UNIVERSITY OF CALIFORNIA SAN DIEGO

“An Investigation of rAAV-Induced Ablation of Hippocampal Adult Neurogenesis”

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

In

Biology

by

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Professor Gulcin Pekkurnaz

The Thesis of Nolan Mac is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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During adult neurogenesis, newborn neurons are selectively produced throughout adulthood in the hippocampus, a brain structure heavily involved in the processes of learning and memory formation. During hippocampal adult neurogenesis, intermediate neural progenitor cells (NPCs) in the dentate gyrus (DG) produce immature adult-born dentate granule cells (abDGCs), which integrate into existing neural circuits and contribute to pattern separation and pattern completion. To visualize the activity of

newborn and developmentally-derived DGCs in-vivo, genetically encoded calcium indicators (GECIs) can be transfected into neuronal genomes with the use of viral vectors such as recombinant adeno-associated virus (rAAV). Although rAAV has become widely used as a viral vector due to its low pathogenicity and low immunogenicity, its effect on cells located in the hippocampus was previously unknown. Our experiments reveal that exposure of the DG to rAAV in experimentally significant doses leads to the death of intermediate NPCs and abDGCs in adult mice. This study demonstrates that the rAAV-induced ablation of adult hippocampal neurogenesis is dose-dependent, cell-specific, and time-sensitive.

Chapter 1. Introduction

The Hippocampus

Everyday, the brain processes hundreds of thousands of sensory inputs from our internal and external environments. Memory is the process by which that information can be stored for later use, and the formation of declarative memories relies on a structure known as the hippocampus. Located in the medial temporal lobe, the hippocampus plays a vital role in emotion, motivation, and spatial learning (Squire 1992). Studies of bilateral hippocampal lesions revealed that the extent of damage was positively correlated with a higher degree of memory loss and increased issues with semantic memory storage (Scoville et al. 1957).

The hippocampus is broken into three main regions: CA1, CA3, and the dentate gyrus (DG). The granule cell layer of the DG is comprised of neurons known as dentate granule cells (DGCs), and impaired function of DGCs leads to temporal lobe epilepsy and significant memory deficits (Hester 2014). Mature DGCs (mDGCs) are produced during embryonic and early postnatal development, and form the basis of neural circuitry that is critical to memory formation and information processing.

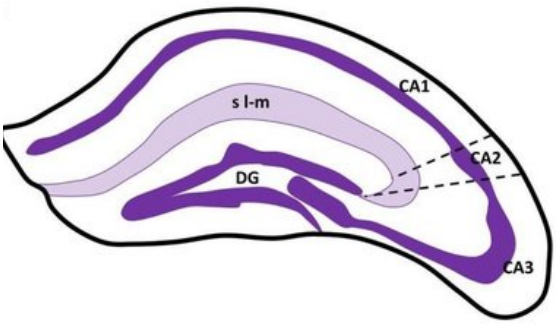

Figure 1. The Hippocampus. The three main regions of the hippocampus include the CA1, CA3, and the dentate gyrus (DG). Adult neurons in the hippocampus form the basis of neural circuits that are critical for the discriminatory processes pattern completion and pattern completion. Figure adapted from Pepin (2017).

The combination of external and internal sensory inputs our brain interprets is first processed by neurons in the entorhinal cortex (EC), which serves as the major cortical input to the hippocampus. Via axonal projections that form the perforant pathway, neurons from the EC transmit sensory information to the DG, and mDGC axons form the mossy fiber tract, which allows mDGCs to communicate with pyramidal neurons in the CA3. Via the Schaffer collaterals, CA3 pyramidal neurons synapse onto CA1 pyramidal neurons, which can then synapse onto neurons located in the EC, completing a loop of connections known as the tri-synaptic pathway (Deng 2013).

While the tri-synaptic pathway plays an important role in learning and memory, the mechanism by which it contributes is unclear. Although the DG contains four to five times more neurons than the EC, DGCs of the hippocampus demonstrate sparse activity and fire action potentials at a significantly lower frequency. Studies demonstrate that the DG may serve as an attenuator of inputs to reduce the signal interference to the CA3, and that DGCs fire in a sparse pattern that is specific to each unique experience (Johnston et al. 2016). Theoretical models of declarative memory indicate that the formation of unique memories relies on two specific discriminatory processes: pattern completion and pattern separation.

