al., 2006; Jessberger et al., 2007; Walter et al., 2007). Whether the maturity of DGCs restricts the types of aberrant reorganization they undergo after SE, however, remains unclear. To better determine how cellular maturity influences DGC plasticity induced by SE, we used a RV reporter to label dividing DGC progenitors and their progeny at specific timepoints before or after SE. This approach offers the advantage of precise temporal control for labeling DGC progenitors, as RV selectively transduces mitotically active cells during S phase, and VSV-G-pseudotyped RV has a half-life of about 4.5 hours at 37°C (Higashikawa and Chang, 2001). Spatial control was achieved by targeting the stereotactic RV reporter injections to the dentate gyrus.

We first focused on DGCs with hilar basal dendrites as prior studies using biocytin cell fills or Golgi stains demonstrated that many more granule neurons with hilar basal dendrites are found after SE than in controls (Spigelman et al., 1998; Buckmaster and Dudek, 1999; Yan et al., 2001). GFP-expressing RV was injected into the dentate gyrus of P7 rat pups and SE was induced with pilocarpine seven weeks
later at P56 (Figure 2.1A). Thus, labeled cells were post-mitotic for about 7 weeks by the time of SE. Animals survived for 4 weeks after pilocarpine-induced SE (P84). Because P7 is near the peak of postnatal neurogenesis, hundreds of progenitors were GFP-labeled and matured into DGCs over the subsequent 7 weeks (Figure 2.1B, 2.1J). However, labeled DGCs showed no structural abnormalities at 1 month after SE, appearing essentially identical to labeled cells in saline treated controls (Figure 2.1B, 2.1F, 2.1J, 2.1N). In contrast, many DGC progenitors labeled at all other time points, including 4 weeks or 2 weeks before SE, or 4 days after SE, showed abnormal hilar basal dendrites at 28 days after pilocarpine treatment (arrowheads in Figure 2.1K-M, O-Q) that were not found in controls (Figure 2.1C-E, G-I). A small subset of DGCs born at P28 (4-weeks-old at SE) showed robust hilar basal dendrites (arrowheads in Figure 2.1K, O), while many born on P42 (2-weeks-old at SE) or P60 (4 days post-SE) had extensive and very long hilar basal dendrites (arrowheads in Figure 2.1L, M, P, Q; Figure 2.2). Saline treated controls receiving RV injections at the 3 later time points rarely had DGCs with hilar basal dendrites (Figure 2.1C-E, 2.1G-I; Figure 2.2).

When we quantified the percentage of GFP-labeled DGCs with hilar basal dendrites, we found that four-week-old DGCs (P28 RV injection) exposed to pilocarpine-induced SE were nearly 3 times more likely to show hilar basal dendrites than DGCs that were fully mature (7-week-old) at the time of SE (13.88 ± 2.39% for 4-week old cells vs. 5.10 ± 1.90% for 7-week old cells) (Figure 2.3A). This finding was even more dramatic for cells born 2 weeks before SE, which were about 6.5 times more likely to form hilar basal dendrites, and cells born 4-days post-SE, which were almost 7 times more likely to form basal dendrites than P7-injected rats (Figure 2.3A).
Animals injected with RV at P28, P42 and P60 that experienced SE also showed significantly greater numbers of DGCs with hilar basal dendrites than their respective control groups (Figure 2.3A). However, mature granule cells (7-weeks old at SE) exposed to SE did not differ from their age-matched controls (Figure 2.3A; p=0.25).

Previous work suggests that hilar-ectopic DGCs that appear after SE in adult rodents derive from newborn DGCs (Parent et al., 1997; Scharfman et al., 2000; Jung et al., 2004; Parent et al., 2006b; Jessberger et al., 2007; Walter et al., 2007), but other findings raise the possibility that mature DGCs migrate ectopically (Heinrich et al., 2006; Nitta et al., 2008). We therefore examined whether GFP-positive DGCs labeled by RV reporter injection at the time points described above appeared in the hilus 4 weeks after SE. RV-GFP labeling of DGC progenitors at P7 that were mature during SE at P56 failed to show GFP-positive ectopic DGCs in the hilus (Figure 2.1J, N). Similarly, DGCs labeled with RV at P28 or P42 and still differentiating at the time of SE did not migrate to the hilus (Figure 2.1K, L, O, P). Examination of DGCs born 4 days after SE (P60 injections), however, revealed many GFP-labeled cells in the hilus at 4 weeks after SE (Figure 2.1M, Q, arrows). These ectopic cells had a typical DGC morphology but a substantial portion exhibited abnormal dendrites similar to GFP-positive cells with hilar basal dendrites in the DGC layer. The ectopic cells were not included in counts of hilar basal dendrites, however, because the cell bodies were already located in the hilus. Quantification of ectopic GFP-labeled cell numbers showed that only cells labeled after SE were significantly more likely to migrate to the hilus (Figure 2.3B). Thus, DGCs generated after SE show multiple structural
abnormalities that likely lead to aberrant integration and marked disorganization of dentate gyrus circuitry (compare panels E and M in Figure 2.1).

**Effect of DGC maturity on MFS at 4 weeks after SE**

The sprouting of granule cell axons, or MFS, is a pathological feature of human mTLE that is replicated by pilocarpine-induced SE and other rodent mTLE models (Sutula et al., 1989; Houser et al., 1990; Mello et al., 1993; Okazaki et al., 1995). Because our RV reporter clearly labels DGC axons, we were able to examine how DGC maturity influences MFS after SE.

In the first series of experiments, we made all observations at P84, 4 weeks after SE (Figure 2.1A). Using this timepoint, we found that similar to non-seizure controls (Figure 2.4E and data not shown), GFP-labeled cells in rats that received RV at P7 and underwent SE at P56 had axons that projected properly into the hilus and towards stratum lucidum of area CA3 when observed 4 weeks after pilocarpine treatment (Figure 2.4A, arrows). In animals receiving RV 2 weeks before SE, in contrast, occasional cells with axons projecting aberrantly into the molecular layer were seen 4 weeks after SE (Figure 2.4B, arrowheads). Rats that received RV-GFP injections 4 weeks before pilocarpine treatment frequently exhibited cells with GFP-labeled processes in the DGC layer and extending into the molecular layer that had an axonal morphology (Figure 2.4F, arrowheads) or small, bouton-like structures (data not shown).

To confirm the axonal nature of the processes, we double-labeled for GFP and NF-M, a medium chain neurofilament that specifically labels axons, but not dendrites,
in the hippocampus (Parent et al., 1997). Confocal microscopy was performed to assess co-localization of immunostaining. Double-labeled axons extending from the cell body and projecting into the inner molecular layer were observed at this time point (arrows in Figure 2.4C, D), further supporting the idea that DGCs generated 2 or 4 weeks before SE project mossy fibers aberrantly at 4 weeks after SE. No processes double labeled for GFP and NFM appeared in the molecular layer of rats injected with RV-GFP at P7 or P60 when observed 28 days after seizure (data not shown), suggesting that only cells born several weeks before and still differentiating at the time of SE significantly contributed to the MFS seen 4 weeks after SE. These data are the first evidence that only developing, and not mature, DGCs contribute to MFS, and are also consistent with our previous finding that ablating DGC progenitors after SE does not suppress MFS 4 weeks following pilocarpine treatment (Parent et al., 1999).

**Transient suppression of neurogenesis at different times with respect to SE attenuates specific forms of aberrant plasticity**

As a complementary approach to the RV reporter labeling, we sought a means to transiently suppress DGC neurogenesis before or after SE and determine the effects on aberrant seizure-induced plasticity. Because proliferating DGC progenitors are very susceptible to ionizing irradiation (Parent et al., 1999; Tada et al., 2000) we chose to use low-dose irradiation to accomplish the suppression. We previously irradiated with a 10 Gray (Gy) dose given in two fractions 1 day before and 4 days after SE to show that DGCs born after SE were not necessary for MFS 4 weeks later (Parent et al., 1999). Here we used a lower fractionated dose to transiently suppress neurogenesis for
only a few weeks. In pilot studies comparing 4, 6 and 9 Gy given over 2 or 3 days, we found that 6 Gy given in 2 divided 3-Gy doses spaced one day apart suppressed DGC neurogenesis for about 2-3 weeks when given at either P21 and P23 or P56 and P58, as demonstrated by loss of proliferating cells and DCx-immunoreactive neuroblasts (Figure 2.5 and data not shown). Both cell proliferation and DCx expression returned to baseline levels at 4 weeks after irradiation (Figure 2.5).

We next administered 6 Gy fractionated radiation doses to rats at specific times before or after pilocarpine-induced SE to transiently suppress neurogenesis. Analogous to the RV reporter studies above, we wanted to deplete (instead of label) cohorts of developing DGCs that would otherwise have been a specific age at the time of SE, or those born after SE. Thus, we irradiated or sham irradiated rats at P21 and P23, P42 and P44, or P60 and P62. Animals received pilocarpine at P56, and were sacrificed 4 weeks after SE at P84 (Figure 2.6A), the same time point used in our prior study of irradiation and mossy fiber sprouting (Parent et al., 1999). Radiation at all time points before SE did not impact the latency or severity of the SE episode, although a slightly greater proportion of animals died in irradiated compared to sham-irradiated groups.

We examined MFS at 4 weeks after pilocarpine treatment by Timm stain in irradiated and sham-irradiated pilocarpine-treated rats. Compared to sham-irradiated controls experiencing SE, supragranular Timm staining was visibly reduced by 6 Gy X-irradiation beginning on P21, but not by irradiation beginning at P42 or after SE at P60 when examined 4 weeks after SE induction (Figure 2.6B-F, black arrows). When we quantified these data using average pixel density units (PDU), we found that
animals irradiated at P21, but not P42, had significantly less MFS at 4 weeks post-SE (Figure 2.6G; P21/23 rad: 48.12 ± 5.09 PDU; P42/P44: 69.85 ± 10.40 PDU, control: 74.96 ± 7.59 PDU, p<0.05 for P21/23 vs. control). Animals irradiated after SE (P60/62) did not experience any decrease in MFS at 28 days post-SE (P60/P62: 88.18 ± 13.05 PDU, control: 74.96 ± 7.59 PDU) similar to prior results using a higher radiation dose (Parent et al., 1999). Our findings are consistent with the RV-GFP labeling data, as neurogenesis after 6 Gy fractionated irradiation at P21 should be inhibited for several weeks before returning to baseline levels by about P49 (Figure 2.5A-E). Taken together, these data suggest that late differentiating (~3-5-week-old) DGCs, but not those born during the 2 weeks before or for several weeks after SE, generate much of the MFS seen at 28 days after pilocarpine treatment. Therefore, a certain level of maturity (late differentiating) is required for cells to sprout mossy fibers within the first 4 weeks after SE.

To further test the hypothesis that DGCs born after SE migrate ectopically, we immunostained for the DGC-specific marker Prox1 to examine hilar ectopic DGCs in rats receiving 6 Gy fractionated irradiation or sham irradiation on days 4 and 6 (P60/62) after SE. Irradiated animals showed a marked reduction of hilar Prox-1-immunoreactive cells after SE compared to pilocarpine-treated, sham-irradiated controls (Figure 2.7). These results are consistent with our RV-GFP findings and indicate that hilar ectopic DGCs arise from progenitors dividing after SE.
Cells born after SE show aberrant MFS with longer-term (10 week) survival after SE

Previous studies have demonstrated the progressive nature of MFS, with a peak at approximately 100 days after pilocarpine-induced SE (Mello et al., 1993). The results of our initial RV labeling and irradiation experiments, however, suggest that a fairly narrow time window exists during which developing, but not fully mature or newborn, cells respond to SE by sprouting aberrantly. These findings seem inconsistent with the evidence that MFS increases for several months after SE. We therefore reasoned that the 28 day post-pilocarpine treatment time point at which we examined GFP labeling and MFS may not have provided sufficient time to observe sprouting from cells generated after SE. Thus, we performed additional experiments in which adult rats underwent pilocarpine-induced SE, were either injected with RV reporter or irradiated 4 days later, and then survived for 10 weeks (instead of 4 weeks).

