UNIVERSITY OF CALIFORNIA SAN DIEGO

In Vivo Reprogramming of Immature Adult-born Dentate Granule Cells

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Yu Cai is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

In Vivo Reprogramming of Immature Adult-born Dentate Granule Cells

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Mesial temporal lobe epilepsy (mTLE) is the most common form of temporal lobe epilepsy in adults, and is characterized by seizure activities and pathological changes in the limbic region, including the hippocampal formation. The dentate gyrus (DG) is thought to be a critical node in the hippocampus, with its unique properties, to prevent hippocampus from overexcitation. DG is also one of the only two places in mammalian brain that has new neurons born during adulthood. In both human and animal mTLE models, dramatic changes in migration and wiring of immature new-born dentate granule cells cause them to form pathological excitatory connections with neighboring DGCs. This has been considered as a major contributor to hippocampal hyperexcitability and epileptogenisis in mTLE. Studies have shown that grafting GABAergic inhibitory interneurons into epileptic mTLE mouse model can significantly reduce seizure activity. However, isolating safe, effective and ethical

source of inhibitory precursor cells could be challenging for human patients. Hence, we hypothesize that using retrovirus *in vivo* to overexpress complement inhibitory transcription factors in aberrant DGCs can reprogram them into GABAergic inhibitory interneurons. In order to reach that goal, we first identified *Sox2* transcription factor as a way to maintain or reverse the development of adult-born immature DGCs into a multipotent state. However, in the process, we have noticed substantial fluorescent signals in the hilus region, which should not contain any dividing cells. With multiple staining, we have discovered that the extensive fluorescent signals largely mossy cells that appeared to be infected with retrovirus. With further experiments, we found that red fluorescent gene *mScarlet* was the root cause. We later replaced *mScarlet* with another red fluorescent reporter gene *dTomato* and did not see any red fluorescent signal in the hilus region. Future work would involve making a retrovirus construct that contains *Sox2* and alternative red fluorescent proteins such as *dTomato* as well as an inducible *Sox2* construct that will give us temporal control. Moreover, we will investigate the possible combination of inhibitory GABAergic and proneural transcription factors *Lhx6*, *Dlx5*, *Ascl2* and *Ngn2*, and co-inject with *Sox2* construct.

CHAPTER 1

INTRODUCTION

MTLE is the most common form of temporal lobe epilepsy in adults affecting about 50 millions people worldwide (Brodie et al. 1997), and is characterized by seizure activity and pathology within the medial temporal limbic areas, including the hippocampal formation (Priel et al. 1996; Hsieh et al. 2015; Ramanathan et al. 2017). Patients suffering from mTLE experience substantial impairment resulting from recurrent seizures, associated cognitive and psychiatric comorbidities and significantly increased mortality (Priel et al. 1996). Patients with mTLE are usually classified as having focal epilepsy and manifest as focal onset seizures with or without impaired awareness (Nayak et al. 2021).

While the current antiepileptic medications can reduce seizure frequency, the effect on epileptogenesis is limited and they have a variety of disabling side effects. Although invasive surgical treatment, such as antereomesial temporal resection (Engle et al. 2001), can control seizures, it brings the possibility of impairing cognitive function. Thus, while there have been improvements in mTLE treatment, novel therapies are much needed.

Evidence points to the hippocampal formation as being a critical site of pathogenesis in mTLE. Hippocampal sclerosis, involving atrophy and astrogliosis of the hippocampal formation, is the most common pathology encountered in mTLE and is associated with a poor prognosis (Priel et al. 1996). In both human and animal studies, evidence show mTLE demonstrates significant alterations in the migration and wiring of immature adult-born DGCs, which form pathological excitatory connections with neighboring DGCs (Hsieh et al. 2015; Gage et al. 2016; Danzer et al. 2016). Hence, understanding the regulations of activities in the hippocampus and

how the impairment of those regulations cause recurrent seizure is critical for epileptogenesis in mTLE.

The Dentate gate theory of mTLE

Sensory information is represented throughout the cerebral cortex and is funnel by entorhinal cortex to the dentate gyrus, which is the first stop in the trisynaptic loop of entorhinal-hippocampal circuitry within the temporal lobe (Esther et al. 2015). Despite continuous input from the entorhinal cortex, the firing rates of DGCs are minimal (Close et al. 2017). This controlling nature of dentate gyrus is thought to be important not only for proper memory formation and recall but also for controlling the activity spread throughout the rest of hippocampus. Recent studies suggest that in epileptic animals, the controlling ability of dentate gyrus is severely impaired (Esther et al. 2015).

Hilar interneurons are essential for the dentate gyrus

In the dentate gyrus, there contains several different types of cell population that are considered important to maintain the dentate gate, including hilar interneurons. Hilar interneurons are found deep to the granular cell layer within the hilus. In both human and animals studies on mTLE, the loss of these inhibitory interneurons are found (Hosford et al. 2016). Since hilar interneurons have intrinsic firing rate and provide inhibitory feedback to DGCs, the loss found in mTLE cases is thought to be a significant pathological change. Moreover, study has shown that through grafting embryonic inhibitory progenitor cells in hippocampus of pilocarpine mice, seizure frequency have been reduced by 92% compare to untreated animal (Hunt et al. 2013).