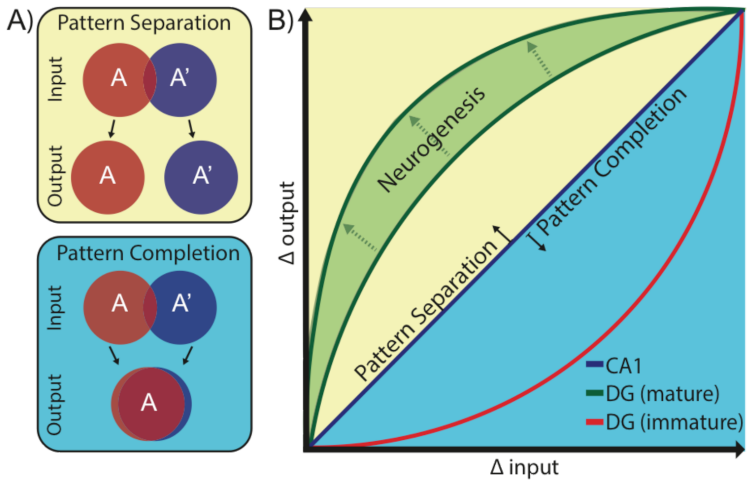

Figure 2. Pattern Separation and Pattern Completion in the Hippocampus. A) Pattern separation abilities allow for the discrimination of similar inputs A and A' into two distinct neuronal representations. Pattern completion allows for the generalization of inputs A and A' into a similar neuronal representation. B) Immature abDGCs are believed to play a more pronounced role in pattern separation. Figure adapted from Stark and Yassa (2011).

Pattern completion allows the brain to complete partial patterns from incomplete data, and pattern separation is the ability of the brain to differentiate similar patterns of activation. The ability to perform pattern separation is crucial to avoiding memory interference, as patients with hippocampal damage performed significantly worse on pattern separation discrimination tasks (Kirwan 2012). fMRI studies revealed that activity consistent with pattern completion was recorded in CA1, while activity consistent pattern separation was recorded in the CA3 (Bakker 2008). In-vivo electrophysiological recordings have associated DGC activity with both pattern separation and pattern completion, and lesion studies in rats show that the DG is a necessary component of pattern separation (Kesner 2004; Leutgeb 2007). However, the cellular mechanisms by which DGCs affect memory formation are still not yet fully understood. Although mDGCs form the basis on which the brain can perform discriminatory processes,

immature neurons produced throughout adulthood can integrate into existing hippocampal neural circuits to improve pattern separation abilities. These neurons can be produced throughout adulthood in a process known as adult neurogenesis.

Neurogenesis

Neurogenesis is the process by which new neurons are generated from undifferentiated neural stem cells in the brain. Previously believed to only occur during fetal and early postnatal development, neurogenesis was discovered to occur throughout adulthood in two distinct brain regions: the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus (Eriksson 1998).

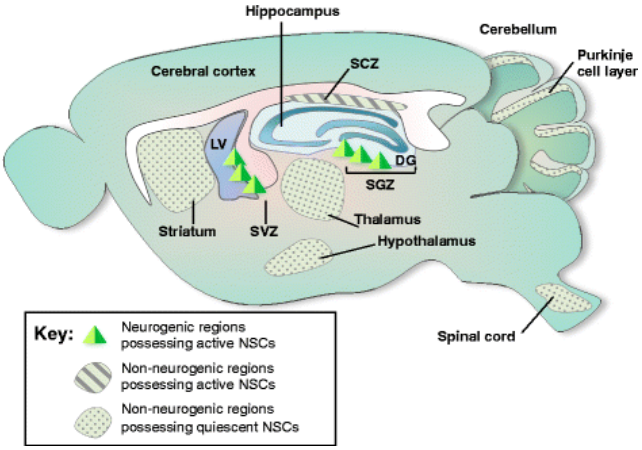

Figure 3. The Subgranular Zone. The SGZ is one of two select regions of the brain in which adult neurogenesis occurs. The SGZ contains neural stem cells (NSCs), which are nestled between the hilus of the DG and the granule cell layer of the hippocampus. During adult neurogenesis, NSCs in the SGZ will divide and produce neural progenitor cells (NPCs), which can divide into non-neuronal cells, such as astrocytes or microglia, or neurons. The neurons produced during this period are known as adult-born DGCs (abDGCs). Figure adapted from Ryu et al. (2016).