In the first set of experiments, rats treated with saline or pilocarpine on P56 were injected with RV-GFP on P60 and their dentate gyri analyzed for GFP expression on P126 (10 weeks after SE). Saline-treated animals, as expected, showed many GFP-labeled axons in the hilus but none in the molecular layer (Figure 2.8A). Rats that underwent SE on P56, however, showed many horizontally aligned, GFP-immunoreactive processes in the inner molecular layer with the same axonal appearance and small mossy fiber bouton-like structures as those seen in the hilus (arrows in Figure 2.8B, C). This finding contrasted with our earlier experiments in which rats experiencing SE on P56 and RV-GFP injections on P60 showed no GFP-positive axons in the inner molecular layer by P84 (data not shown). These data indicate that DGCs generated after SE require more than 24 days to respond to cues in
the post-SE environment by sending axons aberrantly into the dentate inner molecular layer; a duration of 10 weeks after SE, in contrast, is sufficient for the DGCs newly generated after SE to exhibit robust MFS.

In the second set of experiments, we again used X-irradiation to transiently suppress neurogenesis 4 and 6 days (P60 and P62) after inducing SE on P56 with pilocarpine. This time, however, Timm staining was assessed after a 10-week survival. Controls experienced SE and then sham irradiation. In sham-irradiated controls, we found dense MFS (Figure 2.8D) with much darker inner molecular layer Timm staining than in similarly sham-irradiated controls at 4 weeks after SE (compare Figure 2.8D with Figure 2.6C). Unlike the earlier experiments in which MFS at 4 weeks after SE was not decreased by post-SE irradiation, however, animals that were irradiated 4 and 6 days after pilocarpine treatment and survived 10 weeks showed a less dense band of inner molecular layer MFS than the respective sham-irradiated controls (Figure 2.8D, E). Densitometric quantification of Timm staining in the inferior blade at 10 weeks after SE (Figure 2.8F) showed that controls had an average of 46.7 ± 2.7% of the inferior blade granule cell and molecular layers positive for Timm staining, while animals irradiated at P60 and 62 after SE had significantly less granule cell and molecular layer Timm staining, with a mean of 30.2 ± 1.4% (p<0.005). Together with the irradiation and RV reporter studies described above, these findings provide strong evidence that DGCs generated after SE contribute to MFS occurring between 4 and 10 weeks after pilocarpine treatment.
Discussion

In this study, we labeled DGC progenitors in neonatal, juvenile or adult rats with retroviral reporters such that the cells would be at selected stages of maturity during pilocarpine-induced SE or be born after SE. We also depleted DGC progenitor cohorts by irradiating at specific timepoints before or after SE. We found that DGCs born 7 weeks before SE were resistant to injury and exhibited no abnormal plasticity at 4 weeks after pilocarpine treatment. RV-GFP-labeled DGCs that were 4 weeks old at SE developed hilar basal dendrites and MFS when examined 4 weeks after SE, and those generated 2 weeks before pilocarpine treatment showed substantial hilar basal dendrites and some MFS. Many DGCs generated after SE, in contrast, migrated ectopically and developed marked hilar basal dendrites, but did not show MFS, at 4 weeks after SE. When observed 70 days after SE, however, cells born 4 days following pilocarpine treatment showed robust MFS. Along with the irradiation experiments, the data suggest a model in which DGCs show a maturation-specific vulnerability to SE-induced plasticity (Figure 2.9). Mature cells are resistant to aberrant plasticity. Late-differentiating, “juvenile” DGCs (~3-5 weeks old) respond to injury with MFS and to a lesser extent hilar basal dendrite formation. DGCs that are more immature (≤ 2 weeks old) at the time of SE show less MFS but pronounced basal dendrites. Finally, DGCs born after SE develop extensive basal dendrites and migrate ectopically to the hilus by 28 days following SE, and also show robust MFS by 70 days. Thus, the repertoire of plasticity in response to this epileptogenic insult appears restricted to specific critical periods in DGC development.
Interestingly, many GFP-labeled cells appeared normal in all groups receiving RV-GFP injections before SE. The proportion of GFP-positive DGCs that either migrated ectopically or showed hilar basal dendrites after SE was on average 5.6% of those labeled at P7, 14% of those labeled at P28, and 33.7% of DGCs labeled at P42 (vs. 0-5.9% for the 4 control groups). When RV was administered after SE (P60), however, the majority of DGCs appeared abnormal (57.4%) when observed 4 weeks following pilocarpine treatment. A single RV injection labels only a small subset of DGCs compared to the total number generated after SE (Parent et al, 1997); therefore, very large numbers of DGCs born after SE probably develop abnormally. Our results also underestimate the degree of abnormal dendrite formation of RV-labeled cells because only DGCs with normal cell body orientation were included. We excluded misoriented cells to identify basal dendrites extending into the hilus unequivocally. Furthermore, dendrites present on ectopic cells were excluded, as they were already located in the hilus. Others have demonstrated that aberrantly developing adult-born neurons stably integrate into the dentate circuitry (Scharfman et al., 2000; McCloskey et al., 2006) and that altered neurogenesis after SE is long lasting, with ectopic cells forming for many months after seizure induction (Rao et al., 2006b; Jessberger et al., 2007). Thus, the extent of SE-induced neurogenesis abnormalities is likely even more severe than is suggested by our RV labeling.

The finding of MFS only by developing or newborn, and not mature, DGCs has important mechanistic implications for understanding this form of plasticity. It also helps resolve some of the controversial findings from our previous work and studies by others. We originally reported that newborn cells contributed to MFS at 35
days after SE based on double labeling for BrdU and an axon-specific marker (Parent et al., 1997). This led us to hypothesize that newborn cells played a large role in MFS. However, we subsequently demonstrated that ablating newborn cells did not alter MFS, refuting our original hypothesis (Parent et al., 1999). Our current findings indicate that this refutation was incorrect because the four week post-SE timepoint studied in the irradiation experiments was too early to observe MFS by DGCs generated after SE. Our current data instead indicate that a developmental window exists for MFS, and that cells born in the first few weeks after SE contribute to inner molecular layer sprouting by 70 days, but not within 28 days, following pilocarpine treatment.

Rather than recapitulating developmental, SE-induced MFS appears to involve alterations of ongoing development. The timing of MFS from a given DGC in relation to that particular cell’s maturity fits with the idea that DGCs develop vulnerability to a persisting SE-induced signal when they reach 3-5 weeks of age. They respond by aberrantly sprouting axons, and older DGCs appear incapable of responding to these signals. Alternatively, immature DGCs may attain responsiveness to a “sprouting signal” at an earlier age but take longer to sprout. This explanation is unlikely given that adult-born DGCs send mossy fiber axons to area CA3 within a week of birth (Hastings and Gould, 1999; Markakis and Gage, 1999). The idea that successive generations of adult-born DGCs sprout as they develop is consistent with MFS progression for several months after SE (Mello et al., 1993). In fact, the timing of transiently increased neurogenesis for 2-3 weeks after the initial seizures (Parent et al., 1997) followed by a potential chronic suppression of neurogenesis (Hattiangady et al.,
fits well with a model in which most or all newborn DGCs eventually sprout, leading to a peak in MFS at 2-3 months after SE.

Our data are consistent with another study that found no MFS from DGCs labeled after SE by RV-GFP injection when hippocampi were examined 4 weeks later (Jessberger et al., 2007). We also confirmed our previous finding that post-SE irradiation did not suppress MFS 4 weeks later (Parent et al., 1999). As in the current study, one group showed that RV-GFP-labeled DGCs that were 4 weeks old at the time of SE contributed to MFS (Jessberger et al., 2007). In contrast, this group did not observe sprouting from DGCs labeled with RV-GFP one week after SE and examined a year later, whereas we found robust MFS from DGCs born after SE when assayed after 10 (but not 4) weeks. They also observed that RV reporter injections 1 week before SE did not label DGCs with morphological alterations. Although we did not inject RV reporter at 1 week before SE, we found that many DGCs born 2 weeks before or 4 days after SE developed hilar basal dendrites. Several possibilities may explain these discrepancies, including our use of a different rat strain and of pilocarpine rather than kainic acid. An unlikely possibility is that the MFS we found from RV-GFP-labeled cells 10 weeks after SE may not persist for a full year. Our hilar basal dendrite findings are similar to data generated in a study of Thy1-GFP transgenic mice showing that DGCs born several months before SE do not develop hilar basal dendrites, while many DGCs that are immature during SE do form them (Walter et al., 2007).

We found that irradiation before SE does not change the latency to or severity of SE, but increases SE mortality. This effect probably reflects a tendency for
irradiated rats to have more discrete generalized convulsive seizures. Potential non-specific damage to the dentate gyrus from ionizing irradiation is thus one possible limitation. We therefore interpret the data in the context of our RV reporter labeling strategy, thus combining findings from progenitor labeling and ablation methods to reach our conclusions. We also did not monitor seizure frequency. It will be interesting to determine whether transiently suppressing neurogenesis with low-dose irradiation at specific time points chronically suppresses SE-induced structural abnormalities, and whether this will also attenuate epileptogenesis.

Accumulating data supports the hypothesis that MFS, hilar ectopic DGCs, and DGCs with hilar basal dendrites contribute to epileptogenesis (Tauck and Nadler, 1985; Ribak et al., 2000; Scharfman et al., 2000; Buckmaster et al., 2002; Scharfman et al., 2002; Scharfman et al., 2003; Jung et al., 2004; Jung et al., 2006; Morgan and Soltesz, 2008b). The overall contribution of adult-born DGCs to epileptogenesis, however, remains unclear. Adult-born DGCs integrating after SE appear to have complex influences, as recordings from RV-GFP-labeled DGCs in the granular layer after electrically-induced SE suggest that they show reduced excitability (Jakubs et al., 2006). These effects may relate to the substantial proportion of RV-GFP-labeled DGCs that appear to integrate normally at the time points we examined using the pilocarpine model, although electrophysiological studies are needed to determine whether reduced excitability of adult-born DGCs also occurs in this model. In support of an epileptogenic role of seizure-induced neurogenesis, one study showed that DGC progenitor ablation with mitotic inhibitors exerts partial anti-epileptogenic effects after SE (Jung et al., 2004). Future work should aim to determine the functional
implications of suppressing neurogenesis before or after SE by correlating morphological abnormalities with seizures, although such studies may be complicated because ablation methods suppress both aberrant and potentially compensatory neurogenesis. Because the birth of DGCs that migrate ectopically or integrate normally likely temporally overlap, a potential challenge is the ability to ablate one of these populations without also suppressing the other. Interventions that normalize, rather than suppress, neurogenesis should be ideal in this regard.
Notes to Chapter 2

1 A modified version of this chapter was published as: Kron MM, Zhang H, Parent JM (2010) The developmental stage of dentate granule cells dictates their contribution to seizure-induced plasticity. J Neurosci 30:2051-2059.

Helen Zhang produced all viruses and aided in development of stereotactic injection protocol.

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Figure 2.1. RV-GFP reporter labeling of DGCs generated at specific time points before or after SE. A, Timeline for RV labeling of newborn cells in the dentate gyrus born before or after SE. B-Q, Images of GFP-immunoreactive cells after injection of RV at the designated postnatal ages (P7, 28, 42 or 60) in rats receiving saline (B-I) or pilocarpine (J-Q) at P56. Higher magnification views of RV-GFP-labeled DGCs show granule cells with hilar basal dendrites (arrowheads) or hilar ectopic DGCs (arrows) only at specific RV labeling timepoints (P28, 42 or 60) with respect to SE at P56. ml, molecular layer; gcl, granule cell layer; h, hilus. Scale bars: 50 µm (in B) for B-E, J-M; 25 µm (in F) for F-I, N-Q.
Figure 2.2 Spines are present on apical and hilar basal dendrites, but not axons.

A C, Apical dendrites with evident spines (arrows) in control rats injected with RV-GFP at P42 (A) or P60 (C), given saline at P56, and perfused at P84. The axon is evident for one of the cells (arrowhead in C). B, D, Animals injected with RV-GFP at P42 (B) two weeks prior to SE at P56, or 4 days after SE at P60 (D) exhibit apical and basal dendrites with evident spines (arrows). The molecular layer is at the top and bottom is towards the hilus for each panel. Scale bar: 15 μm.
Figure 2.3. Quantification of the percentage of RV-GFP-labeled DGCs with hilar basal dendrites or ectopic migration to the hilus. 

A, Hilar basal dendrites were analyzed in GFP-positive cells in the DGC layer. The percentage of DGCs with hilar basal dendrites labeled with RV-GFP at P7 that were mature at the time of SE (P56) was not significantly increased compared to controls (p= 0.25), but cells labeled by RV injections at the three other time points before or after SE did show significantly greater proportions with hilar basal dendrites (*, p<0.005 for all groups vs. controls; #, p<0.05 for P28, P42 and P60 vs. P7 RV).

B, A significantly increased number of hilar ectopic GFP-labeled DGCs were present compared to controls only when RV was injected after SE at P60. *, p<0.005 for the P60 group vs. P60 control.
Figure 2.4. Only DGCs still developing and not fully mature at SE display MFS. 