Mossy fiber sprouting is considered pro-epileptogenic

Another prominent histopathological finding in mTLE is mossy fiber sprouting (Carvasan et al. 2018). In healthy rodent model, the granular cell axons, also named mossy fibers, extend to the hippocampal area CA3 as well as hilus where they project to excitatory neurons called mossy cells and inhibitory interneurons.(Carvasan et al. 2018). Mossy fibers are thought to have little to no recurrent synapses onto other granule cells. However, in mTLE patient and animal models, abnormalities are observed where mossy fiber from immature DGCs extends to the molecular layer and innervate other DGCs to form excitatory feedback. (Sutula et al. 1989, Carvasan et al. 2018, Jack et al. 2012). This is thought to contribute to dentate gyrus hyperexcitability and seizures in mTLE.

En masse, these changes of DGCs are thought to be major contributing factors for hippocampal hyperexcitability and epileptogenisis in mTLE (Hsieh et al. 2015; Gage et al. 2016; Danzer et al. 2016). In addition, there is a growing body of evidence suggest that much of this hyperexcitability can be reversed by introducing GABAergic interneurons to the dentate gyrus through embryonic stem cell graft or through *in vitro* reprogramming. (Lentini et al. 2021; Colasante et al. 2015; Hunt et al. 2013). Medial ganglionic eminences (MGE) are progenitor cells for GABAergic interneurons during embryonic brain development. After grafting into the hippocampus of pilocarpine mice, seizure frequency was reduced by 92% compared to untreated mice (Hunt et al. 2013). *In vitro* reprogramming is another promising way to reintroduce inhibitory GABAergic interneurons into the hippocampus. Through grafting inhibitory interneuron derived from astroglia, kainate mice has demonstrated dramatic reduction in seizure frequency compare to untreated mice (Lentini et al. 2021). Moreover, Lentini et al. targeted glia

cells for *in vivo* reprogramming. They introduced retrovirus, with multipotent transcription factor and GABAergic inhibitory factor, into the hippocampus of kainite mice has significantly reduced both seizure duration and frequency (Lentini et al. 2021). However, they didn't show any marker for glia cells as their targets.

Isolating a safe, efficacious, and ethical source of inhibitory precursor cells for treatment of mTLE continues to be a challenge. There is a crucial need for effective yet ethical way for treating mTLE patients.

We hypothesized that *in vivo* reprogramming of the immature adult-born DGCs into GABAergic inhibitory interneurons could be an innovative approach to reduce seizures in mTLE. We plan to use retrovirus to overexpress inhibitory neuronal transcription factors in immature adult-born DGCs, turning those cells into GABAergic inhibitory interneurons. In order to accomplish this, we plan to use *Sox2* transcription factor to maintain or reverse the newborn DGCs to multipotent state, which would provide enough time for inhibitory transcription factors to function.

CHAPTER 2

Development of a viral vector to maintain multipotent state of immature DGCs

2.1 Results

Adult C57bl mice were used throughout the experiments and they were injected with retrovirus bilaterally. All experiment sites are shown on the right in the figures below and control injections are shown on the left . The experiment side was injected with retrovirus expressing a transcription factor and a fluorescent protein separated by p2A sequence. The control side was injected with retrovirus containing only a fluorescent protein and driven by the same promoter as the experimental site. Unless stated otherwise, the long terminal repeat (LTR) is used as the promoter. Due to the short size of LTR, it is an ideal candidate for sufficient packaging efficiency while maintaining transcription efficiency.

2.1.1 *Sox2* transcription factor halts development of new-born dentate granule cells

Sox2 is an HMG domain transcription factor that is critical for maintenance of pluripotency and neurogenesis. In previous study, *Sox2* has been used to successfully used in production of GABAergic interneurons through viral vector delivery (Colasante et al. 2015). Due to determined role of *Sox2* in progenitor cell multipotency, it was used to drive fibroblasts towards a multipotent state, followed by expressing GABAergic factors to drive multipotent progenitors along the GABAergic differentiation pathway (Colasante et al. 2015). Under the same schematic, in order to explore if *Sox2* will function in DGCs, we injected retrovirus expressing *Sox2* and the red fluorescent protein *mScarlet* in the murine dentate gyrus and performed histology at different

time points. We have noticed at the experimental site, red *mScarlet*+ cells only appeared in the subgranular zone. While on the contralateral site, because the control virus does not contain *Sox2* transcription factor, we saw *mScarlet*+ cells (in circle) have migrated into granular zone (Figure 1). In addition to that, we didn't discover any visual dendritic development at experimental site. But, at control site, we saw some dendritic development for the cells (in circle) that have migrated in to granular zone (Figure 1). This provides visual evidence for the cells are held at multipotent state with the injection of virus containing *Sox2* transcription factor. Furthermore, there is no DCX signal overlapping with *mScarlet* signal at experiment site, which is consistent with delayed development in cells expressing *Sox2* DGCs (data not shown). Additionally, we have noticed a significant number of red cells in the hilus region, which is odd because there shouldn't be any dividing cells in the hilus region. This observation will be discussed later.