While mature dentate granule cells (mDGCs) are born during early developmental states, remaining hippocampal NPCs can produce newborn neurons known as adult-born DGCs (abDGCs). The process of neurogenesis in the SGZ begins with the maturation of

NSCs into neural progenitor cells (NPCs), which further divide and develop into immature adult-born dentate granule (abDGCs). Compared to their mature counterparts, immature abDGCs exhibit reduced GABAergic inhibition, higher excitability, and a lower threshold for LTP induction. And within 4 -10 weeks, newborn abDGCs fully mature and integrate into the granule cell layer of the DG (Kempermann 2015). Although the majority of newborn neurons produced during adult neurogenesis do not initially survive beyond two weeks, abDGCs that survive have been discovered to contribute to hippocampal dependent pattern separation and pattern completion. Within days, these newborn neurons extend their newly formed dendrites towards the molecular layer and project axons through the hilus towards CA3 (Kuhn 1996; Yassa 2011; Zhao 2006). Even before fully maturing, newborn abDGCs play a more active role in pattern separation, while mDGCs and mature abDGCs play a more prominent role during pattern completion (Nakashiba 2012). Ablation of hippocampus neurogenesis with targeted low dose irradiation resulted in significant impairments to spatial navigation tasks, further suggesting that immature abDGCs play a critical role in normal pattern separation function (Clelland 2009). Though it is clear abDGCs play a role in affecting pattern separation**,** the precise function of these immature abDGCs in the hippocampus is still unclear. To further elucidate the function of abDGCs in these hippocampal circuits, researchers have turned to manipulating and observing abDGCs and mDGCs in-vivo with the use of two-photon microscopy technology and genetically-encoded calcium indicators (GECIs).

Two-Photon Microscopy and Genetically Encoded Calcium Indicators

Rrecent advancements with two-photon microscopy technology have now made in-vivo imaging of deep hippocampal neural networks possible without compromising temporal reor spatial resolution. During two-photon imaging, two photons arrive simultaneously to excite a fluorophore, a molecule that reemits light upon excitation to produce an image. Fluorophores can be attached to various biomolecules, and can be used to monitor the neuronal activity by tracking calcium (Ca^{2+}), an ion necessary for in synaptic transmission (Feilmeier 2000). With the use of genetically encoded Ca^{2+} indicators (GECIs), fluorescent genes can be transfected into target neurons that allows for the observation of neuronal firing and activity. Two-photon microscopy has been proven effective for the use of in-vivo hippocampal imaging of DGC activity in mice through the excitation of various red and green fluorescent protein based GECIs without affecting behavior or memory performance (Goncalves 2016, Pilz 2016). In order to observe neurons in the hippocampus, we turned the viral vector known as recombinant adeno-associated virus (rAAV) to label DGCs.

Recombinant Adeno-Associated Virus (rAAV)

Viruses are infectious particles capable of infecting a variety of organisms and utilizing the cellular machinery of host cells to reproduce. All viruses are composed of two universal components: 1) a protein capsid and 2) a viral genome consisting of either DNA or RNA. Although viruses vary in organization and genomic composition, viruses can be manipulated and modified to yield viral vectors. These genetically modified viruses still have the ability to integrate into the genome of host cells, but viral genomes

can be modified with with genes of interest. Viral vector technology thus allows researchers to manipulate genomes and transfect target cells to alter cell function.

Wild-type adeno-associated virus (AAV) is a replication-defective, single-stranded DNA virus with a low immunogenicity and pathogenicity. Wild-type AAV contains a linear genome of approximately 4.7-kilobases that consists of two open reading frames: the *rep* region and the *cap* region. The genome is flanked by palindromic inverted terminal repeats (ITRs), which are the only genes necessary for the packaging of DNA into viral capsids (Martynov 2016, Muzyczka 1992). Rep and cap genes can be supplied in trans, and portions of the wild-type AAV coding regions can be removed to create space for DNA sequences of interest, yield the viral vector know as recombinant AAV (rAAV). rAAV has become increasingly popular tool for genetic therapy due to its ability to integrate into a multitude of host genomes and its ability to infect both dividing and non-dividing cells (Daya 2008). Previously, rAAV has been used to label cells with sensors for neuronal function, successfully labeling hippocampal DGCs with JRGECO1 (Goncalves 2016). In an effort to further study adult neurogenesis, we turned to rAAV to determine the precise role that abDGCs play in hippocampal dependent pattern completion and pattern separation. However, we determined that administration of rAAV vectors in experimentally relevant titers to the hippocampus during stereotaxic surgeries resulted in the loss of dividing cells in the DG. Intrigued by this previously unseen toxicity of rAAV, we designed a set of experiments to determine how rAAV contributed to the ablation of adult neurogenesis in the hippocampus.

