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Reduced NG2 Cell Differentiation in Hilus in a Pilocarpine Model of Temporal Lobe Epilepsy

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Reduced NG2 Cell Differentiation in Hilus in a Pilocarpine Model of Temporal Lobe Epilepsy

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I. Introduction

a. *Overview of NG2 Cells*

NG2 cells are a cell population in the CNS recently identified as a fourth distinct major glial cell population (Nishiyama et al. 1999), differentiated by the expression of neural/glial antigen 2 (NG2). Initially identified as a cell population exhibiting characteristics that seemed neither entirely glial nor neuronal, these cells exhibit characteristics unique to other glial populations. They express voltage gated sodium channels, and are capable of modest excitation via common neurotransmitters such as GABA and Glutamate (Chittajulla et al. 2004, Lin et al. 2004, Tong et al 2009). Much of their potential is still actively being investigated, and the extent of their abilities is yet to be identified. Nevertheless the major role of the cell type has been well established. The endogenous role of NG2 cells is to serve as precursors to oligodendrocytes, thus they are typically also referred to as oligodendrocyte precursor cells (OPCs). However, they have been shown in some studies to also be capable of differentiation to astrocytes and interneurons under specific environmental manipulations (Belachew et al. 2003, Zhu et al. 2008, Zhao et al. 2009). As the precursors to mature, myelinating oligodendrocytes, NG2 cells have been observed to have a consistent response to a variety of injuries and insults to the CNS, particularly those that are demyelinating. In animal models examining demyelinated lesions, NG2 cells have been shown to proliferate and migrate to the site of injury, where they differentiate to oligodendrocytes and begin to produce novel myelin sheaths in the region (Keirstead et al. 1998, McTigue et al. 2001, Bu et al. 2004). Besides their response to demyelination, studies have indicated that NG2 cells may also respond to an increase in excitation of local neurons (Demerens et al. 1996, Tamura et al. 2004). The combination of these responses has led to

questions regarding the extent of their role, if any, in epileptic brains, given their possession of both these events.

b. *Status Epilepticus*

Temporal lobe epilepsy (TLE) is the most common form of adulthood epilepsy. The disorder is marked by recurrent and spontaneous seizure activities, thought to occur due to an imbalance of excitation and inhibition endowed in part by hippocampal sclerosis (Thom 2014). It is unknown what specific events are responsible for initiating this behavior, and it is likely that a combination of these events are involved, but many cellular and network alterations have been well characterized in both experimental models of epilepsy and clinical cases. The closest replicate for TLE in animal models is known as status epilepticus (SE), and is one of the most commonly used methods to study the disorder. This approach hinges on the idea that "seizures beget seizures", and involves initially inducing seizure activity, commonly through exposure to chemical convulsants (Morimoto et al. 2004). The induced seizure activity initiates events that will ideally cause enough cellular and network changes to elicit spontaneous seizures in the future. There are several cellular events that are well documented in this model, and many imitate cellular changes observed in clinical cases, that are believed to underlie the occurrence of spontaneous seizures. Presence of these events are now typically used to validate the model and to predict the occurrence of future spontaneous seizures. Some of those most commonly exhibited include neuronal loss, neurogenesis, and mossy fiber sprouting. The hippocampal region is tightly associated with epilepsy, and many of the characteristic events associated with the disorder have been thoroughly exhibited in this region in previous studies. The hippocampus is composed of several well-defined layers and regions (Fig. 4A), allowing for fine discernments for the various relevant cellular changes. Some of the layers commonly analyzed for

characteristic indications of seizure activity are the hilus, the dentate gyrus (DG), and the pyramidal layer. The hilus is the innermost region of the hippocampus, and is consistently a site of extensive cellular changes, including significant diminishment of the local hilar interneuron population (Borges et al 2003, Bouillret et al 1999, Riban et al 2002). The loss of this interneuron population is thought to be one of the events that leads to a loss of regulation of excitation. Bordering the hilus is the DG, which is one of the few regions capable of adult neurogenesis. Increased neurogenesis has been shown to take place after SE (Parent et al 1997) and is a standard characteristic used to validate a model. The pyramidal layer, composed of excitatory pyramidal cells, also suffers extensive neuronal loss after seizure activity. Typically the layer is most affected in the CA1, CA3, and CA4 regions of the hippocampus (DeGiorgio et al. 1992, Cavazos et al. 1994, Shetty et al. 2009), and is well documented in both experimental and clinical cases. In addition to population changes in primary and interneuron populations, there is an increase in excitatory input via an event referred to as mossy fiber sprouting. Mossy fibers are the granule neurons of the DG, and studies have demonstrated that seizure activity encourages axonal branching of these cells (Borges et al 2003, Bouillret et al 1999, Shibley & Smith 2002, Chen et al 2013). These arborizations feed back into the dendritic field of the granule cells themselves, and thus begin to excite themselves. This event therefore is believed to further contribute to an overall gain of excitation, leading to spontaneous seizure events. Presence of these cellular events are vital to validate a model in order to examine points that precede the end of the latent period, when spontaneous seizures begin to occur.

c. Current Understanding of Glial Cells' Response to Seizure Activity

Extensive studies on neuronal changes in epilepsy models have culminated into some well-established hallmarks of the models. Comparatively less is known about the roles of the

various glial populations in response to seizure activity, but recent studies have begun to elucidate some of these responses. Several studies have indicated that both astrocytes and microglia proliferate and exhibit an altered morphology in response to seizures, though the particulars of their role remain to be determined (Borges et al. 2003, Bouillret et al. 1999, Bordey & Sontheimer 1998, Avignone et al. 2015). Studies suggest that in addition to these responses, significant loss of mature oligodendrocytes is demonstrated in in vivo studies of epilepsy as well as in clinical cases (Luo et al. 2015, Wang et al. 2018). Accompanying this cellular loss is a widespread decrease in myelination, specifically exhibited in areas of the hippocampus (Ye et al. 2013).