A, Representative image of DGCs labeled with RV-GFP at P7 that have axons extending normally into the hilus (h; arrows) 4 weeks after experiencing SE on P56, with no GFP-labeled axons in the inner molecular layer (iml).  

B, P42 RV-GFP injection labeled a DGC 2 weeks old at SE with GFP+ axon extending through the granule cell layer (gcl) into the iml (arrowheads).  

C-D, GFP/NFM double-labeled axon extending from the cell body towards the iml (arrows) 4 weeks after SE in an animal that received RV injection at P28.  

E, P28 RV-GFP injection into a saline-treated control rat labeled axons only in the hilus.  

F, P28 RV-GFP injection into an animal that subsequently experienced pilocarpine-induced SE on P56 labeled axonal sprouts in the iml (arrowheads) 4 weeks after SE. Scale bar: 75 μm for A, B, E, F; 40 μm for C, D.
Figure 2.5. Low-dose ionizing irradiation transiently suppresses DGC neurogenesis. A-C, Doublecortin (DCx) immunostaining of immature neurons in the DG of rats irradiated (rad) beginning on P21 (P21/23) shows decreased neurogenesis at 7 d (B, arrows) vs. control (A), and recovery 28 d after rad (C). D, E, BrdU labeling shows cell proliferation also recovers 4 weeks after rad at P21/23 (E) vs. control (D). BrdU was given 2 hrs before sacrifice on P49. F-J, Neurogenesis examined by DCx immunolabeling is also transiently suppressed after rad beginning on P56 (P56/58), with knockdown after 2 (G, arrows) and 7 d (H, arrows), partial recovery at 14 d (I, arrows) and nearly total recovery to control levels (F) after 28 d (J). Scale bar: 100 μm.
Figure 2.6. Effect of irradiation before or after SE on MFS. A. Timeline for irradiation studies. Hatched areas show predicted timing of suppressed neurogenesis after 6 Gy X-irradiation (rad). B-F. Representative images of dentate gyrus Timm stain (arrows) in a naïve control (B), a control receiving sham rad with SE (C) and rats receiving rad at specific timepoints before or after SE (D-F). Higher magnification views are shown in insets. G. Quantitative analysis of pixel density revealed that 6 Gy fractionated irradiation administered 5 weeks before SE (P21) significantly decreased the amount of MFS (*, p<0.05). Irradiation either 2 weeks before or 1 week after SE did not have a significant effect on MFS at 4 weeks after SE. ml, molecular layer; gcl, granule cell layer; h, hilus. Scale bars: 100 μm for B-F, 25 μm for insets.
Figure 2.7. Effect of irradiation (rad) given after SE on hilar ectopic DGCs. 
A, A’, Prox1 immunostaining 28 d after SE (on P56) shows many hilar Prox1+ cells in a rat sham irradiated 4 days after SE. B, B’. Representative images from a rat that received 6 Gy rad beginning 4 d after SE (P60/62) shows few Prox1-immunoreactive cells in the hilus (h). gcl, granule cell layer. Scale bar: 50 μm.
Figure 2.8. Cells born after SE contribute to MFS 10 weeks following pilocarpine treatment. A, GFP immunolabeling of a control injected with RV-GFP 4 days after saline treatment and sacrificed 10 weeks later shows axonal labeling in the hilus (h) but only GFP+ dendrites in the inner molecular layer (iml). B, C, At 10 weeks after SE, animals that received pilocarpine and then RV-GFP 4 days later display many GFP+ axonal processes in the iml (arrows) that appear identical to those seen in the granule cell layer (gcl) and hilus (arrowheads). D, E, Denser iml Timm staining is seen at 10 weeks after SE in a sham-irradiated control (D) than in a rat irradiated beginning 4 days after pilocarpine treatment (E). G, Densitometric analysis of the percentage area of inferior blade gcl and molecular layer that is Timm stain-positive. A dose of 6 Gy fractionated irradiation administered 4 and 6 days after SE significantly decreased the amount of Timm staining (*=p<0.005). Scale bars: 50 μm (in A) for A-C; 200 μm (in D) for D, E, and 50 μm for insets.
Figure 2.9. Diagrammatic model showing the influence of granule cell maturity on vulnerability to SE-induced structural plasticity. In this model, fully mature cells (born at P7, top row) are resistant to SE-induced remodeling. Some DGCs born at P28 (4-weeks-old at SE, second row), however, show abnormal hilar basal dendrites (HBDs; b) and mossy fiber sprouting (MFS; s). Those born on P42 (2-weeks-old at SE, third row) display extensive HBD formation 4 weeks after SE and to a lesser extent contribute to MFS seen 4 weeks after pilocarpine treatment. DGCs born 4 days after SE (P60 injections, bottom row) exhibit both HBDs and ectopic migration without MFS at 4 weeks after SE, but they develop MFS by 10 weeks. The model assumes that changes seen at 4 weeks remain stable for RV-GFP injections prior to SE (top 3 rows) as only animals administered RV-GFP after SE (bottom row) were examined at the 10 week time point in this study.
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Chapter 3

Disabled-1 is Required for Proper Migration and Maturation of Adult-born Hippocampal Neurons

Abstract

Reelin, a large molecule secreted into the extracellular matrix, is important for neuronal migration and process development. Binding and internalization of Reelin leads to phosphorylation of disabled-1 (Dab1), an adaptor protein, activating downstream signaling and Dab1 degradation. Evidence implicates the Reelin/Dab1 pathway in early dentate granule cell (DGC) development. Mice with Reelin, Dab1, or Reelin receptor mutations have markedly abnormal DGC layers as well as DGCs with abnormal dendrites, similar to those seen in experimental medial temporal lobe epilepsy (mTLE). Interestingly, DGC layer abnormalities in mice with deficient Reelin signaling also resemble DGC layer dispersion seen in human mTLE, and dentate gyrus (DG) Reelin expression is markedly decreased in experimental mTLE.

To better understand the role of Dab1 in neonatal and adult DGC neurogenesis, we crossed mice with two floxed Dab1 conditional knock-in (cki) alleles (Dab1<sup>cki/cki</sup>) with a Nestin-CreER<sup>T2</sup>/ROSA-YFP bitransgenic mouse line and used tamoxifen (TMX) to conditionally delete Dab1 in DGC progenitors beginning at postnatal day
(P) P7 or P28. We also injected a retroviral Dab1 shRNA vector (RV-pU6-shDab1-CAG-GFP-WPRE) into the DG of adult rats to suppress Dab1 expression in adult-born DGCs. In conditional Dab1 deficient mice, immunolabeling for Prox1 (a DGC marker) and YFP revealed an increase in hilar ectopic DGCs after postnatal Dab1 deletion similar to that seen in experimental mTLE. The DG of Dab1 deficient mice also contained DGCs with impaired dendritic maturation and axonal disorganization. Expression of Dab1 shRNA in adult rat DGC progenitors also markedly decreased DGC dendritic arborization and led to increased numbers of labeled glia in both the hilus and DGC layer compared to rats injected with a control shRNA vector.

Our findings indicate that Dab1 signaling plays a critical role in migration and dendritic development of neonatal- and adult-born DGCs. Additionally, Dab1 appears necessary for directing DGC progenitors to a neuronal, rather than glial, phenotype. Abnormalities seen after conditional Dab1 deletion resemble those seen in experimental mTLE, suggesting that restoring Reelin signaling through Dab1 after epileptogenic insults may ameliorate aberrant plasticity associated with mTLE.

Introduction

Neurogenesis persists throughout adulthood in the mammalian hippocampal dentate gyrus (DG) (Altman and Das, 1965; Kuhn et al., 1996; Eriksson et al., 1998) DG neuroblasts arise in the subgranular zone and migrate a short distance into the granule cell layer, where they differentiate into mature dentate granule cells (DGCs) and form appropriate synaptic connections (Cameron et al., 1993; Kuhn et al., 1996; Ge et al., 2008; Toni et al., 2008). In experimental mTLE, however, these normal
processes are disrupted, as neuroblasts often migrate aberrantly into the hilus and display characteristic abnormalities including axonal (mossy fiber) sprouting and hilar basal dendrites (HBDs) (Mello et al., 1993).

Little is known about the precise mechanisms governing the sequential developmental process that leads to proper migration and maturation of neonatal- or adult-born neurons. Disrupted in schizophrenia-1 (DISC1), a schizophrenia susceptibility gene, was recently found to cause abnormal morphological development and aberrant migration of adult-born DGCs (Duan et al., 2007). Other molecules, including cyclin-dependent kinase 5 (cdk5) and NeuroD, are also implicated in regulating several steps of DGC neurogenesis, including neuronal survival, maturation and synapse formation (Jessberger et al., 2008; Lagace et al., 2008; Gao et al., 2009). Wnt signaling is important for differentiation, synaptic maintenance, and function of adult-born DGCs (Inestrosa and Arenas, 2010).

A critical player in the Reelin pathway, Disabled-1 (Dab1), is an intracellular adaptor protein activated by Reelin binding to its receptors, apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR) ((Rice et al., 1998 reviewed in (Rice and Curran, 2001)). Developmental DGC layer abnormalities in mice with deficient Reelin, Reelin receptor or Dab1 signaling (Howell et al., 1997; Drakew et al., 2002) resemble DGC layer dispersion seen in human mTLE (Houser, 1990). Mice with defective Reelin signaling also show DGCs with dendritic abnormalities similar to those seen in mTLE (Drakew et al., 2002). Reelin expression persists into adulthood in gamma-aminobutyric acid (GABA)-ergic interneurons (Pesold et al., 1998) that are susceptible to epileptogenic injury, and we and others
have found that Reelin expression in the adult decreases markedly in experimental and human mTLE (Haas et al., 2002; Heinrich et al., 2006; Gong et al., 2007). In addition, the degree of DGC layer dispersion in hippocampi from patients with pharmacoresistant mTLE inversely correlates with Reelin mRNA levels (Haas et al., 2002; Haas and Frotscher, 2009). In vitro studies also provide evidence that Reelin influences the migration of postnatally-born DGCs (Gong et al., 2007). Together, these data suggest that Reelin signaling serves to regulate adult neurogenesis, and dysfunction of this pathway may play a role in DG morphological abnormalities seen in mTLE.

To study how loss of Dab1 function influences early postnatal and adult rodent DGC neurogenesis, we used conditional (“floxed”) Dab1 knock-in (Dab1-cki) mice or intrahippocampal injection of a retroviral (RV) Dab1 shRNA vector in rat. Dab1 deletion in mice beginning at either postnatal day (P) 7 or P28 caused multiple DGC morphological abnormalities, including granule cell layer dispersion, ectopic DGCs in the hilus and molecular layer, decreased dendritic complexity and axonal disorganization. In adult rats injected with Dab1 shRNA, we also observed decreased maturation of DGC dendrites and found increased numbers of labeled glia in the granule cell layer compared with controls. Taken together, these data suggest a role for Dab1 in multiple aspects of postnatal and adult DGC neurogenesis, including migration, cell fate decisions and process development.
Materials and Methods

Animals: All animal procedures were performed in accordance with protocols approved by the University Committee on Use and Care of Animals of the University of Michigan. All rats were purchased from Charles River. The Nestin-Cre-ER<sup>T2</sup> line on a C57/6J background was crossed with the ROSA26-yellow fluorescent protein (R26R-YFP) reporter mice, which is a mixture of 129x1/SvJ and C57/6J backgrounds (Lagace et al., 2007). Either bigenic mice or Nestin-Cre-ER<sup>T2</sup> single transgenic mice were then crossed with the Dab1-conditional knock-in (cki) line on a C57/B6 background with which contains floxed Dab1 alleles (Pramatarova et al., 2008). Three genotypes of mice were generated: mice with wild-type Dab1 (Dab1<sup>+/+</sup> mice), mice heterozygous for the Dab1 cki gene (Dab1<sup>cki/+</sup> mice) and mice homozygous for the Dab1 cki gene (Dab1<sup>cki/cki</sup> mice). All animals were kept under a constant 12 h light/dark cycle and had access to food and water ad libitum.

Generation of plasmid DNA constructs: To generate pDab1-IRES-mCh-WPRE, a Dab1 overexpression vector, Dab1 cDNA was cloned into a RV vector under the control of the CAG promoter. IRES, mCherry (mCh) and WPRE were sub-cloned downstream of Dab1, and proper orientation verified by sequencing.