Chapter 2. Materials and Methods

Animal Use and Housing

6-7 week old male, wild type C57BL/6J mice (Jackson Laboratories) were used for all experiments. Up to 5 mice from the same litter were placed in regular cages on a 12-hour light–dark cycle, with open access to food and water.

Viral Injection

Mice were first anesthetized with isoflurane before their heads were immobilized using a stereotax. Isofluorane was continually administered to the mouse throughout the surgery with the use of a nose cap. The mice were placed into a stereotaxic frame and onto a heating pad that maintained a body temperature of 37°C for the entire procedure. Intraperitoneal (IP) injections of dexamethasone were administered to each mouse before surgery to decrease inflammation. Ophthalmic ointment is applied generously over both eyes before surgery to prevent desiccation and hair on the mouse's scalp was removed with electric shavers. For pre-surgical skin disinfection, betadine and alcohol were applied in an alternating fashion to the scalp three separate times. A pair of surgical scissors is used to make an incision in the mouse scalp spanning from its ears to just before its eyes. A pair of sterile cotton-tipped applicators is then used to dry and expose the skull. A small hole of 1 mm in diameter is drilled into the skull with a dental drill above the right hemisphere of the mouse brain. Viral vector solutions are then delivered to the DG of the hippocampus with the use of a Nanoject III microinjector. After completion of the surgery, the mouse's scalp is closed with cyanoacrylate adhesive. IP injections of carprofen and buprenorphine were administered for inflammation and pain relief to the mice before being returned to their home cages. The following viral vectors

were used: AAV1-CAG::flex-eGFP-WPRE-bGH (Zeng – Addgene Plasmid #51502, U Penn Vector Core & Addgene), AAVretro-CaMKIIa::NES-jRGECO1a-WPRE-SV40 (Gage, Salk), AAV8 empty capsid (Salk).

Tissue Collection

Mice were euthanized by lethal injection with a combination of ketamine and xylazine. The mouse is secured in a supine position before an incision is made along the thoracic midline, spanning the mouse's pelvis to its neck. Without damaging any underlying organs, surgical scissors are used to split the sternum and cut the peritoneal sac to expose the heart. A feeding needle attached to a solution of 0.9% phosphate buffered saline is then inserted into the left ventricle of the heart and an incision is simultaneously made to the right atrium. The mouse is then perfused transcardially until the fluid exiting the right atrium is completely clear and free of blood. Complete perfusion with saline is followed by perfusion with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for a minimum of 5 minutes. The head of the mouse is then removed and the brain is carefully dissected out and left to soak in a test tube containing 4% PFA in a 4 °C fridge. After 24 hours, fixed brain brains were transferred to a 30% sucrose solution to soak for at least 24 hours before sectioning. Fixed brains are then frozen on the stage of a sliding microtome and sectioned to obtain 40-μm thickness coronal sections. Sections are stored in 48-well plates with tissue collection solution in a –20 °C freezer until staining.

Immunochemistry

40-μm coronal brain sections are first blocked in tris-buffered saline (TBS) for 5 minutes, three separate times. Sections are then blocked with 0.25% Triton X-100 in TBS

with 3% horse serum (TBS++) for 1 hour. Sections were then incubated with select primary antibodies in a well plate covered with aluminum foil and placed on a shaker in a 4°C cold room. After 72 hours, sections were washed in both TBS and TBS++ before incubation in fluorophore-conjugated secondary antibodies for 2 hours. Following secondary antibody incubation, sections were blocked with DAPI for 15 minutes to label nuclei before sections were then mounted onto microscope slides (Fisherbrand) with Immu-Mount mounting media.

To label proliferating cells, brain sections stained with Bromodeoxyuridine (BrdU) are first washed in TBS three times for 5 minutes, then incubated in 2N HCL in a 37°C water bath for 30 minutes, followed by a wash in 0.1M Borate buffer for 10 minutes. Sections are then washed in TBS six times for 5 minutes then blocked in TBS++ for 1 hour. Secondary antibody incubation is as follows above. Mounted slices were left overnight in a 4°C fridge before analysis. A confocal microscope (Olympus) was used to obtain immunofluorescence images, which were processed with ImageJ software.