Despite some of this recent progress in investigation glial cells in epilepsy, relatively little is known about how the NG2 cell population responds to seizure activity. As previously mentioned, it is consistently observed that NG2 cells exhibit responses to both demyelination and excitation of local neurons. Thus it is anticipated that NG2 cells may demonstrate a similar response to seizure activity, since the disorder displays both these types of insult. A modest number of studies suggest that NG2 cells may indeed respond to seizures similar to the manner in which they respond to other insults. Some of these recent investigations have demonstrated that the NG2 cell population increases in vivo in SE models (Luo et al. 2015, Hu et al. 2016). However, this finding has been contradicted (You et al. 2011), and thus additional support is needed to confirm this observation. And despite an initial idea of how the population may change in response to these seizures, the role that the cells are serving remains to be determined. This study thus aimed to accomplish both goals, to reaffirm an increase in the NG2 cell population's size and to investigate the fate of these cells in response to seizure activity.

II. Methods

a. Animals/SE Induction

Mice were kept on a regular 12 hour light/12 hour dark cycle and provided food and water ad libidum. All animals were analyzed between 6 and 8 weeks of age. The primary mouse line investigated in this study is an NG2creER:YFP line, acquired by the crossing of an NG2creER line and ROSA-YFP reporter line. All animals were administered 0.1mg/g tamoxifen intraperitoneally daily for five consecutive days, beginning 5-7 days prior to the anticipated day of seizure induction. On the day of SE, mice were first administered 34mg/kg Atropine 30 minutes prior to administration of pilocarpine. 320mg/kg pilocarpine was then given IP to each animal. Seizure activity was allowed to persist for up to 3 hours before being chemically attenuated with diazepam. Seizure events were monitored and evaluated using a modified Racine scale (Racine, 1972). Seizures were then relieved by administration of either 10 or 5mg/kg diazepam, if mice did or did not progress beyond stage 3 seizures. After completing the SE procedure, the animals' recovery was aided by provision of wet food and daily IP injections of 5% dextrose in Lactate Ringer's solution for up to 5 days after SE.

In addition to the NG2 reporter line, both pure C57BL/6 and FVB mice were utilized in this study and underwent the SE procedure, with some alterations. C57BL/6 mice underwent seizure induction similarly to the NG2creER:YFP mice, however rather than a single dose of 320mg/kg pilocarpine, these mice were initially given 280mg/kg pilocarpine a half hour after receiving atropine, and then repeated subsequent doses of 10% the initial amount were administered every half hour until a stage 4 seizure or higher was observed. The remainder of the protocol used for SE in the C57BL/6 mice was identical to that of the NG2 reporter line. Similarly, FVB mice received a similar protocol. These mice were also given an initial dose of

280mg/kg pilocarpine a half hour after receiving atropine, but received 20% every half hour after the initial dose until stage 4 seizures were observed. The subsequent doses were increased from 10 to 20% in hopes of increasing the amount of time animals spend experiencing seizures of higher severity without needlessly extending the time spent seizing at a low grade.

b. Perfusion and Sectioning

On the day of intended sacrifice, all animals were administered 0.05 mg/g 5-ethynyl-2'deoxyuridine (EdU) 4 and 2 hours before perfusion. Animals were then anesthetized with isoflurane and fixed transcardially with 4% paraformaldehyde (~50mL/animal). Tissue was then post fixed in 4% PFA for 2 hours and rinsed repeatedly in 0.2M sodium phosphate buffer and finally stored in the buffer until sectioning, no later than 7 days post fixation. Sections were then taken on a vibratome (Leica VT1000 S) at 50 micrometers and sections containing hippocampus stored in cryostorage solution until staining.

c. Immunofluorescence

All sections undergoing immunofluorescence were first blocked for 1 hour in 0.1% Triton and 5% NGS or NDS. Sections were then incubated in primary solution for 1 hour at room temperature on a shaker, then stored at 4C overnight. Primaries used include: 1:1000 mouse anti- NeuN IgG1, 1:1000 chick anti-GFP, 1:100 mouse anti-MBP, 1:500 rabbit anti-NG2, and 1:12000 mouse anti-Gad67. Sections were then incubated in secondaries for 1 hour at room temperature on shaker. Secondary antibodies used include: 1:500 goat anti-mouse IgG2a Cy3, 1:500 donkey anti-rabbit Alexa 488, 1:200 goat anti-mouse IgG2a Alexa 568, 1:500 donkey anti-chick Alexa 488, 1:500 donkey anti-mouse IgG Cy3, 1:300 goat anti-rabbit Alexa 633, and 1:500 goat anti-mouse IgG1 Cy3. Tissue intended for EdU analysis was then stained using a

Homemade Click-iT EDU reaction. Sections were then mounted and allowed to dehydrate before coverslipped with Vectashield Mounting Solution with DAPI.

d. Analysis

Tissue was analyzed and imaged using the Zeiss Axiovert 200M and Leica DMR microscopes. Regions of interest for quantification were acquired using Zeiss software. Quantifications were correlated to the highest stage seizure reached by each animal and grouped by color. For quantitative analyses, a P value of less than 0.05 was considered significant.