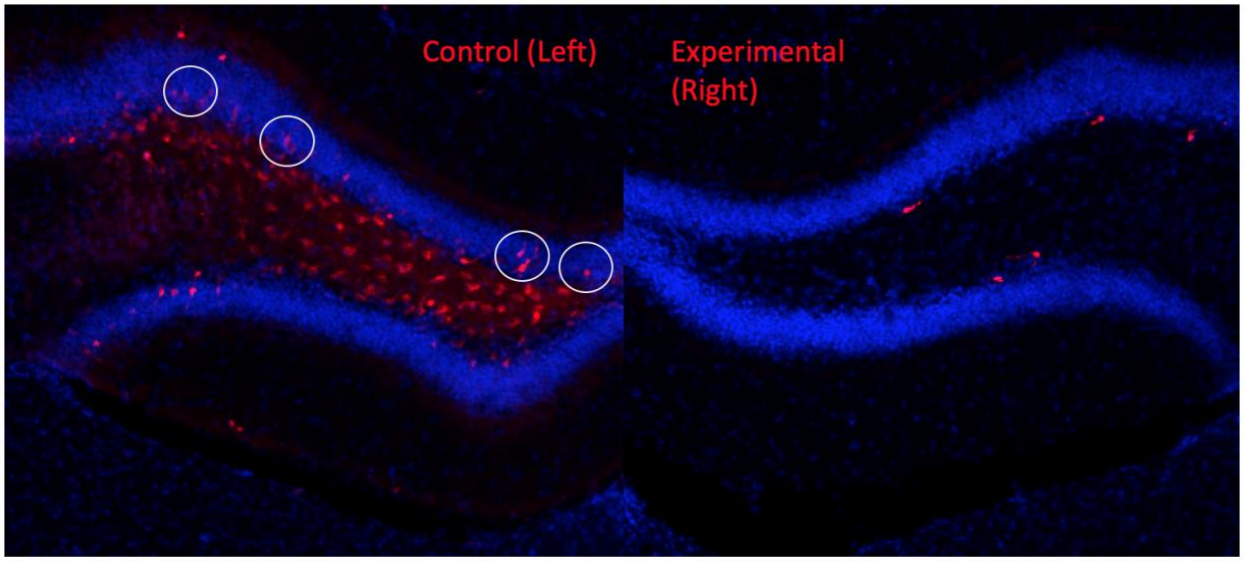

Figure 1: Visual inspection of retrovirus expression after injection

Mice were bilaterally injected with RV-LTR-mScarlet at control site and RV-LTR-Sox2-mScarlet at experiment site within the WT C57bl mouse hippocampus. Mouse was perfused 2 weeks post injection. Red signal represents *mScarlet*. Blue signal represents Dapi staining.

2.1.2 Confirmation of cells in the subgranular layer are *Sox2* positive

Since we have seen visual evidence that immature DGCs are held in an immature state at the side with virus injection, we need to confirm that the change happened at experiment site is caused by *Sox2* transcription factor. In order to do that, we stained the slices with *Sox2* antibody. We discovered that for both 3 days and 2 weeks post-injection, *mScarlet* fluorescent didn't overlap well with *Sox2* antibody staining at the control site but overlapped well at experiment site (Figure 2.1 and 2.2). At 3 days, we saw limited signal at experimental site (Figure 2.1). This could due to the short period of time of expression. At 2 weeks, we saw increased amount of signals overlap at experimental site (Figure 2.2). This indicates that the change of cells at experiment site is caused by the expression of *Sox2* transcription factor.

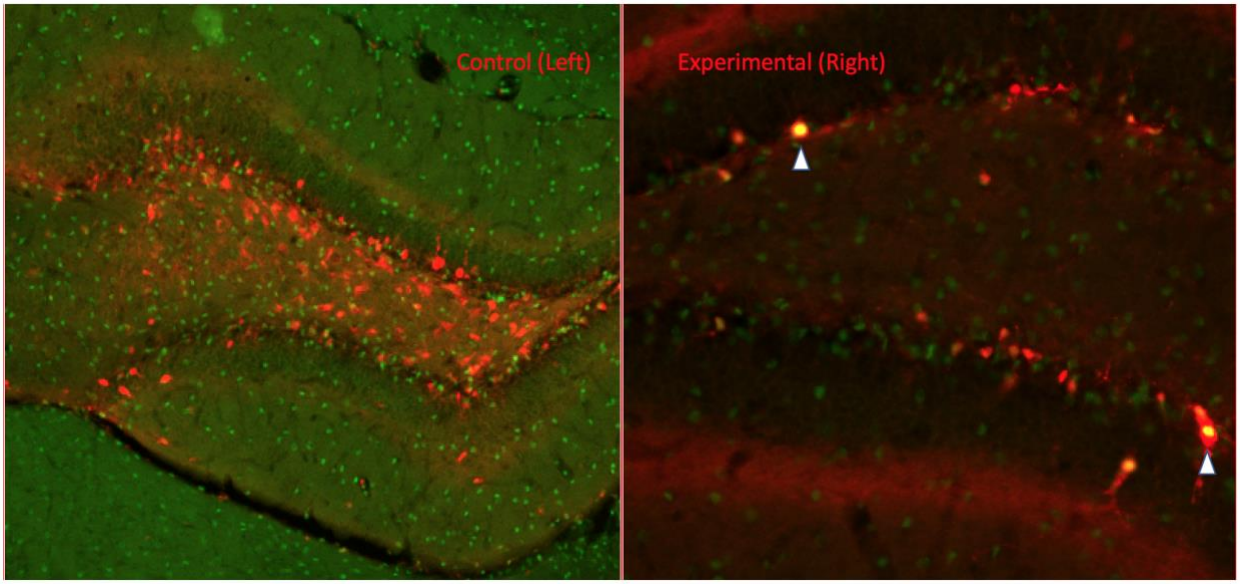

Figure 2.1: Confirmation of Sox2 expression with Sox2 antibody staining for 3 days post injection mice

Mice were bilaterally injected withRV-LTR-mScarlet at control site and RV-LTR-Sox2-mScarlet at experiment site within the WT C57bl mouse hippocampus. Mouse was perfused 3 days post injection. Red signal represents *mScarlet*. Green signal represents *Sox2* antibody.