To generate pSiE-shDab1-EF1α-GFP and pSiE-shDsRed-EF1α-GFP, the target sequence of Dab1, AATCACAGATTTGTGGCCATC (21-mer), was inserted downstream of the human U6 (hU6) promoter (constructs provided by H. Song). EGFP was inserted under the control of an EF1α promoter. As a control, shDab1 was replaced by shDsRed to generate pSiE-shDsRed-EF1α-GFP.
**In vitro studies:** 293T cells were cultured onto 4-well slides in DMEM with 10% FBS for 20 hours at 37°C. Two sets of experiments were performed. In the first, cells were first transfected with plasmid DNA constructs pSiE-shDab1-EF1α-GFP or pSiE-shDsRed-EF1α-GFP using lipofectamine 2000 (Invitrogen, CA), incubated for 6 hours at 37°C, followed by lipofectamine transfection with the pDab1 DNA construct. In the second experiment, cells were simultaneously transfected with either pSiE-shDab1-EF1α-GFP or pSiE-shDsRed-EF1α-GFP and pDab1-IRES-mCh-WPRE DNA.

Cells were subsequently cultured at 37°C for 48 hours, briefly fixed in 4% paraformaldehyde (PFA) at room temperature for 20 minutes, blocked in PBS with 10% normal goat serum, 1% BSA and 0.05% triton X-100 for 1 hour at room temperature and incubated overnight at 4°C with rabbit anti-Dab1 primary antibody (1:2000; (Pramatarova et al., 2008)) and mouse anti-GFP (1:400, Molecular Probes). Cells were washed twice in PBS, incubated with anti-rabbit IgG secondary antibody conjugated to Alexa Flour 350 (Molecular Probe, CA) for 90 minutes at room temperature, washed and imaged with a confocal microscope.

**Western blotting:** For western blotting, 293T cells were cultured in DMEM at 1.5x10^6 cells per 60 mm Petri dish for 20 hours at 37°C. Cells were transfected as previously described, protein extracted using lysis buffer and RIPA, and protein concentration determined using a spectrophotometer. Protein (50 ug) was resolved on a 10% SDS-PAGE gel (1.5 M Tris, pH 8.8, 30% acrylamide/bis-acrylamide, 10% SDS, 10% ammonium persulfate, and N,N,N’,N’-tetramethyl-ethylenediamine) using
an electrophoresis apparatus (Bio-Rad, Hercules, CA). Proteins in the gel were transferred onto a 0.45 µm nitrocellulose membrane using a Trans-Blot semi-dry blotter (BioRad). The membrane was then blocked in a solution of 5% nonfat dry milk in Tris Buffered Saline Tween (TBST) for 30 minutes at room temperature with slow agitation, probed with goat anti-Dab1 primary antibody (1:500, Santa Cruz, CA) in blocking buffer overnight at 4°C with agitation, washed in TBST and then incubated in secondary antibody conjugated with horseradish peroxidase (HRP) at 1:2000 in blocking buffer for 1 hour. The membrane was washed and ECL reagent (Thermo, CA) was applied as an HRP substrate. Immunoreactivity was detected and analyzed using the Gel-Doc XR System (Bio-Rad, CA). The same membrane was washed and re-probed with anti-β-actin monoclonal antibody (1:2000; Santa Cruz, CA) as an internal control to normalize Dab1 signal intensity.

**Production of high titer RV stocks and intrahippocampal RV injections:** The GP2-293 packaging cell line (Clontech, CA) was transfected with a RV backbone (RV-CAG-GFP-WPRE, gift of S. Jessberger and F. Gage, Salk Institute, or RV-U6-dsRed-EF1α-GFP or RV-pU6-shDab1-EF1α-GFP, gift of H. Song, Johns Hopkins) and VSV-G envelope protein to generate high-titer (10^7-10^9 cfu/ml) replication-incompetent RV vectors as previously described (Kron et al., 2010). For intrahippocampal injections, animals were anesthetized with a ketamine/xylazine mixture and placed on a water-circulating heating blanket. After positioning in a Kopf stereotaxic frame, a midline scalp incision was made, the scalp reflected by hemostats to expose the skull, and bilateral burr holes drilled. Either shDab1 (RV- pU6-shDab1-
EF1α-GFP) or control virus (RV-pU6-shdsRed-EF1α-GFP) was injected (2.5 µl of viral stock solution/site) into the left and right dentate gyri over 20 minutes using a 5 µl Hamilton Syringe, and the micropipette left in place for an additional 2 minutes. Coordinates for injections (in mm from Bregma and mm depth below the skull) were: caudal 3.5, lateral 2.1, depth 3.8.

**Tamoxifen Administration:** To conditionally delete Dab1 in adult DGC progenitors, mice received daily intraperitoneal (ip) injections of tamoxifen (TMX; dissolved in 10% EtOH/90% sunflower oil) at 180 mg/kg/d for 5 days on P28-32 or 100 mg/kg/d on days P7-8.

**Tissue Processing, Histology and Immunohistochemistry:** At either 2 or 3 months of age, all mice and rats were perfused with 4% PFA, brains removed and postfixed, cryoprotected in 30% sucrose and frozen. For some animals, a 0.37% sulphide fixative was also perfused for subsequent Timm staining (Kron et al., 2010). Coronal sections (40 µm thick) were cut with a cryostat and fluorescence (single and double-label) immunohistochemistry performed on free-floating sections as described previously (Parent et al., 1997; Parent et al., 1999; Kron et al., 2010). For some animals, a cresyl violet stain was performed. Briefly, sections were mounted onto Superfrost Plus Slides (Thermo Fisher Scientific) and dehydrated and rehydrated in graded ethanol, immersed in Cresyl Violet, dehydrated in graded ethanol and coverslipped with permount (Sigma-Aldrich).
Sections were immunostained for rabbit (Rb) anti-Prox1 (1:10,000- gift of S. Pleasure, University of California at San Francisco) rabbit (Rb) or mouse (Ms) anti-GFP (1:1000 or 1:400, respectively, Molecular Probes), Rb anti-Dab1 (rabbit polyclonal; (Howell et al., 1997)), mouse IgM anti-PSA-NCAM (1:500; 2-2b clone, Chemicon), mouse anti-GFAP (1:250, Sigma) or Ms anti-NF-M (1:500, Chemicon). In some cases, Tyramide-Plus signal amplification (catalog #SAT705A, 1:50; PerkinElmer Life Sciences, Boston, MA) was used to ensure that all dendritic processes were brightly labeled to allow accurate quantification. Some slides were counterstained with the nuclear stain bisbenzamide (1:10,000, Sigma-Aldrich) and all immunofluorescence slides were coverslipped with anti-fade (Invitrogen).

**Unbiased Stereology:** All quantification was performed blind to experimental condition. Stereological estimates of cell number were performed with a computer-aided microscopy system (Stereoinvestigator, MBF Bioscience, Williston, VT). Every 6th coronal section through the entire DG was examined at high power by using a 60x oil immersion objective. Cells were considered ectopic only if they showed immunoreactivity for Prox1, a DGC marker, and were located at least 2 cell body widths outside the DGC layer and into the hilus. Three animals were used in the control group, and 5 animals in each transgenic group (total of 6-8 dentate gyri per animal). For these analyses, wild-type animals with and without TMX were pooled in the control group (n=6), as TMX administration in Dab1+/+ mice did not impact the number of hilar ectopic cells (data not shown). Blinded cell counts were then estimated using an optical fractionator probe (West et al., 1991). The coefficient of
variation in the set of sections from each mouse was calculated (Gundersen et al., 1999) and found to be less than 5%.

**Epifluorescence and Confocal Microscopy:** Single-label images were captured using a Leica DSM/IRB epifluorescence microscope attached to a SPOT-RT digital camera. For double-labeling, images were acquired using a Zeiss LSM 510 confocal microscope as 1 μm thick z-stacks, colored in Adobe Photoshop, and analyzed for co-localization. For confocal stacks, maximum intensity z-projections obtained in Image J (NIH) were used for analysis.

**Quantification of Dendritic Length:** Rats receiving dsRed shRNA (n=4) and Dab1shRNA (n=5) were analyzed for dendritic length and dendritic complexity. Tissue from animals injected with retrovirus was immunolabeled for GFP using TSA amplification. For each animal, a minimum of 4 cells that had well-labeled and properly located cell bodies and dendrites that extended to the hippocampal sulcus were analyzed. Confocal z-stacks were reconstructed and Imaris software (Bitplane Scientific Software, St. Paul, MN) used to reconstruct and flatten the images for further analysis using the NeuronJ plug-in (Meijering et al., 2004) for Image J. All observations were made by an observer blinded to experimental condition.

**Statistics:** Comparisons among counts in multiple treatment groups were performed using a one-way ANOVA with Tukey’s posthoc t-tests. All values are reported as mean ± SEM, with p ≤ 0.05 considered statistically significant.
Results

Conditional Dab1 deletion alters integration of postnatally generated DGCs

Previous work demonstrated that the Dab1-cki mouse is a hypomorph but that the DG forms normally in the absence of TMX treatment (Pramatarova et al., 2008). We therefore crossed wild-type Dab1 mice (Dab1<sup>+/+</sup>), Dab1-cki heterozygotes (Dab1<sup>cki/+</sup>) and Dab1-cki homozygotes (Dab1<sup>cki/cki</sup>) with the Nestin-Cre-ER<sup>T2</sup> line and examined DG morphology in each of the different bi-transgenic lines. The presence of granule cell layer disorganization and increased number of hilar and molecular layer ectopic DGCs was associated with a dose-dependent reduction of Dab1 and, as expected, was partly dependent upon the age of TMX administration. Nestin-Cre-ER<sup>T2</sup>/Dab1<sup>+/+</sup> mice that received TMX either at P7-8 or at P28-32 and survived until P56 showed a normal DG morphology (Figure 3.1A, D). In contrast, Nestin-Cre-ER<sup>T2</sup>/Dab1<sup>cki/+</sup> mice (Figure 3.1B, E), and to a greater extent Nestin-Cre-ER<sup>T2</sup>/Dab1<sup>cki/cki</sup> mice (Figure 3.1C, F), displayed a more dispersed DGC layer and increased numbers of ectopic granule cells as assessed by immunolabeling for the DGC marker Prox1. We quantified the number of hilar ectopic cells using unbiased stereology, and found significant increases in hilar ectopic DGCs in Dab1<sup>cki/cki</sup> mice with early and late TMX, and in Dab1<sup>cki/+</sup> mice with early TMX compared to Dab1<sup>+/+</sup> littermate controls (Figure 3.1C, F, G). For example, Dab1<sup>cki/cki</sup> mice treated with TMX at P7-8 had a greater than 6-fold increase in hilar ectopic cells (mean ± SEM of 13,010 ± 1233/mouse) than Dab1<sup>+/+</sup> controls (mean ± SEM of 1840 ± 376/mouse).
We next directly examined recombined cells with conditional Dab1 deletion by generating Nestin-Cre-ER\textsuperscript{T2}/R26R-YFP/Dab1-cki triple transgenic mice and immunostaining for YFP. The timing of TMX delivery and survival were the same as described above. After P7-8 TMX treatment followed by a 52-day survival, YFP-immunoreactive DGCs in Nestin-Cre-ER\textsuperscript{T2}/R26R-YFP/Dab1\textsuperscript{+/+} controls were well-organized, normally oriented and mainly located in the inner granule cell layer (Figure 3.2A). In Nestin-Cre-ER\textsuperscript{T2}/R26R-YFP/Dab\textsuperscript{cki/cki} mice, however, we found many YFP-positive neurons abnormally oriented in the granule cell layer or located ectopically in the hilus or molecular layer (Figure 3.2B, arrows). We also noted a decrease in dendritic complexity of labeled DGCs and increased numbers of YFP-expressing hilar cells with glial morphology (Figure 3.2B, arrowhead). A minority of labeled DGCs appeared to integrate more normally. With later (P28-P32) TMX injections, the same abnormalities are present compared to control, albeit less severe than with earlier TMX injections (Figure 3.2B-D). These findings indicate that many, but not all, neonatal- or juvenile-born DGCs with decreased Dab1 levels integrate aberrantly, and are consistent with a role for Reelin signaling through Dab1 to influence DGC progenitor migration, dendritic development and possibly neuronal differentiation.

**Abnormal DG neuroblast migration and process outgrowth after conditional Dab1 deletion**

To further study the role of Dab1 in postnatal DGC progenitor migration, we immunostained brain sections for poly-sialated neural cell adhesion molecule (PSA-NCAM), a marker of neuroblasts in the DG. We found that neuroblasts in Nestin-Cre-ER\textsuperscript{T2}/Dab1\textsuperscript{+/+} controls receiving TMX at P7-8 were properly located in the
subgranular zone (Figure 3.3A, C), whereas those in Nestin-Cre-ER$^{T2}$/Dab1$^{ck/ck}$ mice appeared disorganized, showing abnormal placement in the hilus and outer granule cell layer (Figure 3.3B, D). These mice also displayed clusters or chains of presumptive migrating neuroblasts extending into the hilus from the subgranular zone (Figure 3.3D).