	Description
Stage 1	Motionless staring
Stage 2	Head nodding
Stage 3	Full body hops
Stage 4	Falling to side
Stage 5	Remaining on side, unable to right self or maintain posture, twitching

Table 1*: Modified Racine scale used for qualitative analysis of severity of seizures underwent by induced animals.*

III. Results

a. Seizure analysis

After administration of pilocarpine, mice typically exhibited a delay in seizure activity for 30 minutes. Once seizure activity began the severity consistently increased for another hour, before beginning to wane again in severity. Approximately half of the surviving NG2creER mice reached up to stage 4 seizures. 15% exhibited only as high as stage 3 seizures, and another 15% demonstrated stage 5 seizures. The remaining animals died either during initial seizure induction or soon after before the desired time points, and were excluded from analysis (Fig. 1).

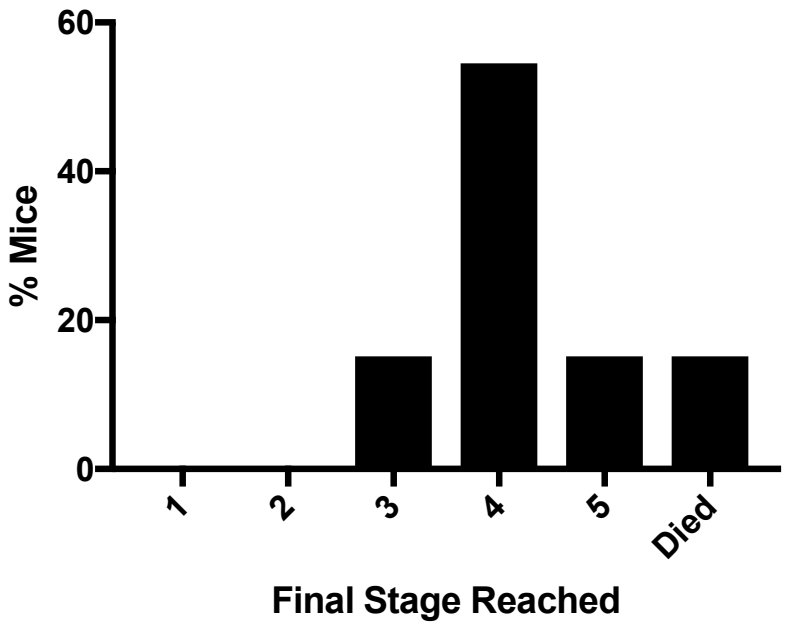

Figure 1*: Distribution of highest stages reached by NG2creER:YFP mice. Over half of induced animals reached a maximum of stage 4 seizures, but did not progress further. Equal amounts of induced animals reached a maximum of stage 3 and stage 5 seizures. A comparatively low number of induced animals, approximately 15%, died during or shortly after SE, before the desired time points and were excluded from further analysis accordingly. There were no NG2creER mice treated with pilocarpine that did reach at least stage 2 seizures. N=33*

b. Cell loss

NeuN stains indicated no obvious loss of cells in the pyramidal layer at either 2 or 7 days post induction of SE (dpSE). Preliminary analysis suggests that similar lack of pyramidal cell loss is seen at 125dpSE (Fig. 2). Similarly, stains for Gad67 exhibited no significant loss of interneurons in the