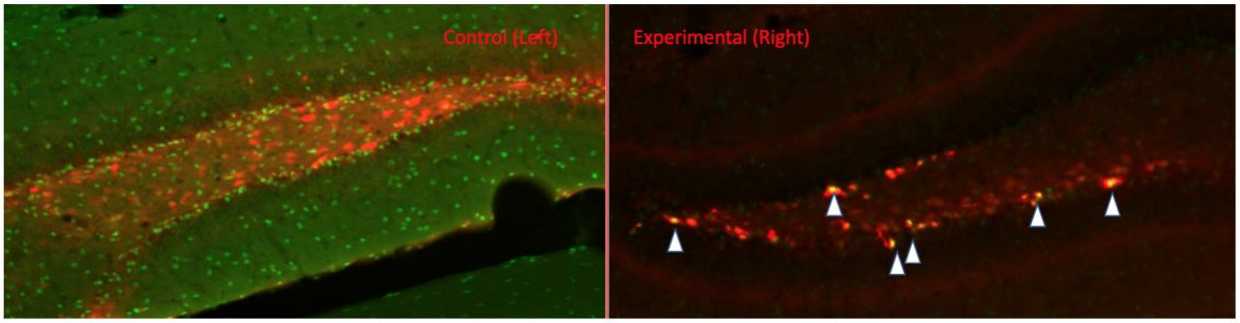

Figure 2.2: Confirmation of *Sox2* expression with *Sox2* antibody staining for 2 weeks post injection mice

Mice were bilaterally injected with RV-LTR-mScarlet at control site and RV-LTR-Sox2-mScarlet at experiment site within the WT C57bl mouse hippocampus. Mouse was perfused 2 weeks post injection. Red signal represents *mScarlet*. Green signal represents *Sox2* antibody.

2.1.3 *Sox2* positive cells are not dividing

We have the morphological evidences for immature DGCs expressing *Sox2* transcription factor are held at immature stage. However, we still need to test for its dividing ability. Hence, we stained the slices against Ki67. At both 3 days and 2 weeks post injection, there is little to none overlapping with Ki67 signal and *mScarlet* signals (Figure 2.3 and 2.4). Similarly, at experiment site at both time points, the red and green signals were well separated with no overlapping (Figure 2.3 and 2.4). With these images we can say the immature DGCs are not dividing, hence held in a quiescent state with the expression of *Sox2* transcription factor.

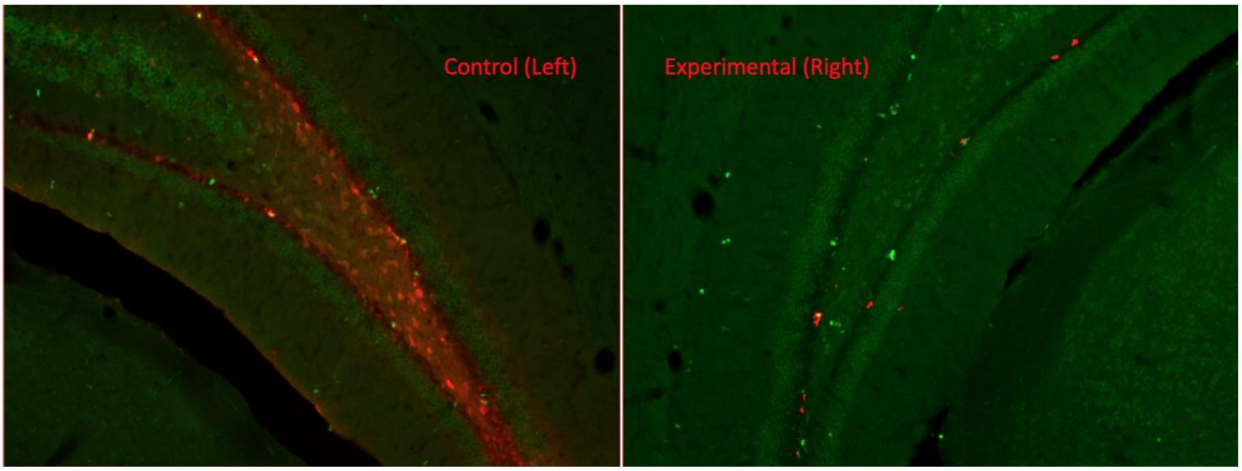

Figure 2.3: Confirmation of cell proliferation status with *Ki67* staining for 3 days post injection mice

Mice were bilaterally injected with RV-LTR-mScarlet at control site and RV-LTR-Sox2-mScarlet at experiment site within the WT C57bl mouse hippocampus. Mouse was perfused 3 days post injection. Red signal represents *mScarlet*. Green signal represents Ki67.