Migration in clusters or chains is typical of neuronophilic tangential, but not radial, migration. It is possible, however, that loss of Dab1 in radial glia indirectly alters DGC progenitor migration. The absence of Reelin signaling during development can interfere with radial glial scaffold formation in the DG, and others have suggested that this alteration may underlie the defective DGC layer organization seen in mice with defective Reelin/Dab1 signaling (Forster et al., 2002; Weiss et al., 2003). Therefore, we used glial fibrilaary acidic protein (GFAP) immunostaining to examine the effects of conditional Dab1 deletion on the radial glia scaffold. No obvious abnormalities in GFAP immunolabeling were present (Figure 3.4), suggesting that the ectopic DGCs seen after postnatal loss of Dab1 are unlikely due to secondary influences on neuroblast migration caused by defects in the radial glial scaffold. In addition, Nestin-Cre-ER$^{T2}$/Dab1$^{ck/ck}$ mice treated with TMX at P7-8 displayed normal lamination of the hippocampus proper and cortex as assessed by Nissl stain (data not shown). This finding suggests that altered inputs to postnatally-born DGCs are unlikely to be responsible for their abnormal placement. Reelin signaling through Dab1 is important for normal dendritic maturation for a variety of neurons generated during embryonic development (MacLaurin et al., 2007; Matsuki et al., 2008; Niu et al., 2008). Therefore, we examined process development of DGCs after postnatal
Dab1 deletion. Comparing dendrites of YFP-labeled neurons in Nestin-Cre-ER<sup>T2</sup>/R26R-YFP/Dab1<sup>+/+</sup> mice and Nestin-Cre-ER<sup>T2</sup>/R26R-YFP/Dab1<sup>ckl/ckl</sup> animals showed that loss of Dab1 decreased dendritic complexity (Figure 3.5A, B; see also Figure 3.2). DGCs in control mice injected with TMX at P7-8 had dendrites that appeared well-arborized (Figure 3.5A), but DGCs of the same age from Nestin-Cre-ER<sup>T2</sup>/Dab1<sup>ckl/ckl</sup> mice displayed much more simplistic dendritic architecture 52 days after TMX treatment (Figure 3.5B). Furthermore, there were consistently fewer cells labeled in Nestin-Cre-ER<sup>T2</sup>/Dab1<sup>ckl/ckl</sup> mice despite the same dose of TMX (Figure 3.5A, B; see also Figure 3.2), suggesting that loss of Dab1 may impair survival of developing DGCs.

Axonal fasciculation also appeared disorganized in Nestin-Cre-ER<sup>T2</sup>/Dab1<sup>ckl/ckl</sup> mice compared to controls. A well-organized pattern axonal fasciculation pattern was revealed by immunohistochemistry for neurofilament-M (NF-M), which labels DGC mossy fiber axons, in TMX-treated Nestin-Cre-ER<sup>T2</sup>/Dab1<sup>+/+</sup> controls (Figure 3.5C, white arrows and inset). In contrast, axonal organization was disrupted in Nestin-Cre-ER<sup>T2</sup>/Dab1<sup>ckl/ckl</sup> mice treated with TMX at P7-8 (Figure 3.5D, white arrows and inset). Despite this axonal abnormality in the hilus and CA3, Timm staining did not reveal inner molecular layer mossy fiber sprouting after Dab1 deletion (data not shown).

**shRNA-mediated Dab1 knockdown in adult rat DGC progenitors alters their maturation**

To confirm and further explore the effects of Dab1 knockdown in adult animals and in a different species, we used RV-mediated delivery of a Dab1 shRNA to suppress Dab1 expression in the dentate gyri of adult rats. We generated a RV vector
carrying a Dab1 shRNA sequence against the central fragment of Dab1 and an EF1α-driven GFP reporter (RV-shDab1-EF1α-GFP). An shRNA to the invertebrate gene DsRed (RV-shDsRed-EF1α-GFP) was used as a control. We first confirmed the ability of RV-shDab1-EF1α-GFP to knock down Dab1 expression by co-infecting HEK cells with a Dab1-overexpressing RV vector carrying an mCh reporter (RV-Dab1-IRES-mCh) and either RV-shDab1-EF1α-GFP or RV-shDsRed-EF1α-GFP. After 48 hours, the cells were examined by confocal microscopy to detect Dab1 and GFP immunoreactivity, and native mCh fluorescence. We also performed Western blotting to semiquantitatively assay for Dab1 protein expression levels. Triple labeling of cells revealed decreased Dab1 expression in GFP/mCh double-labeled cells infected with RV-shDab1-EF1α-GFP as compared to those expressing RV-shDsRed-EF1α-GFP (Figure 3.6A-F). As expected, mCh expression was relatively reduced after infection with the control shRNA to DsRed (Figure 3.6C, F).

We performed Western blotting after a simultaneous transfection, in which 293T cells were transfected with Dab1 or dsRed shRNA at the same time as Dab1 overexpression vector, and after sequential transfection, in which shRNA was transfected 6 hours before Dab1 overexpression vector. Western blotting for Dab1 using protein showed that the Dab1 shRNA reduced Dab1 protein levels. Normalization for β-actin protein revealed Dab1 reductions of approximately 73% after sequential transfection and 29% after simultaneous transfection (Figure 3.6 G, H).

We next injected high-titer RV-shDab1-EF1α-GFP or RV-shDsRed-EF1α-GFP control vector stereotactically into the DG of adult rats and examined the effects
on GFP-labeled cells 4 weeks later. Similar to conditional genetic deletion of Dab1 in mouse DGC progenitors (Figures 3.2, 3.5), we found that shRNA-mediated suppression of Dab1 in adult rat DGC progenitors decreased dendritic complexity, as 4-week old DGCs showed more extensive dendritic arborization patterns after infection with control shRNA vector compared to those expressing RV-shDab1-EF1α-GFP (Figure 3.7A, B). We quantified dendritic arborization of GFP-labeled cells using reconstructed confocal z-stacks and found that Dab1 shRNA significantly decreased mean dendritic length by about 40% (Figure 3.7C), and also significantly decreased mean numbers of secondary and tertiary dendritic branches by 52% and 28%, respectively (Figure 3.7D, E). Thus, Dab1 knockdown attenuates dendritic maturation in postnatal or adult-born DGCs of both mouse and rat.

In addition to decreased dendritic arborization, we observed an increase in labeled glia after Dab1 suppression in adult rat DGC progenitors. Injection of control vector led to GFP-labeling nearly exclusively in neurons (Figure 3.8A), but animals receiving RV-shDab1-EF1α-GFP injections showed frequent GFP labeling of cells with glial morphology (Figure 3.8B). Some, but not all, of the glia-like cells were immunoreactive for GFAP (data not shown). Strikingly, many sections from rats injected with Dab1 shRNA had almost exclusively glia labeled, including both in the hilus and granule cell layer (Figure 3.8B-D). We also noted a decrease in overall numbers of GFP-labeled cells in Dab1shRNA-injected rats compared to controls, supporting a role for Dab1 in adult-born DGC survival (Figure 3.7A, B; Figure 3.8A, B).
Discussion

Our data from RV-shRNA-mediated Dab1 suppression in rats and TMX-induced deletion in Dab1-cki mice suggest that Dab1 influences multiple stages of adult hippocampal neurogenesis. Aspects of DGC development and integration affected by loss of Dab1 include neuronal migration and laminar organization, dendritic maturation, axonal patterning, neuronal differentiation, and potentially cell survival. A substantial fraction of Dab1-deficient cells in both the mouse and rat failed to develop appropriately complex dendritic processes. Aberrant migration and disruption of axonal fasciculation were also evident in the transgenic mice, and alterations in cell fate decisions were observed in Dab1 shRNA-infected cells in rat and to a lesser extent after conditional Dab1 deletion in mice. Our findings thus identify critical roles of Dab1 in the maturation and integration of neonatal and adult-born hippocampal neurons.

Previous studies indicate that the Reelin-Dab1 pathway is involved in regulating many events during embryonic hippocampal development, including axonal targeting, dendritogenesis, laminar organization and cell migration (reviewed in (Rice and Curran, 2001)). In the adult rodent DG, Reelin expression persists in interneurons (Pesold et al., 1998; Haas et al., 2002; Abraham and Meyer, 2003; Gong et al., 2007) and Dab1 is expressed by neuroblasts and putative interneurons (Gong et al., 2007). The abnormalities caused by postnatal loss of Dab1 parallel those seen when Reelin/Dab1 signaling is disrupted during embryonic development. For example, the disorganized mossy fiber projections induced by loss of Dab1 in our transgenic mice resemble the irregular, broad axonal projections observed in reeler mice and
ApoER2/VLDLR double knockout mice (Drakew et al., 2002). Abnormal axonal projections in these mice are thought to arise from the irregular distribution of granule cell somata. This mechanism may also underlie axonal defects the Dab1-cki line.

Others have reported a role for Reelin and Dab1 in dendritogenesis. Decreased dendritic complexity occurs in mice lacking Reelin both in vivo and in vitro, and this abnormality is rescued by addition of exogenous Reelin (Niu et al., 2004). Inhibitors of Dab1 activation also suppress dendrite outgrowth in embryonic hippocampal neurons (Niu et al., 2004), and early inactivation of a conditional Dab1 allele decreases dendritic complexity in the hippocampal CA1 region (Matsuki et al., 2008). Recent work also suggests that Reelin overexpression in the adult has opposite effects, including increased dendritogenesis, excessive synaptic contacts and hypertrophy of dendritic spines, further implicating the Reelin pathway in developmental processes that remain active in the adult brain (Pujadas et al., 2010). We observed decreased dendritic complexity in cells infected with RV-shDab1. This effect was likely even more pronounced than reported, as our conservative analysis only included cells that extended dendrites all the way to the hippocampal sulcus; many more cells infected with Dab1shRNA had nearly absent dendrites, but these were excluded to avoid cells with dendrites that went out of the plane of sectioning. Many of the YFP-positive cells in the TMX-treated Nestin-Cre-ER<sup>T2</sup>/ R26R-YFP/Dab1<sup>cki/cki</sup> mice also appeared to have more simplistic dendritic architecture. The previously described phenotypes coupled with our data thus strongly support a role for Dab1 in proper dendrite maturation.
The Reelin/Dab1 pathway is important for both radial and tangential cell migration. Reeler or Dab1 mutant mice display overtly abnormal lamination in the cortex and DG (D'Arcangelo et al., 1995; Sheldon et al., 1997). The Reelin/Dab1 pathway is thought to mediate tangential SVZ cell migration by directing chain-migrating progenitors to disperse from their chains (Hack et al., 2002). In mice lacking either Reelin receptors or Dab1, chain formation is severely compromised in the rostral migratory stream, and neuroblasts accumulate in the subventricular zone (SVZ) (Andrade et al., 2007). Conversely, loss of Reelin in the epileptic DG leads to aberrant chain migration of DGC precursors, implicating the Reelin/Dab1 pathway as necessary to maintain normal migration of adult-born DGCs (Gong et al., 2007). We noted a significant increase in the number of hilar ectopic cells in mice after conditional Dab1 deletion, possibly due to aberrant chain migration (Figure 3.3). Interestingly, we did not observe an increase in the number of ectopic cells in rats injected with Dab1 shRNA at 4 weeks after injection. At one week after injection, however, we did find occasional hilar ectopic cells (data not shown); these cells had likely died by 4 weeks post-injection, as fewer labeled cells persisted at the 4 week time point compared to controls.

In addition to aberrant chain migration, abnormal radial migration may arise secondary to a defective radial glial scaffold. In reeler mice, the majority of radial glial cells are reported to be poorly differentiated and decreased in number and length in the DG (Forster et al., 2002), and scrambler and ApoER2/VLDLR-deficient mice also demonstrated alterations in the radial glial scaffold (Weiss et al., 2003). Our data do not support an overtly abnormal radial glial scaffold (Figure 3.4), and Nissl stains
of the transgenic mice appear normal (data not shown). However, experiments with neuroblast-specific conditional Dab1 deletion are needed to definitively rule out this possibility.