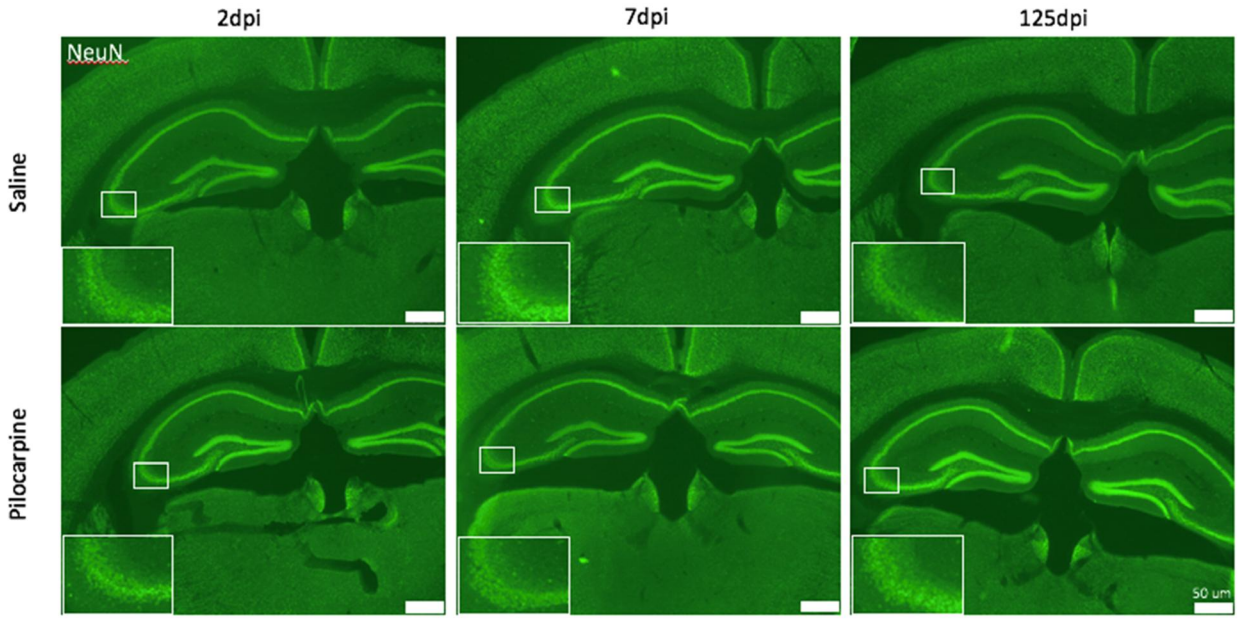

***Figure 2**: No significant loss of pyramidal cells after seizure induction in NG2creER:YFP mice at any examined time point. The pyramidal cells in CA1, CA2 and CA3 regions of the hippocampus remain intact despite undergoing seizure activity.*

hippocampus at either 2 or 7dpSE, and likely at the 125dpSE time point, as suggested by preliminary quantifications (Fig. 3). Because the hippocampus is divided into well-defined layers and regions, the Gad67+ population was quantified in each molecular layer in regions CA1,

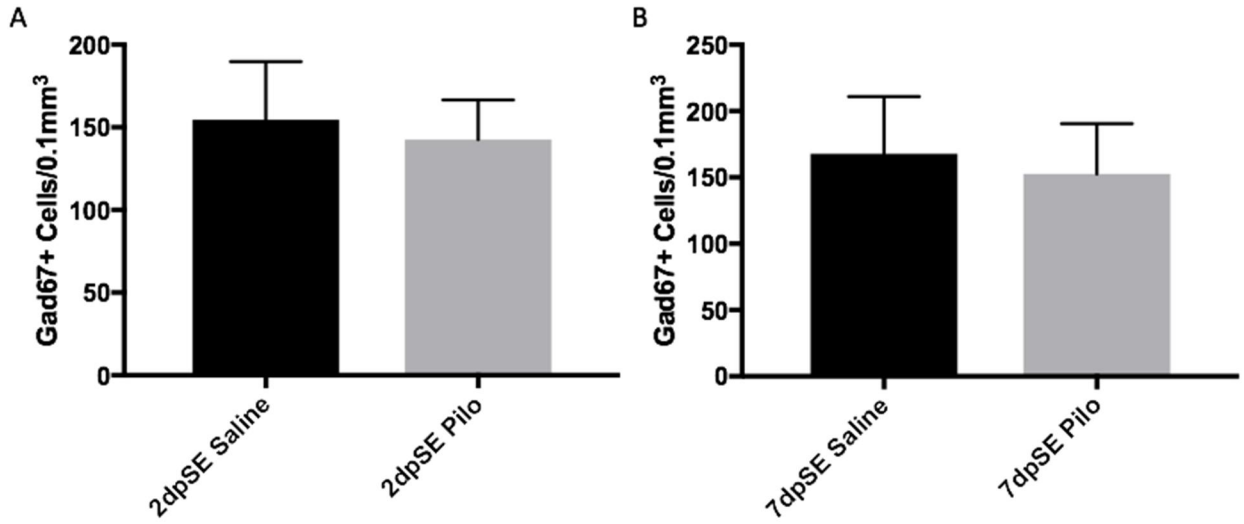

***Figure 3**: No significant changes in hippocampal inhibitory neurons after seizure activity. **A.** quantifications of Gad67+ cells in the molecular regions of the CA1, CA2, and CA3 regions indicate no decrease in inhibitory neurons at 2 days after seizure induction. **B.** A similar lack of interneuron loss was exhibited in the same molecular layers at 7 days after seizure induction. N=21*

CA2, and CA3 to determine if there any significant changes to the interneuron population were exhibited in more restricted regions of the hippocampus. Similar to the quantification of the hippocampus overall, there were little significant changes detected in the Gad67+ population (Fig. 4). However, a significant decrease in Gad67+ cells was detected in the stratum lacunosum moleculare (SLM) of the CA1 region at 2dpSE, indicating a loss of interneurons in this layer after induction of seizures.