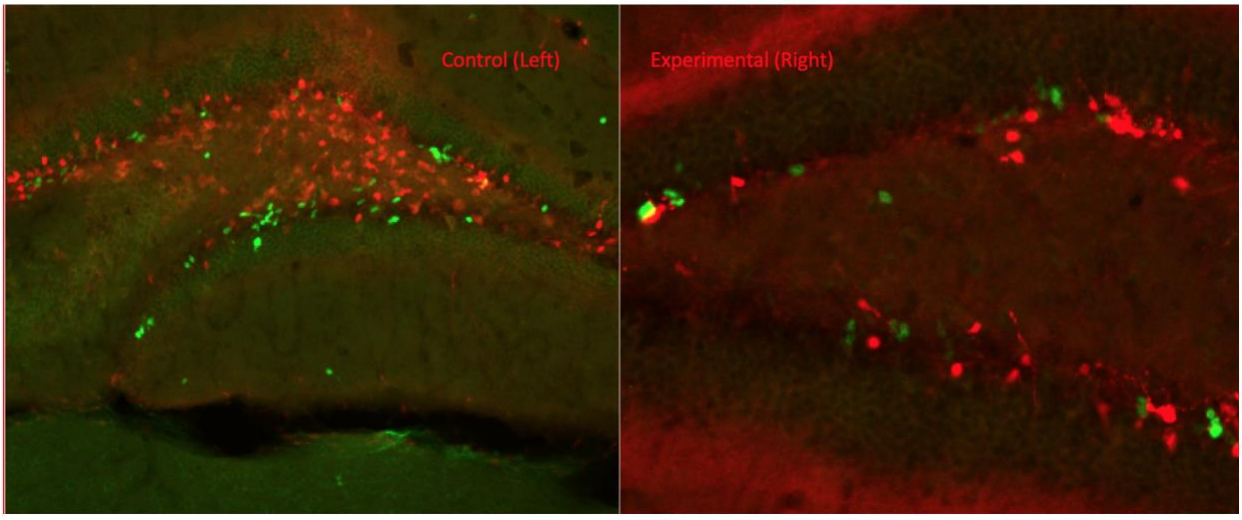

Figure 2.4: Confirmation of cell proliferation status with *Ki67* staining for 2 weeks post injection mice

Mice were bilaterally injected of RV-LTR-mScarlet at control site and RV-LTR-Sox2-mScarlet at experiment site within the WT C57bl mouse hippocampus. Mouse was perfused 2 weeks post injection. Red signal represents mScarlet. Green signal represents Ki67.

2.1.4 Red fluorescent signals in the hilus are not mature DGCs

Thus far all images on the control side , demonstrate significant number of red fluorescent cells in the hilus region However, retrovirus is known to only infect dividing cells, which should be rare in this region that contains a number of cells types including inhibitory neurons, moss cells, and dentate granule cells with aberrant migration. Thus we hoped that by identifying these we could better understand the nature of *mScarlet* labeling in this region. We first hypothesized that the immature DGCs infected with the retrovirus might exhibit aberrant migration into the hilus as is seen after seizures and some neurodevelopmental disorders. In order to test our theory, we stained injected brains for *Prox1*, a marker specific for dentate granule cells. As we can see in Fig. 3, there is no overlapping of *Prox1* signal and *mScarlet* signal 20 days post injection at both control and experiment sites. This shows the retrovirus didn't infect DGCs. We also stained for inhibitory markers such as GAD67 and there was no overlap with *mScarlet+* hilar cells (data not shown), indicating that these were not inhibitory neurons.

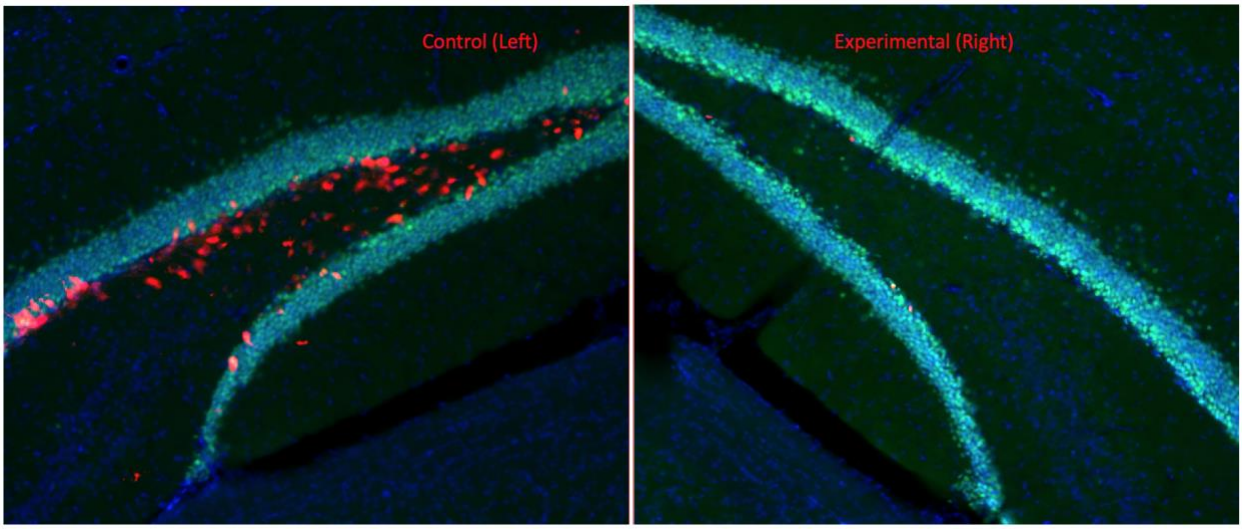

Figure 3: *Prox1* staining revealed infected cells in the hilus are not mature DGCs