In adult reeler mice injected with bromodeoxyuridine (BrdU) to label dividing cells, the number of BrdU-labeled, GFAP-positive astrocytes is increased compared to wild type mice, indicating that adult neurogenesis is altered and that newly generated cells preferentially differentiate into astrocytes (Zhao et al., 2007). Furthermore, neurospheres from the Dab1<sup>−/−</sup> mouse, yotari, and in the yotari brain in vivo GFAP expression increases at the expense of neurons, further suggesting a role for Dab1 in suppressing astroglial differentiation (Kwon et al., 2009). These results are consistent with our finding of increased labeled glia after RV-shRNA-mediated Dab1 knockdown. Some of those animals also had numerous GFP-positive glial cells in the DGC layer, which were rarely observed in adult rat DG infected with dsRedshRNA or RV reporters (Kron et al., 2010).

In our mouse model, we noted that Nestin-CreER<sup>T2</sup>/Dab1<sup>cki/cki</sup> mice given early TMX treatment appeared to have more narrow DGs than littermate controls. We also saw fewer labeled neurons in Nestin-Cre-ER<sup>T2</sup>/R26R-YFP/Dab1<sup>cki/cki</sup> mice and in adult rats 4 weeks after Dab1 shRNA injection. These findings may reflect increased cell death after Dab1 knockdown. Recent evidence suggests that Reelin prevents retinoic acid-induced apoptotic cell death (Ohkubo et al., 2007), implicating the Reelin/Dab1 pathway in cell survival.

Our data further support a role for Reelin signaling through Dab1 in the morphological abnormalities observed in human and experimental mTLE. Neonatal or
adult Dab1 knockdown recapitulates several abnormalities that develop after chemoconvulsant-induced status epilepticus in adult rats, including the appearance of hilar ectopic DGCs and altered dendritic development. Previous work from our lab has demonstrated that prolonged seizures lead to a loss of hilar and molecular layer Reelin expression, which correlates with the appearance of aberrant neuroblast chain migration (Gong et al., 2007). The recapitulation of these abnormalities in a non-injury environment is consistent with a Dab1-mediated mechanism for the seizure-induced migration abnormalities that are associated with decreased Reelin expression in experimental mTLE. Interestingly, we did not observe spontaneous seizures after conditional Dab1 deletion in the adult mice. A more careful assessment with prolonged video/EEG monitoring may detect non-convulsive or infrequent behavioral seizures, and challenge with chemoconvulsants or electrical stimulation may reveal an altered seizure threshold. Consistent with the latter idea, reeler mice show a lowered threshold for seizure induction and a higher incidence of behavioral seizures when animals subjected to electroshock stimulus. Neocortical and hippocampal slices from reeler mice were also more likely to generate spontaneous epileptiform activity after bicuculline application compared with controls (Patrylo et al., 2006).

Our studies are the first to use an inducible model to conditionally knock down Dab1 levels specifically in neural progenitors and examine the role Dab1 plays in neonatal and adult DGC neurogenesis. With this model and shRNA-mediated knockdown, suppression of Dab1 is precisely timed and only impacts a specific subset of cells. These features are similar to experimental, and possibly human, mTLE in which DG Reelin expression decreases after a precipitating injury (Gong et al., 2007).
Our data, coupled with previous work, implicates Reelin/Dab1 signaling abnormalities in epileptogenic plasticity. Manipulations to restore this pathway may therefore prove useful for attenuating the development of epilepsy after brain insults.
Notes to chapter 3:

A modified version of this chapter will be submitted for publication as:


Cell culture experiments and western blot analysis and quantification were performed by Helen Zhang. Diane Lagace in the Eisch lab developed the Nestin-CreERT2 x ROSA-YFP mouse line, Xin Duan in the Song lab created the Dab1 overexpression vector, and Dab1-cki mice were generated by the Howell lab.

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Figure 3.1: Conditional Dab1 deletion induces aberrant DGC migration. A-F: Prox1 immunostaining of DGCs after tamoxifen (TMX) treatment at P7-8 (top row) or P28-32 (bottom row) of Nestin-CreERT2 mice crossed with Dab1 wild-type (A, D), Dab1<sup>cki/+</sup> (Dab1 heterozygous conditional knock-in) (B, E) or Dab1<sup>cki/cki</sup> (Dab1 homozygous conditional knock-in) (C, F) mice. Insets show higher magnification views of the dentate granule cell layer (gcl) and hilus (h). Dab1 levels inversely correlate with the appearance of ectopic DGCs, as heterozygous cki mice show more ectopic cells than controls, and homozygous cki mice show even greater numbers of ectopic cells, as well as gcl dispersion (C, F). Scale bar (in A), 75 μm. G: Quantification of hilar ectopic DGCs showed significant increases in Nestin-CreERT2/Dab1<sup>cki/cki</sup> mice treated with TMX at P7-8 (*, p<0.01) or P28-32 (*, p<0.001), and in Nestin-CreERT2/Dab1<sup>cki/+</sup> given TMX at P7-8 (*, p=0.001) versus TMX-treated Nestin-CreERT2/Dab1<sup>++</sup> (Con).
Figure 3.2: Many DGCs with conditional Dab1 knockdown migrate ectopically.

Triple transgenic mice with wild-type Dab1 (Nestin-CreER<sup>T2</sup>/R26R-YFP/Dab1<sup>+/+</sup>; A, C) or homozygous Dab1-cki (Nestin-CreER<sup>T2</sup>/R26R-YFP/Dab1<sup>cki/cki</sup>; B, D) were treated with tamoxifen (TMX) at P7-8 (A, B) or P28-32 (C, D). YFP-labeled DGCs appear normally organized in the inner granule cell layer (gcl) in Dab1 wild-type controls (A, C). After P7-8 TMX treatment of homozygous Nestin-CreER<sup>T2</sup>/R26R-YFP/Dab1<sup>cki/cki</sup> pups, in contrast, many YFP-immunoreactive DGCs appear misoriented and many ectopic cells accumulate in the hilus (h) and molecular layer (ml) by P60 (B, white arrows). Similar, albeit less severe, abnormalities are seen with P28-32 TMX injection (D, arrows). homozygous Dab1-cki mice also appear to have more labeled glia-like cells in the hilus after early or late TMX treatment (arrowheads in B and D). Scale bar (in A), 75 μm.
Figure 3.3: Neuroblast migration is altered after postnatal Dab1 deletion. Nestin-CreER$^{T2}$/Dab1$^{+/+}$ mice given TMX at P7-8 had properly located neuroblasts in the dentate subgranular zone and inner granule cell layer (gcl) as assessed by PSA-NCAM immunoreactivity 52 days later (A, C). In Nestin-CreER$^{T2}$/Dab1$^{ckl/ckl}$ mice treated with the same TMX regimen, PSA-NCAM immunolabeling is more disorganized and many PSA-NCAM-positive cells appear in the hilus (h; B, D). In addition chains or clusters of presumptive migrating neuroblasts extend from the gcl into the h (arrowheads in D). Scale bar (A,B) 50 μm (C,D), 35 μm.
Figure 3.4: DG glia are not altered following conditional Dab1 deletion. GFAP immunostaining of dentate gyri from P60 Nestin-CreER\textsuperscript{T2}/Dab1\textsuperscript{+/+} control mice (A, C) and Nestin-CreER\textsuperscript{T2}/Dab1\textsuperscript{cki/cki} cki mice (B, D) treated with TMX at P7-8 (A, B) or P28-32 (C, D). Labeled glia, including radial glia-like cells in the granule cell layer (gcl), are only modestly affected by conditional loss of Dab1 (compare with altered neuronal architecture in Fig. 2). Scale bar 50 μm.
Figure 3.5: Postnatal Dab1 deletion alters DGC dendritic complexity and axonal organization. A, B: The complex dendritic architecture seen in DGCs of P60 Nestin-CreERT2/R26R-YFP/Dab1+/+ mice treated with TMX at P7-8 (A) is absent in similarly treated Nestin-CreERT2/R26R-YFP/Dab1ckicki mice (B). C, D: Immunostaining for neurofilament-M, an axonal marker in the DG, revealed disorganized axons in P60 homozygous Dab1 deficient mice compared to controls (C, D 10x magnification; inset, 20x). The well-defined and organized axonal borders in Nestin-CreERT2/R26R-YFP/Dab1+/+ mice (C, arrows and arrowheads in inset) were disrupted in Nestin-CreERT2/ Dab1ckicki mice given TMX at P7-8 (D, arrows and arrowheads in inset).
Figure 3.6: shRNA-mediated knockdown of Dab1 protein expression. A-F: Cells were co-transfected with Dab1 overexpression vector (pDab1-IRES-mCh-WPRE) and either pSiE-shDab1-EF1α-GFP (A-C) or pSiE-shDsRed-EF1α-GFP (D-F), and 48 hour later fixed and triple labeled for GFP, mCh and Dab1. Yellow cells co-expressing mCh and GFP have lower Dab1 expression after knockdown with shDab1 (arrows in A-C) compared with similar cells in control cultures (arrows in D-F). Arrowheads in A-F denote cells with strong Dab1 expression in the setting of low or absent GFP. Note that mCh expression is lower in the shDsRed-expressing cultures (compare F with C). Scale bar (in F), 25 μm. G, H: Immunoblot (G) and densitometric quantification (H) of Dab1 protein expression in cultured 293T cells 48 hours after transfection with varying combinations of Dab1 overexpression (OE) vector and shDab1 or shDsRed. Transfections were either performed simultaneously or with RNAi vector followed 6 hours later by Dab1 OE vector (sequential). Sequential transfection of Dab1shRNA and then Dab1OE vector resulted in a >70% decrease in Dab1 protein levels compared to the control condition (G, lanes 4 and 5; right-sided bars in H).
Figure 3.7: Less complex dendritic arbors form on adult-born DGCs after shRNA-induced Dab1 suppression. A, B: RV-mediated expression of Dab1 shRNA in DGC progenitors markedly decreased dendritic arborization of GFP-labeled cells in the granule cell layer (gcl) of an adult rat (B, arrows) compared to control DsRed shRNA infected DGCs (A, arrows). RV shRNA vectors were injected 4 weeks earlier. Scale bar 50 μm. C-E: Quantification of dendritic complexity showed that Dab1 shRNA-expressing DGCs had significantly decreased mean dendritic length (C, *p=2.1 x 10^{-5}), and significantly fewer secondary (B, *p<0.005) and tertiary branches (C, *p<0.05).
Figure 3.8: shRNA-mediated Dab1 deletion induces glial differentiation of infected DGC progenitors. A, A representative section from adult rat DG 4 weeks after control RV-U6-shDsRed-EF1a-GFP injection shows normal appearing, GFP-labeled DGCs and no labeled glia (A). After RV-U6-shDab1-EF1a-GFP injection, in contrast, many labeled cells with glial morphology were evident in the DG (B-D). Arrows indicate putative glial cells in the hilus (h), and arrowheads denote others in the granule cell layer (gcl). Scale bar (in D), 50 μm. for A and B; 25 μm. for C and D.
References


Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727-3738.


Chapter 4

Thesis Summary and Future Directions

Summary of Dissertation Work

The data presented in this thesis provide insight into alterations of dentate granule cell (DGC) development induced by status epilepticus (SE) and into the possible role of the Reelin/Dab1 pathway in these SE-induced alterations. Using stereotactic retroviral injections at several timepoints before or immediately after SE and complementary irradiation studies, we found that the age of a granule cell dictates its response to prolonged seizures. That is, only neurons of certain ages respond to SE with distinct cellular abnormalities. Whereas mature neurons at the time of SE are resistant to SE-induced plasticity, immature neurons sprout mossy fibers and form hilar basal dendrites (HBDs) by one month after SE. Newborn neurons migrate ectopically and form HBDs by one month after seizures. We also found that the length of time after SE impacts which abnormalities neurons display, as aberrant mossy fiber sprouting (MFS) was not seen in cells born 4 days after SE when examined 1 month after SE, but was observed at 10 weeks after SE.

This work with the rat pilocarpine model corresponds to previous data in the literature suggesting that cells at varying developmental stages respond differently to
pilocarpine-induced SE in the mouse (Walter et al., 2007). Furthermore, it confirms what others have found regarding the ectopic migration of DGCs born after SE (Jessberger et al., 2007), although the timepoints at which we found DGCs responding to SE by forming HBDs are not identical. This may reflect us of a different epilepsy model. Lastly, this work is the first to show that developing, but not mature cells, undergo MFS, and helps to resolve a controversy in the field regarding the age of DGC that contributes to MFS (Parent et al., 1997; Parent et al., 1999; Jessberger et al., 2007).