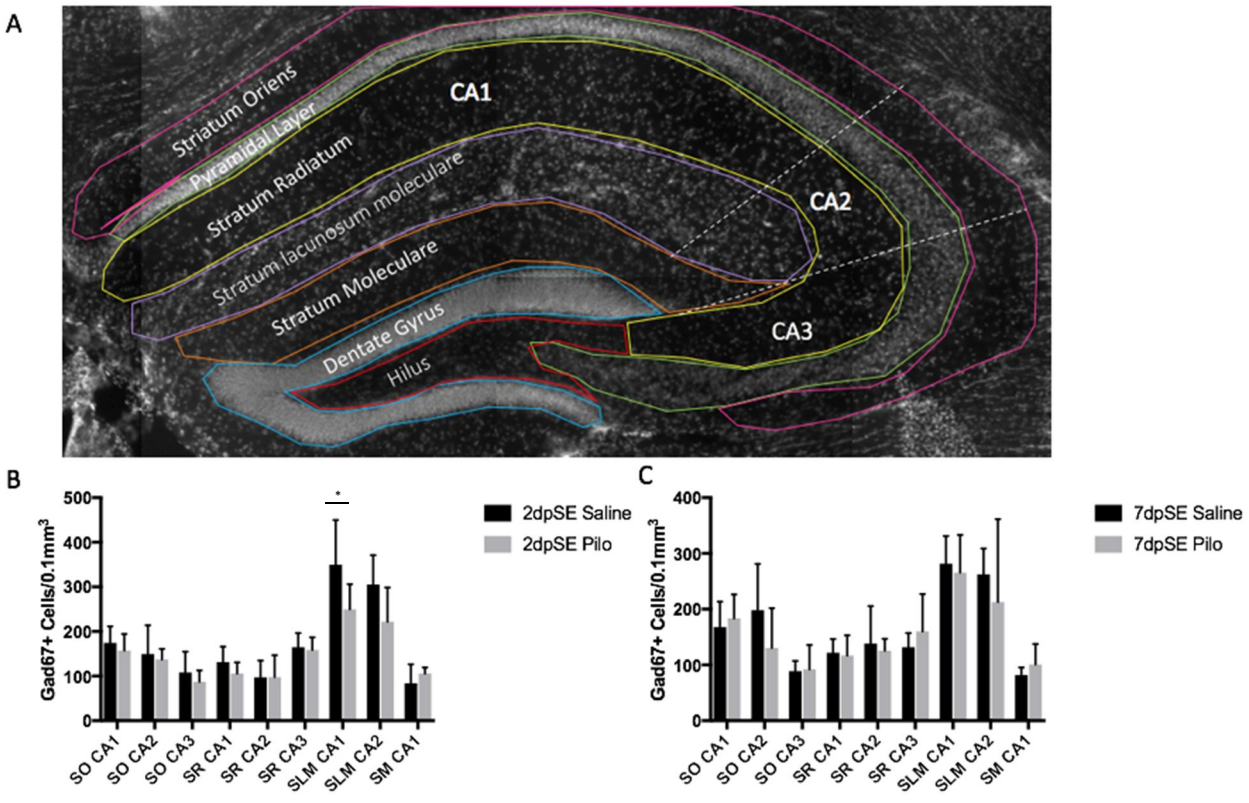

***Figure 4**: Regional changes in density of hippocampal Gad67+ cells after seizure activity. **A.** Mapping demonstrating the manner in which regions and molecular layers of the hippocampus were divided for quantifications. **B.** At 2 days after seizure induction, all layers in each region exhibited no significant changes in density of Gad67+ cells except for the SLM of the CA1 region. N=5 **C.** Similar quantification*

*of Gad67+ cells the same regions indicated no significant changes to cell density in any region at 7 days after seizure induction. N=6. *p<0.05,*

c. Proliferation in the DG

Assays for proliferation in the hippocampus revealed a drastic increase in the density of EdU+ cells in the DG of animals enduring seizures at 2dpSE, particularly in the subgranular zone (SGZ) of the DG, suggesting neurogenesis of granular cells was doubled in response to seizure activity on the second day after induction. However this proliferation was lost by 7dpSE (Fig. 5).