Mice were bilaterally injected with RV-LTR-mScarlet at control site and RV-Sox2-mScarlet at experiment site within the WT C57bl mouse hippocampus. Mouse was perfused 20 days post injection. Red signal represents *mScarlet*. Green signal represents Prox1

2.1.5 Mossy cells in the hilus were infected with retrovirus with *mScarlet*

In order to test if *mScarlert+* cells were mossy cells, we stained with the mossy cell marker GluR2/3.. Fig 4.1 and Figure 4.2 show that the GluR2/3 signal overlapped well with *mScarlet* fluorescent at control site for both 3 days and 2 weeks post-injections mice but those two signals didn't overlap well in the contralateral side injected with retrovirus expressing Sox2 and *mScarlet* (Figure 4.1 and Figure 4.2). These experiments are consistent with mossy cells in the hilus being infected with retrovirus.

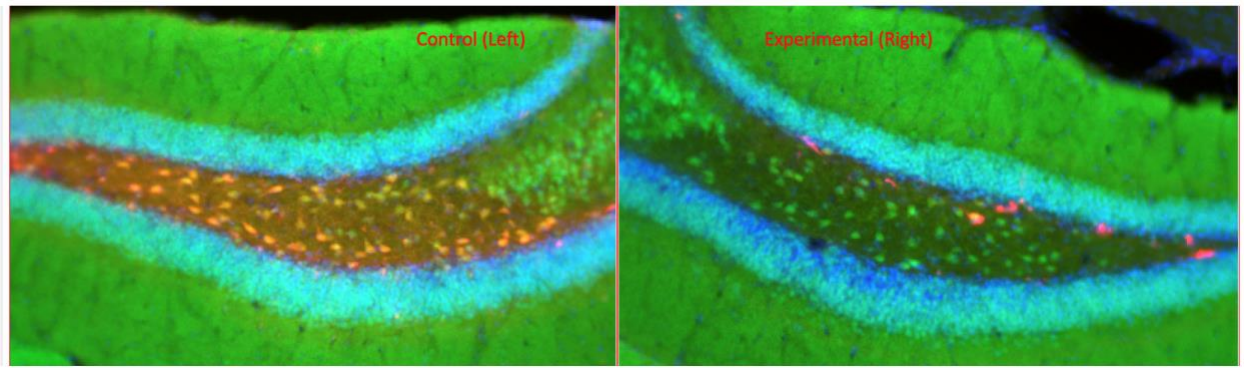

Figure 4.1: Confirmation of stained cells in the hilus are mossy cells with 3 days post injection mice

Mice were bilaterally injected with RV-LTR-mScarlet at control site and RV-LTR-Sox2-mScarlet at experiment site within the WT C57bl mouse hippocampus. Mouse was perfused 3 days post injection. Red signal represents *mScarlet*. Green signal represents GluR2/3

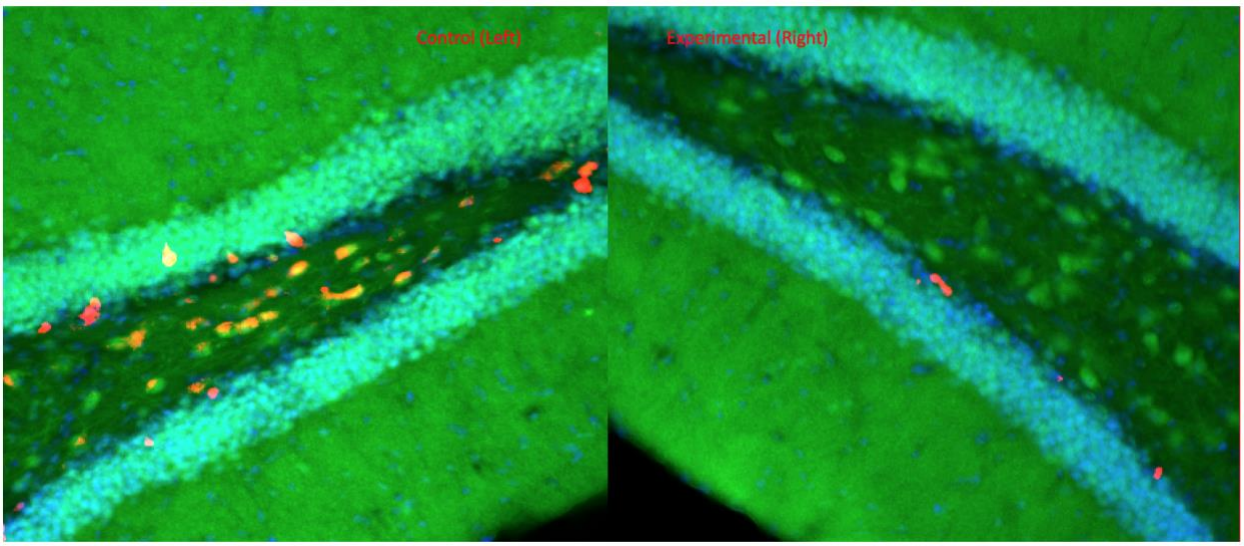

Figure 4.2: Confirmation of stained cells in the hilus are mossy cells with 2 weeks post injection mice

Mice were bilaterally injected with RV-LTR-mScarlet at control site and RV-LTR-Sox2-mScarlet at experiment site within the WT C57bl mouse hippocampus. Mouse was perfused 2 weeks post injection. Red signal represents *mScarlet*. Green signal represents GluR2/3