Previous work from our laboratory and others implicated the Reelin/Dab1 pathway as important for cellular migration and proper process development. We took two approaches for further study. We first used a Dab1-cki mouse to conditionally knock down Dab1 expression in neonatal or young adult mice. We found increased numbers of hilar ectopic cells in Dab1\textsuperscript{cki\textbackslash cki} mice (conditional homozygote deletion) receiving tamoxifen (TMX) at P7-8 or P28-32 and Dab1\textsuperscript{cki/+} (conditional heterozygote deletion) receiving TMX at P7-8. We also noted a subtle increase in axonal disorganization and a decrease in dendritic complexity.

We next injected a retrovirus containing an shRNA to Dab1 into the adult rat dentate gyrus (DG) to assess its impact on postnatal neurogenesis and neuronal development. At four weeks after injection, we found that cells infected with this shRNA exhibited a significantly less complex dendritic morphology, and that fewer GFP-positive cells were present as compared to control shRNA injection. Interestingly, we also noted an increase in the number of labeled glia in the DG, notably in the granule cell layer.
Our work is an important first step in establishing a causal relationship between the Reelin pathway and the DGC response to epilepsy. Our study was the first use of an inducible model to conditionally knock down Dab1 levels specifically in neural progenitors in transgenic mice. The findings from those studies were recapitulated by the shRNA-mediated knockdown, resulting in a precise Dab1 knockdown in DGC progenitors. Our work is consistent with the current literature regarding experimental and human mTLE in which DG Reelin expression decreases after a precipitating injury (Gong et al., 2007) or is decreased in hippocampi resected from patients with intractable TLE (Haas and Frotscher, 2009).

From the experiments described in chapter 2, two main questions arise. First, what makes DGCs of different ages respond differently to SE? On that same note, why do DGCs that are the same age at the time of SE respond differently to SE? That is, not every cell migrates aberrantly or displays an abnormality, why is that the case? From chapter 3, the main issue that remains to be addressed is establishing a definitive causal link between Reelin and epilepsy.

In the following section these questions are addressed and future studies proposed to further explore the role of seizure-induced neuronal plasticity in epileptogenesis.

**Why do DGCs of Different Ages Respond Differently to SE?**

In chapter two, we found that the age of a DGC at the time of SE dictates its response. This could be due to cell intrinsic or cell extrinsic factors. To address intrinsic factors, an initial study to compare the RNA profiles of DGCs labeled with GFP that display abnormalities versus those that do not would be helpful to identify factors that may contribute to the observed abnormalities.
To answer the question of what cell-intrinsic factors influence DGC responses to SE, CAG-GFP carrying RV should be injected into the DG at timepoints before and after SE. Cells of different ages that display different abnormalities should be assessed for their RNA profiles. Instead of perfusing the animals at 4 weeks after SE as in chapter 2, we would instead prepare thick sections using a vibratome (as for electrophysiology), and collect RNA from GFP-positive cells displaying different morphological abnormalities. Performing a microarray analysis on the RNA from these cells to assess changes in gene expression would undoubtedly highlight pathways that are altered in cells that migrated ectopically versus those that migrated properly. If HBDs were visible, analyzing the profiles of similarly aged cells both with and without HBDs would be an important follow-up study. A better understanding of the molecular basis for the morphological abnormalities in epilepsy is crucial for the advancement of the field, and this experiment would identify future promising candidate genes and molecules for study.

To address potential cell intrinsic and extrinsic factors, microarrays of the entire DG, and not just the intracellular contents of GFP-positive cells, would aid in highlighting potentially important genes. In that vein, a recent study addressing a molecular portrait of epilepsy found a group of 128 genes consistently hyper-expressed throughout epileptogenesis (Okamoto et al., 2010). Genes found to be elevated at several timepoints after SE included the mammalian target of rapamycin (mTor) signaling pathway, and genes from the TGF-beta and IGF-1 pathways.

Mammalian target of rapamycin (mTor) is an evolutionarily conserved serine/threonine kinase with a central role in the control of cell growth.
and Hall, 2000). Many tissues, including brain, express mTOR (Kim et al., 2002) and it is specifically inhibited by rapamycin (Sabatini et al., 1994). Rapamycin can act as an anticonvulsant (Kwon et al., 2003) or an antiepileptogenic agent in some rodent models of epilepsy (Zeng et al., 2008), and unilateral rapamycin infusion into the dorsal hippocampus for 1 or 2 months after pilocarpine-induced SE reduced aberrant Timm staining in the GCL and molecular layer (Buckmaster et al., 2009). Thus this study fits with previous research highlighting the mTOR pathway as a potential therapeutic target. Performing similar microarray analysis in GFP-positive cells would further narrow the genes responsible on the cellular level for SE-induced DGC abnormalities and help to discern why DGCs of different ages respond differently to SE.

**Why do DGCs that are the Same Age at the Time of SE Respond Differently to SE?**

Although similarly aged cells at the time of SE presumably express similar genes at the time of SE, only a subset respond to SE by displaying abnormalities. This is especially evident in rats injected 4 days after SE, in which 58% of cells display an abnormality (migrate ectopically or have a persistent HBD). One way to address this issue would be to perform microarrays as explained in the last section to focus on the differences in RNA profiles of similarly aged cells that display abnormalities versus those that do not. As a corollary to these experiments, it is important to assess how the cells that migrate aberrantly, versus those that do not, function in the network. To that end, retroviral reporter labeling paired with electrophysiological recordings to assess intrinsic properties of cells of certain ages that do or do not display
abnormalities are important. From these experiments, we could determine how cells that migrate aberrantly versus those that do not function in the network.

We performed preliminary electrophysiological experiments in collaboration with Dr. Geoffrey Murphy who recorded from hippocampal slices from Nestin-CreERT2 x ROSA-YFP mice and compared the recordings to those made from rats in which we had injected retrovirus. In rats, we injected at P28, waited either 17 or 28 days after retroviral injection, and recorded from GFP-positive cells, filling them with Alexa Fluor 594 during recording (Figure 4.1A-A4). We saw that, as expected, cells exhibited electrophysiological responses characteristic of maturing neurons (Figure 4.1B, C).

The results from our Nestin-CreERT2 x ROSA-YFP mice were more complex. After administering TMX at P28, we waited 3 weeks and then recorded from GFP-positive cells. Immunofluorescence for YFP and DCx at this time point revealed many double labeled cells in the dentate gyrus of bi-transgenic, TMX treated mice (Figure 1D). We found, however, that responses of GFP-positive cells were heterogenous and included repetitive spiking (E1), single spikes (E2) or no spiking (E3) (Figure 4.1E). Repetitive spiking cells are likely mature DGCs, single spiking cells are likely early post-mitotic neurons, and those cells lacking action potentials are likely transit-amplifying cells or neuroblasts. Thus, our data suggest that YFP-positive cells in Nestin-CreERT2 x ROSA-YFP mice represent a heterogeneous population born at different times after Cre-mediated recombination.

In summary, when we used whole-cell patch clamp recordings to assess firing properties of labeled neurons, we found that while there was a predictable response
based on neuronal age in rats injected with retrovirus, the firing of cells in our bitransgenic mice was significantly more varied. Thus, using retroviral injections to label cells and analyze them for both gene expression profiles and electrophysiological properties should be a powerful approach to characterize the molecular and functional profile of aberrantly integrating DGC progenitors.

As the Nestin-CreERT2 mice produced data that was slightly difficult to analyze, a potential next step would be to instead use a Doublecortin-CreERT2 (Dcx-CreERT2) mouse and cross it with the YFP-reporter mouse. Dcx is a microtubule-associated protein expressed by immature neurons for approximately the first 3 weeks after birth. Thus, Dcx is present in immature neurons but not earlier progenitors, such that Dcx positive cells (Type 2b and Type 3 cells) are committed to a neuronal phenotype and normally proceed within a well-characterized timeline to mature neurons. Performing corollary studies to those performed with the Nestin-CreER\textsuperscript{T2} mice with the Dcx-CreER\textsuperscript{T2} would help solidify our hypothesis and more precisely birthdate neurons. To that end, it would also serve as additional support for the retroviral injections we performed in rats. Furthermore, the Dcx-CreER\textsuperscript{T2} mouse crossed with the floxed Dab1 mouse would allow for knockout of Dab1 only neurons and not radial glia as in the Nestin-CreER\textsuperscript{T2} mouse. These preliminary experiments helped establish the feasibility of assessing cells via electrophysiology born at specific times on a cell by cell basis. Future experiments that use these techniques after SE will help to analyze how cells of different ages contribute to network function.
Additional experiments to assess the impact of the differential response of DGCs to SE can also be performed using X-irradiation. Using X-irradiation to ablate different cohorts of cells as in Chapter 2 and pairing those studies with *in vitro* electrophysiology to assess changes in network properties would undoubtedly shed light onto the role of the ablated cells in abnormal hippocampal excitability.

Although we saw morphological amelioration of MFS when animals were irradiated 5 weeks before SE, and a decrease in ectopic cell number when rats were irradiated after SE, the impact on seizure phenotype was not analyzed. This issue would be best answered with follow-up studies of the animals irradiated at various timepoints using continuous video recording paired with electroencephalography (EEG) to analyze the impact of irradiation on seizure frequency, severity and duration. Others have demonstrated that cytosine-b-D-arabinofuranoside infusion decreased the development of spontaneous recurrent seizures when infused after SE, and that this decrease in seizure frequency occurred without a change in MFS (Jung et al., 2004). That same group found that the cyclo-oxygenase 2 inhibitor celecoxib attenuated the likelihood of developing spontaneous recurrent seizures and inhibited the generation of ectopic granule cells in the hilus after pilocarpine-induced SE (Jung et al., 2006). A single injection of the synaptic blocker botulinum neurotoxin E was also recently shown to decrease spontaneous recurrent seizures (Antonucci et al., 2009), and mathematical modeling suggests that even weak MFS can induce robust hyperexcitability in the dentate gyrus (Santhakumar et al., 2005). Pairing our irradiation timepoint that ameliorates MFS with video/EEG is therefore an important first experiment, and is currently being pursued in the Parent laboratory. If decreasing
MFS can substantially ameliorate the severity of the epilepsy phenotype, it would implicate MFS as a crucial subject for future study.

**Is There a Causal Link Between Altered Reelin Signaling and Epilepsy?**

We found that altered Reelin signaling is associated with abnormal plasticity that occurs during epileptogenesis. To address whether there is a causal relationship between suppressed Reelin signaling and epilepsy developments, two important initial experiments would be to delete Dab1 in DGC progenitors and perform seizure susceptibility experiments and continuous video/EEG recording to assay for spontaneous seizures. A more careful assessment with prolonged video/EEG monitoring may detect non-convulsive or infrequent behavioral seizures, and challenge with chemoconvulsants or electrical stimulation may reveal an altered seizure threshold. Consistent with the latter idea, reeler mice with spontaneous null Reelin mutations show a lowered threshold for seizure induction and a higher incidence of behavioral seizures when animals are subjected to electroshock stimulus. Neocortical and hippocampal slices from reeler mice were also more likely to generate spontaneous epileptiform activity after bicuculline application compared with controls (Patrylo et al., 2006).

To shed light onto the role of Reelin in epilepsy, we made a Dab1 overexpression vector and inserted it into a retrovirus, and injected it into the DG in the post-SE rat. Our Dab1 overexpression experiments (described in Appendix A) revealed what appeared to be a polarity reversal of fluorescently-labeled neurons by one month after injection as compared to animals injected with control retrovirus.
Cells infected with the retroviral Dab1 overexpression vector displayed short but thick dendrites aberrantly projecting into the hilus. There were also many fewer labeled cells in rats injected with Dab1 overexpression vector at 4 weeks after injection, although the one week timepoint was again inconclusive in definitively demonstrating a cell death phenotype. Follow-up studies to these are warranted to more fully explore the role of Dab1 in the DGC abnormalities observed after SE, as it is unknown where, when, and in what quantity Reelin signaling normally occurs in the DG.

**Future Direction of the Field**

Despite the progress made recently in the field, it is still unknown if the SE-induced changes observed in rodent mTLE models precisely mimic the human condition. Thus experiments to correlate the two are warranted. Furthermore, I think that more focus should be shifted to studying the latent period, as it presents a defined window in which interventions could be aimed to cure instead of simply treat mTLE.

Both humans and rodent models of epilepsy are reported to have a latent period between initial insult such as SE and the development of spontaneous recurrent seizures (Mello et al., 1993; Aridaa et al., 1999). Further study of changes in the latent period should be pursued by pairing early post-SE video/EEG recordings with our irradiation studies. If irradiation at certain timepoints could lengthen the latent period, cells born at those timepoints would be ideal therapeutic targets.