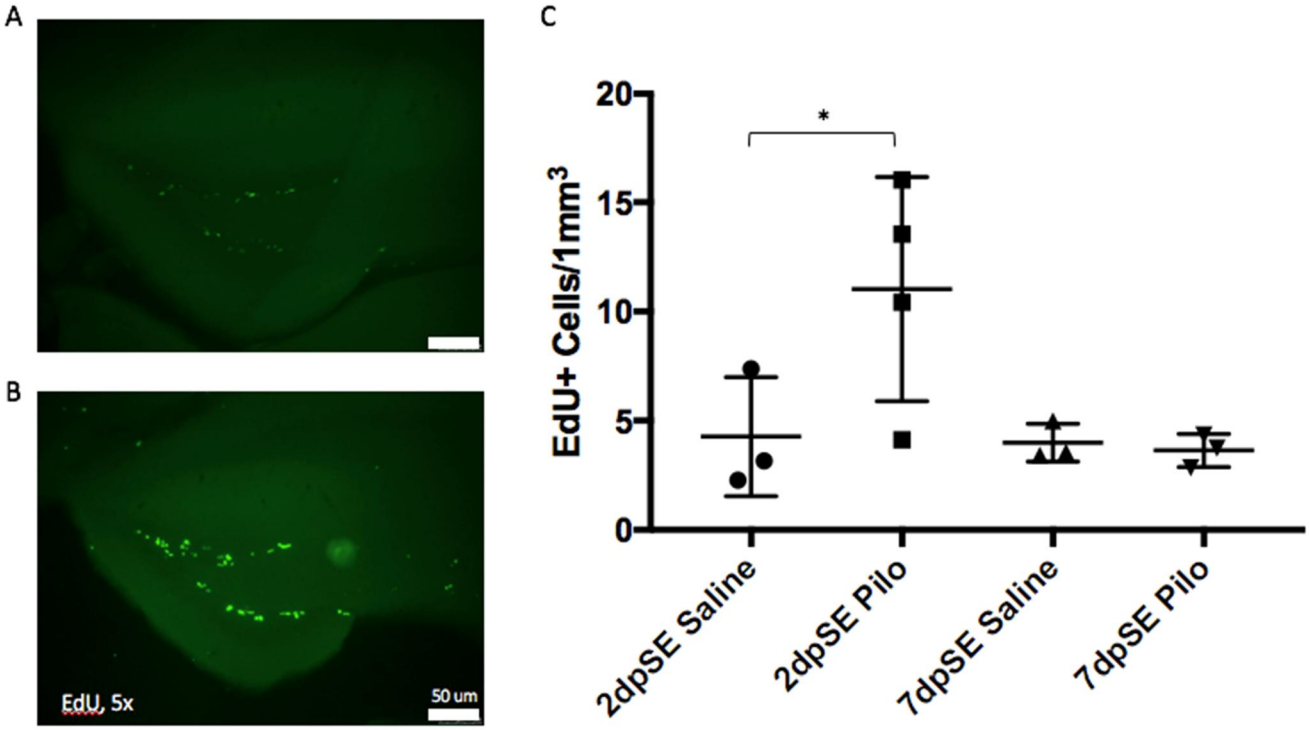

Figure 5*: Analysis of proliferation DG* ***A.*** *EdU+ cells in DG at 2dpi in saline treated NG2creER:YFP controls. Nearly all EdU+ cells localized to the SGZ.* ***B.*** *Density of EdU+ cells in the DG of NG2creER:YFP animals after seizure activity is increased at 2dpSE. Nearly all EdU+ cells again are localized to the SGZ.* ***C.*** *Quantifications of the density of EdU+ cells in the DG at 2 and 7 days after seizure induction indicate that there is a significant increase in dividing cells in the DG at 2 days, but not 7 days after induction. 2dpSE N=4; 7dpSE N=3. *p<0.05.*

d. NG2 Cell Population

IF staining for NG2 suggests a significant increase in the NG2 cell population in the hilus at 2dpSE that is then lost by 7dpSE, which falls in line with reports from previous studies on the response of NG2 cells to seizure activity (Fig. 6). However, staining for GFP, which in this model marks all cells in the lineage having at any point expressed NG2, was also performed.

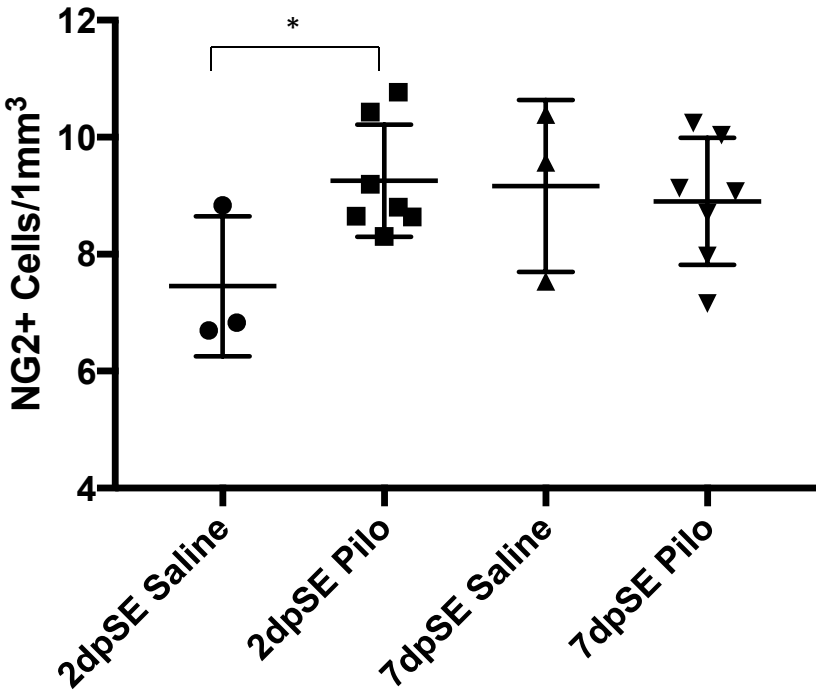

*Figure 6: NG2 cell population quantifications. Analysis suggests that there is an increase of NG2 cell density in the hilus 2 days after induction of seizures, but no similarly significant change is observed at 7dpSE. N=7. *p<0.05.*

Surprisingly, this analysis indicated there were no significant GFP+ population changes in the hilus at either time points (Fig 7). The combination of these results would suggest that there is an increase in NG2 cells in the hilus that is not resultant of an increase of cells in the lineage itself, and is accomplished in a manner other than by proliferation of NG2 cells.