2.1.6 *mScarlet* could be the cause for extensive retrovirus infection in the hilus

Mossy cells are born during late embryonic and early postnatal development and do not continue to proliferate during adult hood. Thus, the labeling of mossy cells with retrovirus was unexpected and the reason for this phenomenon is unknown. One possible explanation is that the viral LTR promoter may be inducing aberrant expression in our system. Alternatively, we hypothesized that the *mScarlet* gene or protein might causes this phenomenon. In order to investigate whether *mScarlet* causes this atypical labeling, we later injected a retrovirus with the same backbone and promoter, but substituted *GFP* for *mScarlet* (RV-LTR-eGFP) bilaterally to explore if changing the fluorescent protein would eliminate this effect. Both 4 weeks and 5 weeks post-injection images showed minimal *GFP*+ cells in the hilus region (Figure 5). *GFP*+ cells demonstrated clear dendritic trees and appropriate migration to the subgranular zone (Figure 5), which suggests retrovirus infecting the correct target. These results are consistent with *mScarlet*, and not the LTR promoter, causing aberrant retroviral infection of cells in the hilus region.

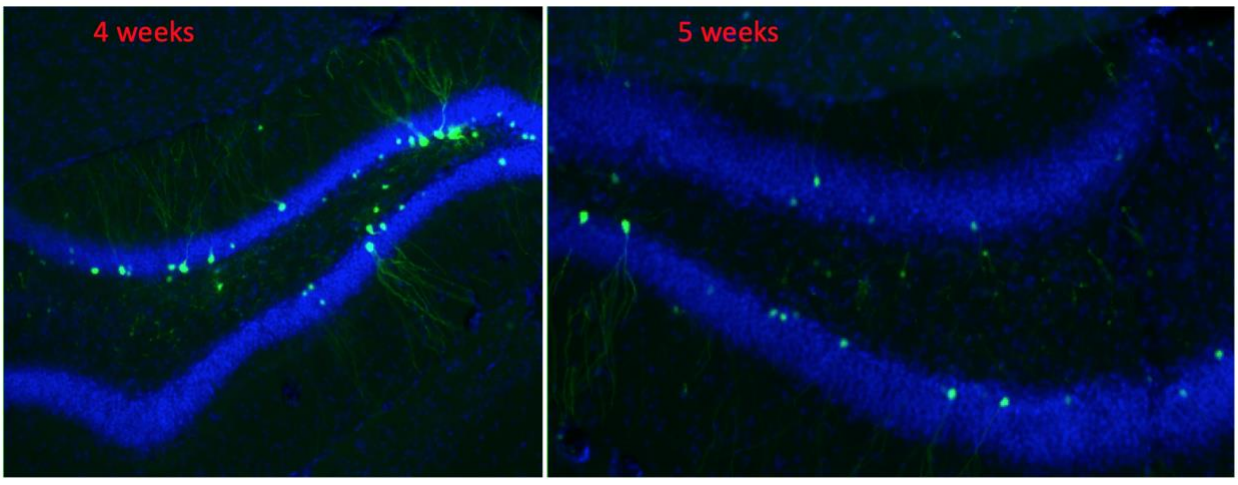

Figure 5: *mScarlet* is the root cause for abnormal infection pattern in the hilus

Mice were injected with RV-LTR-eGFP within the WT C57bl mouse hippocampus. Mice were perfused at various time points. Blue signal represents Dapi. Green signal represens GFP.

2.1.7 *dTomato* would be a possible replacement for *mScarlet*

Future studies aiming to reprogram DGCs using a 2 step approach with *Sox2* and a combination of inhibitory transcription factors, will require infection with multiple retroviruses expressing different fluorescent tags. Thus, there is a need to replace *mScarlet* with another red fluorescent reporter. an alternative candidate is *dTomato*. *dTomato* with its monomer structure provides good expression and stable bright fluorescence with a modest size, allowing sufficient space for *Sox2* and inducible elements. Also, LTR driven expression can be silenced in neurons after 4 weeks (data not shown). Thus, we also introduced a more stable promoter with relatively short length, CBh. Hence we constructed RV-CBh-dTomato and injected it bilaterally to investigate if CBh and *dTomato* would be an appropriate replacements for LTR and *mScarlet* respectively. Fig. 6 shows that red fluorescent cells were constrained within the subgranular zone with strong fluorescence and no expression in the hilus region. This is a good indicator that *dTomato* can replace *mScarlet* as a replacement red fluorescent protein and we can expect it to function properly for future experiments.

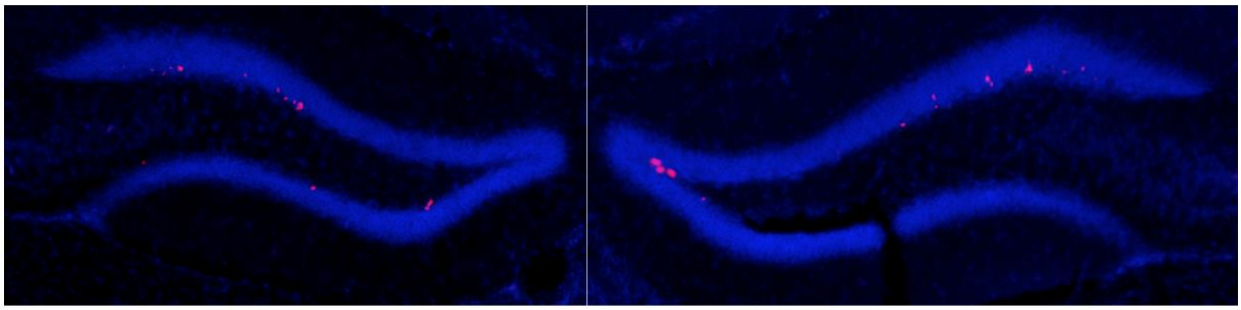

Figure 6: *dTomato* as a replacement for *mScarlet*

Mice were injected with RV-CBh-dTomato within the Cas9 mouse hippocampus. Mice were perfused at 10 days after injection. Blue signal represents Dapi. Red signal represents *dTomato*