On that same note, some animals that undergo SE do not exhibit MFS, and during my thesis work I noted that about 1% of animals that undergo a clear episode
of SE do not progress to epilepsy. I believe that studying these animals by RNA analysis of the entire DG or hippocampus is incredibly important, as it may reveal clues about why these animals did not progress to overt epilepsy and shed light onto potential clinical interventions in humans.

**Conclusion**

Our understanding of epilepsy and neurogenesis has dramatically increased over the past decade. In epilepsy, the stimulation of endogenous neural stem cells may have opposing consequences for epileptogenesis after acute insults depending upon whether they integrate normally or aberrantly, and a better understanding of the role of various SE-induced abnormalities is important to help guide the development of future treatments. Rigorous studies in animal models must be conducted to ascertain the precise roles of these abnormal DGCs, as only then can we translate experimental advances to the clinical disorder.
**Figure 4.1:** Fluorescently labeled adult-born DGCs of different ages exhibit varying firing patterns. A, RV-GFP-labeled DGC filled with Alexa 594 during recording (A1). A2–4 show Alexa 594 and GFP labeling the filled neuron. B, C, Whole cell recordings under current clamp from GFP+ DGCs made 17 (B) or 28 (C) days after RV-GFP injection. D, Dentate gyrus from Nestin-CreER<sup>T2</sup>/R26R-YFP mouse labeled for YFP (green) and DCx (red). Lower panels: higher magnification views of double-labeled cells (white arrows). E, Whole-cell current clamp recordings from newborn DGCs from Nestin-Cre-ER<sup>T2</sup> x ROSA-YFP mice. Scale bar: A, 20 μm; D, 150 and 50 μm; E, 10mV/10ms
References


Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727-3738.


Appendix A

Dab1 Overexpression in the Intact and Epileptic Hippocampus

Abstract

Reelin is a molecule secreted into the extracellular matrix that binds apolipoprotein E receptor 2 and very low density lipoprotein receptor. Binding and internalization of Reelin leads to phosphorylation of disabled-1 (Dab1), an adaptor protein, activating downstream signaling and Dab1 degradation. Evidence implicates the Reelin/Dab1 pathway in dentate granule cell (DGC) migration and dendritic formation. Mice with Reelin, Dab1, or Reelin receptor mutations have a markedly abnormal DGC layer as well as DGCs with abnormal dendrites similar to those seen in experimental mesial temporal lobe epilepsy (mTLE).

In rats injected with the Dab1 overexpression vector post-SE, labeled cells were often improperly oriented and displayed more abnormal dendrites than controls, but fewer appeared in ectopic locations than cells infected with control vector. In the intact hippocampus, Dab1 overexpression accelerated dendritic maturation at 1 week after injection, but caused improper dendritic formation by 4 weeks post-injection.

Aberrant neurogenesis is a proposed cause of seizures and cognitive deficits common in mTLE. This work supports a role for Dab1 overexpression in
ameliorating the ectopic cells, but not the abnormal dendrites, observed in experimental mTLE.

**Introduction**

Reelin is a large, secreted protein important for neuronal migration (reviewed in (Rice and Curran, 2001)) and dendritic development (Niu et al., 2004). It binds apolipoprotein E receptor 2 and very low density lipoprotein receptors expressed on neurons or radial glia. Reelin binding and internalization leads to phosphorylation of Dab1, an adaptor protein, activating downstream signaling and Dab1 degradation. Evidence implicates this pathway in several aspects of DGC development. Mice with Reelin, Dab1, or Reelin receptor mutations have markedly abnormal DGC layers as well as DGCs with abnormal dendrites, including hilar basal dendrites (HBDs) similar to those seen in experimental mesial temporal lobe epilepsy (mTLE) (Drakew et al., 2002). DGC layer abnormalities in mice with deficient Reelin signaling resemble those in human mTLE, and the degree of DGC layer dispersion in samples from patients with pharmacoresistant mTLE inversely correlates with Reelin mRNA levels (Haas et al., 2002).

We previously found that Reelin expression decreases markedly after chemoconvulsant-induced status epilepticus in rats (Gong et al., 2007), and that transient Dab1 knockdown induces granule cell dispersion, hilar ectopic cells, mild axonal disorganization and alterations in dendritic complexity (see Chapter 3). These findings led us to hypothesize that DGCs migrate to ectopic locations in experimental temporal lobe epilepsy due to a loss of Reelin signaling through Dab1 and that
restoring Reelin signaling will attenuate seizure-induced abnormalities in DGC neurogenesis. We therefore used a Dab1-overexpression vector to transiently overexpress Dab1 in the intact and epileptic dentate gyrus to determine its impact on DGC development, migration and process extension.

Materials and Methods

Generation of Dab1 overexpression DNA construct: pDab1-IRES-mCherry-WPRE- Dab1 cDNA was cloned in a retroviral vector under the control of constitutive promoter CAG. Sequentially, IRES, mCherry and WPRE were cloned downstream of Dab1, respectively. The correct orientation of the cDNA was verified by sequencing.

Production of high titer viral stocks: To generate high-titer, replication-incompetent retroviral (RV) vectors, the GP2-293 packaging cell line (Clontech, CA) was transfected with the RV backbone (RV-CAG-Dab1-IRES-mcherry-WPRE) and VSV-G envelope protein (10^7-10^9 cfu/ml) viral stock as previously described (Kron et al., 2010).

Retroviral Injections: Animals were anesthetized with a ketamine/xylazine mixture and placed on a water-circulating heating blanket. After positioning the animal in a Kopf stereotaxic frame, a midline scalp incision was made, the scalp reflected by hemostats to expose the skull, and bilateral burr holes drilled. Injections were performed using a Hamilton syringe that was slowly lowered into the dentate gyrus
(DG) and left in place for 5 minutes prior to and 20 minutes after the 2.5 µl/site injection.

In one group of rats, we injected either Dab1 overexpression (RV-CAG-Dab1-IRES-mcherry-WPRE) or control retroviral vector bilaterally into the DG at P32. In a second group of rats, we unilaterally injected a Dab1 overexpression vector into the dentate gyrus (DG) after SE (P56 SE, P60 injection). Control vector (RV-CAG-GFP, gift of S. Jessberger) was injected into the contralateral DG. Rats survived for either 7 or 28 days after in vivo injections.

**Tissue Processing, Histology and Immunohistochemistry:** Either one or four weeks after injection, all rats were perfused with 4% paraformaldehyde, postfixed, cryoprotected in 30% sucrose and frozen. Coronal sections (40 µm thick) were cut with a sliding microtome and fluorescence immunohistochemistry performed on free-floating sections (Parent et al., 1997; Parent et al., 1999). Sections were immunostained for rabbit (Rb) or mouse (Ms) anti-GFP (1:1000 or 1:400, respectively, Molecular Probes) or Rb anti-dsRed (1:1000, Clontech).

In some cases, Tyramide-Plus signal amplification (catalog #SAT705A, 1:50; PerkinElmer Life Sciences, Boston MA) was used to ensure that all dendritic processes were brightly labeled to allow accurate assessment. Some slides were counterstained with a nuclear counterstain, bisbenzamide (1:10000, Sigma-Aldrich).
**Epifluorescence and Confocal Microscopy:** Single-label images were captured using a Leica DSMIRB epifluorescence microscope attached to a SPOT-RT digital camera.

**Results**

**Dab1 overexpression after seizure decreases the number of labeled hilar ectopic cells, but increases aberrant dendritic projections**

We used a Dab1-overexpression retroviral vector to increase Dab1 expression in the rat DG after SE. We found that although rats injected with control RV 4 days after SE had a significant number of labeled hilar ectopic cells (Figure A.1A), this abnormality was ameliorated in after injection with a Dab1-overexpression RV (Figure A.1B).

In addition to a decrease in the number of ectopic cells, we also observed an apparent reversal of cellular polarity in the DG. Animals without SE had no hilar ectopic cells and properly located dendrites (Figure A.2A), whereas animals with SE displayed ectopic cells and hilar basal dendrites (Figure A.2B). Interestingly, cell polarity appeared reversed in rats injected with Dab1 overexpression RV after SE, with numerous dendrites projecting into the hilus (Figure A.2C, A.2D white arrows).

**In the intact hippocampus, Dab1 overexpression accelerates dendritic maturation at 8 days after injection, but causes cell death and aberrant projections by 4 weeks**

After finding that Dab1 overexpression impacted dendritic development in the epileptic rat hippocampus, we sought to determine the impact of Dab1 overexpression in the non-epileptic DG. To that end, we injected 1-month old rats and assessed the
impact 7 days and 4 weeks later. We found that GFP-positive cells in controls displayed a characteristic level of immaturity at 8 days, whereas cells infected with Dab1-overexpression RV had longer and much more complex dendritic architectures (Figure A.3A, A.3B). Labeled cells overexpressing Dab1 also tended to have migrated further into the molecular layer aspect of the DGC layer than the GFP-positive cells in controls (Figure A.3A, B). Strikingly, however, at 28 days after SE, cells infected with Dab1-overexpression vector were much less numerous and had almost no dendritic architectures projecting towards the molecular layer (Figure A.3C). Instead, the dendrites appeared to project into the hilus, similar to those we observed 4 weeks after SE.

**Discussion**

In this study, we used a retrovirus-based strategy to characterize the function of Dab1 overexpression in the context of adult hippocampal neurogenesis. Cells that overexpressed Dab1 after SE were less likely to migrate ectopically after pilocarpine-induced SE, although neuronal polarity appears altered. Dab1 overexpression in the intact hippocampus initially accelerated dendritic maturation and perhaps migration, but later inhibited proper process maturation.

Our findings further support a critical role of Dab1 in the migration and process development of newborn granule cells within the adult DG. As the impact of transient Dab1 overexpression ameliorates the ectopic cell phenotype observed after SE, our data also support a role for Dab1 in the morphological abnormalities observed in epilepsy.
Previous work established a role for Dab1 in several aspects of adult neurogenesis in the dentate gyrus including dendritogenesis and cell migration. To that end, others have reported that Dab1 activation inhibitors prevent dendrite outgrowth (Niu et al., 2004) and that there is decreased dendritic complexity in mice lacking Reelin (Niu et al., 2004). Recent work also suggests that Reelin overexpression increases dendritogenesis, synaptic contacts, causes hypertrophy of dendritic spines, and causes migration deep into the GCL (Pujadas et al., 2010) further implicating the Reelin pathway in developmental processes that remain active in the adult brain and are altered in mTLE. Our work supports this idea, as Dab1 overexpression initially increased dendritic development, however we did not see this phenotype in the long-term, as dendrites appeared blunted, thicker than normal, and often projected aberrantly into the hilus by 4 weeks after injection. This difference may be explained by the fact that we looked at a later timepoint than previously published reports, or could indicate a dose-dependent effect of Dab1.

The Reelin/Dab1 pathway is also important for cell migration, with mouse mutants displaying overtly abnormal lamination in the cortex and DG. Our data support a role for this pathway in the epileptic brain, as rats injected with the Dab1 overexpression RV had fewer ectopic cells 1 month after SE than rats injected with control RV. These observation also correlate with our previous work (Chapter 3), in which we noted a significant increase in the number of hilar ectopic cells in mice with a conditionally deleted Dab1 gene.

Further studies to explore the use of Dab1 overexpression as a means to ameliorate the seizure phenotype are warranted, as the data from this experiment,
coupled with previous knowledge, implicate Reelin signaling through Dab1 as an important pathway in the epileptic brain.
Notes to Appendix

Helen Zhang produced the Dab1 overexpression virus, and we thank K. Kron, C. Collins and N. Khanna for technical assistance.
Figure A.1: Animals injected with control retrovirus (CAG-GFP) 4 days after SE have cells with hilar basal dendrites that are often ectopically located (A, yellow arrows). In animals injected with Dab1 overexpression vector 4 days after SE, fewer ectopic cells were observed (B).
Figure A.2: In intact animals, control RV injection at P60 labels DGCs with normal morphology (A), whereas cells labeled with GFP 4 days after SE have hilar basal dendrites and are often ectopically located 24 days later (B, yellow arrows). In animals injected with Dab1 overexpression vector 4 days after SE, fewer ectopic cells were observed (C, D), although cell polarity appears altered in these animals, with dendrites projecting into the hilus (C,D).
Figure A. 3: Dab1 overexpression alters dendritic development. Dab1 overexpression (OE) accelerates dendritic maturation at 8 days after injection (B) compared to control (con, A). By 28 days after injection, neuronal polarity appears reversed, with short, thick dendrites extending into the hilus (C).
References


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