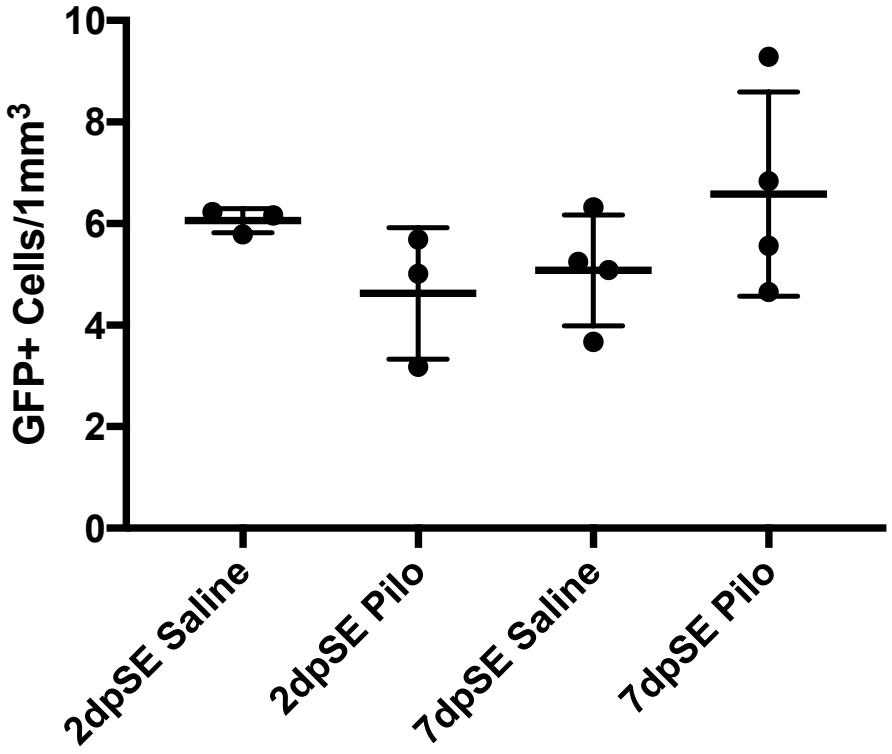

Figure 7: No significant changes in the density of GFP+ cells in the hilus at either 2 or 7 days after seizure inductions. 2dpSE N=3; 7dpSE N=4

e. Altered differentiation of NG2 cells

After the observation that an increase in NG2 cell number in the hilus occurred, without subsequent changed in the GFP+ cell population, we hypothesized that rather than an altered rate of proliferation, NG2 cells may undergo an alteration in their rate of differentiation in response to seizures. To analyze this possibility, sections were double labeled for GFP and MBP, a marker for mature oligodendrocytes. Indeed, quantifications suggest that there is a decrease in the likelihood of NG2 cells to differentiate away from their precursor state after seizure activity. This observation was made at both 2 and 7dpSE (Fig 8).

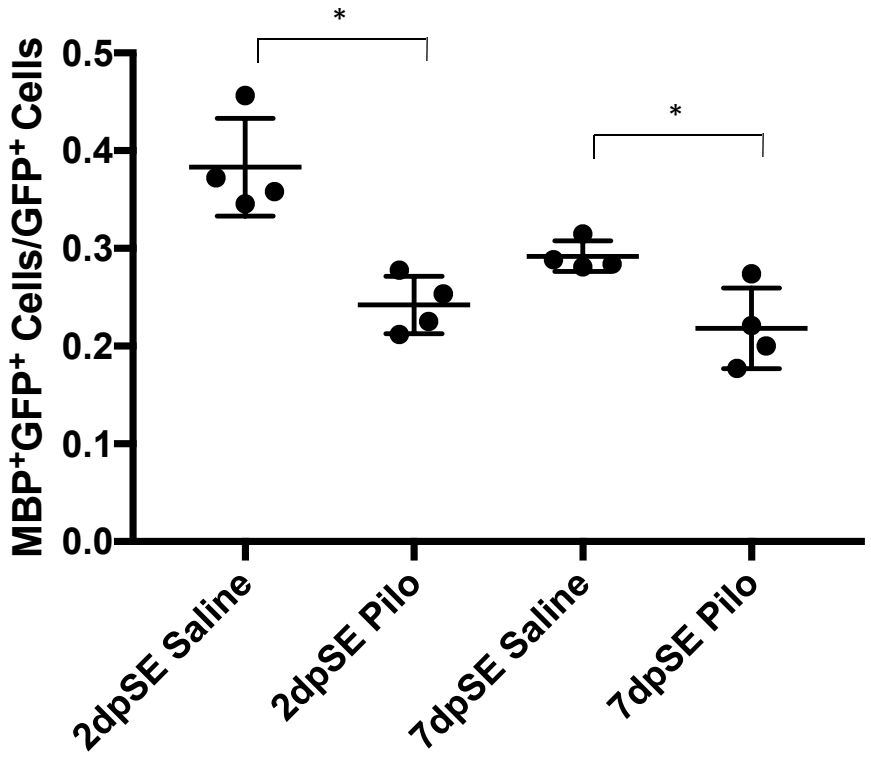

*Figure 8: Altered differentiation of NG2 cells in the hilus in response to seizure activity. At both 2 and 7 days after SE induction, a significant decrease in MBP+/GFP+ cells among all GFP+ cells is exhibited. This would suggest that more NG2 cells remain in their precursor state in response to seizures, and fewer differentiate into a mature oligodendrocyte fate in the hilus. N=4. *p<0.05.*

IV. Discussion

1. NG2creER:YFP line as model for SE

The studies performed in this text suggest some potential difficulties in the use of the NG2 reporter line for modeling SE. Despite administering pilocarpine dosages nearly double that reported in previous literature, the severity of seizures and cellular damage observed in treated animals was minimal if not absent entirely. Initially even higher doses of pilocarpine were attempted, but despite increasing the mortality to rates to those more comparable to previous studies, the severity of seizures experienced by surviving animals was not increased. The C57BL/6 background has been previously reported to present difficulty in seizure induction

(Kosobud et al. 1990, Ferraro et al. 2002, Schauwecker et al. 1997), and the results presented would suggest the NG2creER:YFP line is not an exception to this observation.