2.2 Methods

2.2.1 Molecular cloning

Transcription factors were expressed under control of LTR promoter, together with *mScarlet* or *eGFP* as fluorescent reporter to visualize transduced cells. We generated a retroviral backbone allowing for polycistronic expression of *Lhx5* and *Dlx5* (connected via 2A sequence) under control of LTR promoter together with *eGFP* for visualization. We also generated a retroviral backbone for *Sox2* expression under control of LTR promoter with *mScarlet* as fluorescent visualization. For control experiments, we used containing *eGFP* and *mScarlet* only plasmids driven by the same LTR promoter: RV-LTR-eGFP and RV-LTR-mScarlet. Later, we changed LTR promoter to CBh promoter. *mScarlet* was also replaced with *dTomato* through standard PCR cloning techniques to generate plasmid RV-CBh-dTomato.

2.2.2 Retrovirus production and cell culture

Human embryonic kidney 293 cell derivative 293T cells were grown in MEF medium seeded. Once the cells were 70% confluent, transfection complex including cloned plasmid,

MSCV retroviral vector and PEI (1mg/ml) was given into the medium. 8 hours later fresh MEF medium was used for medium change. First collection of medium happened at 48 hours after the medium change. Second collection of medium happened at 72 hours after the medium change. Retrovirus was separated from the medium through ultracentrifuge spinning, and was resuspended in DPBS+Glucose.

2.2.3 Injection of retrovirus

Stereotactic injection was used to deliver retrovirus into the hippocampus of C57/BL mice. Bilateral injection was performed on single mice. Mice were housed in enrichment cage at Sanford Consortium mouse colony after injection and sacked at different time point. Different recombinant retrovirus were paired for different experimental groups.

2.2.4 Slice preparation and immunochemistry

Immunostaining was performed as described previously (Heinrich et al., 2010). Mice were anesthetized and perfused with 4% PFA. The brains were removed and post-fixed in 4% PFA at 4℃ for 4 hr. The brains were then sliced in coronal sections using microtome and stored in PBS. Brain slices were stained against different antibodies for different targets and were looked at under fluorescent microscope. All color channels were later combined with ImageJ.

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Chapter 2 contains unpublished material coauthored with Ryba, Bryan E.; Wang, Shuhe; Kim, Stacy and Martinez, Adrian J.. The thesis author was the primary author of this chapter.

CHAPTER 3

DISUCSSION

Throughout these experiments, we did have a lot of negative results such as *mScarlet* signal saw in the hilus where there shouldn't be any dividing cells for retrovirus to infect. With comparison to images from *eGFP* and *dTomato, eGFP* and *dTomato* images show significantly less signal in the hilus region. With that we can conclude it was the *mScarlet* fluorescent protein that causes overexpressed signal in the hilus region. Moreover, staining with *GluR2/3* allowed us to find that *mScarlet* signal overlapped well with *GluR2/3* signal at the control sites, which could be an indication of mossy cells. But we don't understand how could retrovirus infect those post-mitotic. We suspect mossy cells under certain circumstances could have higher affinity to those virus, alongside with possible change in the biology of retrovirus brought by *mScarlet,* in combination, they caused retrovirus to infect mossy cells in the hilus. However, we need to do further experiments to confirm this theory.

We noticed that there is a significant fluorescent signal difference between control site and experiment site, where control site had significant higher amount of signal than experimental site. We suspect it's because of the combination of *Sox2* and *mScarlet* that leads to this issue, but we need to conduct further experiment to confirm this theory.

For the future experiment setup, we plan to make RV-CBh-Sox2-dTomato construct and repeat the previous experiments. From previous images, we showed that cells infected with retrovirus containing *Sox2* didn't show apical dendrite progression and didn't migrate to granular zone, which are indicators that *Sox2* functions properly. Moreover, we plan to make an inducible

Sox2 construct with tetracycline on/off system that would give us temporal control of the expression of *Sox2*. Furthermore, we will test several proneural and GABAergic-potentiating transcription factors including: *Ascl2* and *Ngn2*, with combination of *Lhx6* and *Dlx5*. Stereotactic bi-lateral injections of retrovirus into the mouse DG will be performed with inducible retrovirus and non-inducible retrovirus injected both individually into separate mice and co-injected into different mice. Mice injected with inducible constructs will be fed a doxycycline diet to induce *Sox2* expression for variable time periods prior to perfusion. At varying time point from 3 days to 5 weeks post-injection, mice will be perfused, and fluorescent microscopy will be used to confirm red fluorescent and *eGFP* expression. Standard immunohistochemical techniques will be used to stain hippocampal sections with antibodies against inhibitory interneuron markers.

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