The experiments begun in the FVB and C57BL/6 mice presents preliminary data to support this theory, as the FVB mice appeared far more susceptible to seizure activity, experiencing higher stage seizures more rapidly and with a lower dose than that given to the NG2 reporter line. The C57BL/6 mice however, demonstrated similar resistance as the NG2creER:YFP mice. A kindling approach was used in these mice in an attempt to increase the severity of cellular damage without increasing mortality to an undesirable level. Despite these mice rarely progressing beyond stage 3 seizures, 2 of the 4 initial animals died or were humanely euthanized in the following days. These results are similar to those observed with the NG2creER:YFP, a low severity of seizures accompanied by a surprisingly high mortality rate. These results would indicate that more success may be gained in attempting to induce seizures in NG2 reporter mice with a FVB background, as these mice seem to demonstrate less inherent resistance to seizure induction. Also of interest, the cellular changes observed in response to seizure activity had no detectable correlation to the severity of seizures experienced by the respective animals.

2. Increased differentiation but little neuronal loss detectable

Analysis for cellular events and damage resulting from seizure induction in NG2creER:YFP mice indicate a similar difficulty in use of the line for modeling SE. For an animal to be validated as a representative of the disorder, there must be the reasonable expectation that these animals would present spontaneous and recurrent seizures after the initial induced seizures. Of course, with the need to examine time points early after initial SE, it is impossible to use the spontaneous seizures as validation of the model in such cases, as there is a

prolonged latent period that precedes the occurrence of spontaneous seizures. Therefore, observation of early cellular changes is vital to validate animals as true models for SE.

The failure of the NG2creER mice to exhibit substantial neuronal loss thus casts doubt on how faithfully these animals replicate the disorder. The occurrence of spontaneous seizures is believed to be reliant on a loss of inhibition and subsequent gain of excitation. All analysis performed in this study presents little indication of loss of interneurons, an event consistently presented in previous models of SE and thought to be necessary for the loss of inhibition underlying seizure activity. Interestingly, the only region that experienced a significant reduction in Gad67+ cell density was the SLM of the CA1 region in the hippocampus. There is literature to suggest that interneurons in this region play a role in gating the input of the EC to the pyramidal cells of the CA1 regions (Lacaille et al 1988, Capogna 2011). Loss of these interneurons presents some implications that these animals may experience a reduction in inhibition, but this is a modest event at best and still is drastically less than the interneuron loss exhibited in previous studies. Furthermore, there is a substantial amount of diversity among interneurons in the CA1 SLM alone, and further characterization of the interneurons lost in this region would be needed before making any such claims on the implications of their loss.

The only cellular event exhibited by the NG2creER:YFP mice after seizures that provides any amount of confidence in their potential to experience spontaneous seizures is the increased proliferation observed in the DG at 2dpSE, marked by the increased density of EdU+ cells quantified in the SGZ of the DG. Mature granule cells are excitatory, and the proliferation and branching that they are observed to undergo after seizures is thought to contribute to the over-abundance of excitation underlying the future occurrence of spontaneous seizures. Without allowing the mice to survive past the latent period, it is not possible to determine if this

proliferation would be sufficient to endow spontaneous seizures without the subsequent loss of inhibition.

The occurrence of increased DG proliferation with a lack of neuronal loss presents an interesting interpretation on the role of NG2 cells in epilepsy. As is reported in previous studies, an increase in the NG2 cell population was indeed observed in the hilus after seizure induction. However the results of this study suggest that neuronal loss may not be necessary for eliciting a response from the NG2 cell population, and an increase in differentiation of excitatory cells may be sufficient on its own. The interpretations of this study therefore cannot be made for the response of NG2 cells to SE, but may still present novel information on their response to induced seizures.

3. Decreased differentiation of NG2 cells

The dual observations of increased NG2 cell density in the hilus with no subsequent increase in GFP+ cells led to the examination of the rate of NG2 cell differentiation in response to seizure activity, rather than NG2 cell differentiation. As theorized, stains for MBP, a marker for mature oligodendrocytes, and GFP suggest that differentiation of NG2 cells is reduced both at 2 and 7 days after seizure induction. This decrease in differentiation presumably is the underlying cause of the increase in NG2 cells in the hilus at 2 days, rather than a change in their proliferative potential as initially hypothesized. This reasoning does not however explain the cellular events quantified at 7dpSE. As is the case at 2dpSE, differentiation of NG2 cells to mature oligodendrocytes is reduced at 7dpSE. Quantifications for GFP+ cell densities at 2 and 2dpSE also indicate similar responses, neither presenting any significant changes in cell density. However the reasoning for the increased NG2 cell population at 2dpSE is contradicted by the lack of a similar change in NG2 cell density at 7dpSE. If differentiation of NG2 cells to

oligodendrocytes is still reduced at 7 days, it would be expected that a similar increase in NG2 cells in the hilus would be observed. The eventual fate of these NG2 cells is yet to be determined, it is known that NG2 cells are capable of migration, and under specific manipulations to be capable of differentiation to astrocytes or interneurons. These possibilities need further investigation to resolve this discrepancy.

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