Chapter 3. Results

rAAV Affects Dividing Cells in the DG

Looking to further elucidate how immature abDGCs contribute to hippocampal-dependent discriminatory processes, we turned to rAAV to transfect DGCs fluorescent genes for in-vivo imaging. However, stereotaxic injections of rAAV at doses below or equivalent to experimental doses necessary for the visualization of DGCs consistently reduced the number of proliferating cells in the hippocampus. Our experimental goal was to not only determine which cells were most affected by rAAV, but also determine the mechanism by which rAAV ablated adult neurogenesis.

Wild type C57BL/6 male mice were given daily intraperitoneal (IP) injections of bromodeoxyuridine (BrdU) (0.1mL/g) over a 3-day period. To quantify rAAV-induced ablation of adult neurogenesis, 1μL of 3E12 GC/mL of abDGCsAAV1-CAG-flex-eGFP (U. Penn. & Addgene) was unilaterally injected into the dentate gyrus (DG) of the right hemisphere in non-cre expressing wild-type C57BL/6 mice zero days, 7 days, or 14 days after the conclusion BrdU injections. Mice were then euthanized after viral injections and brain tissue was collected and stained for BrdU expression.

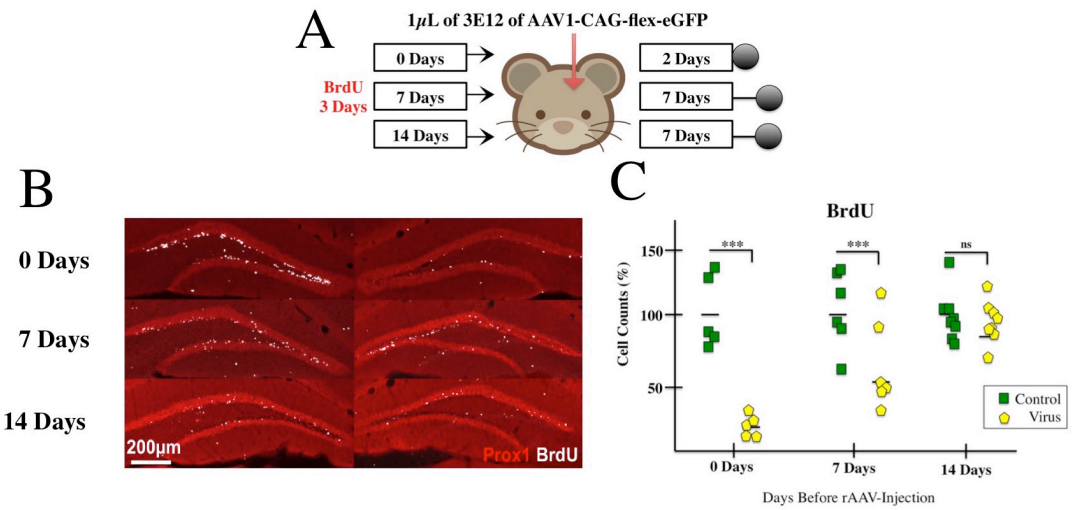

Figure 4. rAAV Reduces BrdU Expression in the Hippocampus. A) Experimental schematic to determine if injections of rAAV affect proliferating cells in the hippocampus. B) Confocal images taken of coronal slices of the mouse hippocampus, stained for BrdU+ cells (white). C) Mice injected immediately after BrdU injections showed an extensive elimination of cells three days or younger. Mice injected 7 days after showed a moderate elimination of cells 8-10 olds, while mice injected 14 days after BrdU injections showed minimal elimination of cells 16-18 days old.

Mice injected zero days after BrdU IP injections exhibited a near complete loss of cells three days or younger (-83.9% ± 6.7%, $p < 0.0001$). Mice injected 7 days after BrdU contained a population of cells 8-10 days old that were moderately protected (-41.3% ± 6.3%, $p < 0.0001$), and mice that expressed cells 16-18 days old that were largely protected (-15.4% ± 6.3%, $p = 0.0731$; **Figure 4C**). This data demonstrated that unilateral injections of rAAV significantly affected proliferating and dividing cells in the hippocampus.

rAAV-Toxicity is Dose-Dependent

Next, we looked to determine the effect of viral vector concentration. rAAV viral vector solutions were diluted with sterile saline and injected immediately after the completion of 3-days of BrdU IP injections. Mice underwent unilateral stereotaxic viral

injections to the DG of the right hemisphere with 1μL of AAV1-CAG-flex-eGFP in titers of 3E12 GC/mL, 1E12 GC/mL, or 3E11 GC/mL.

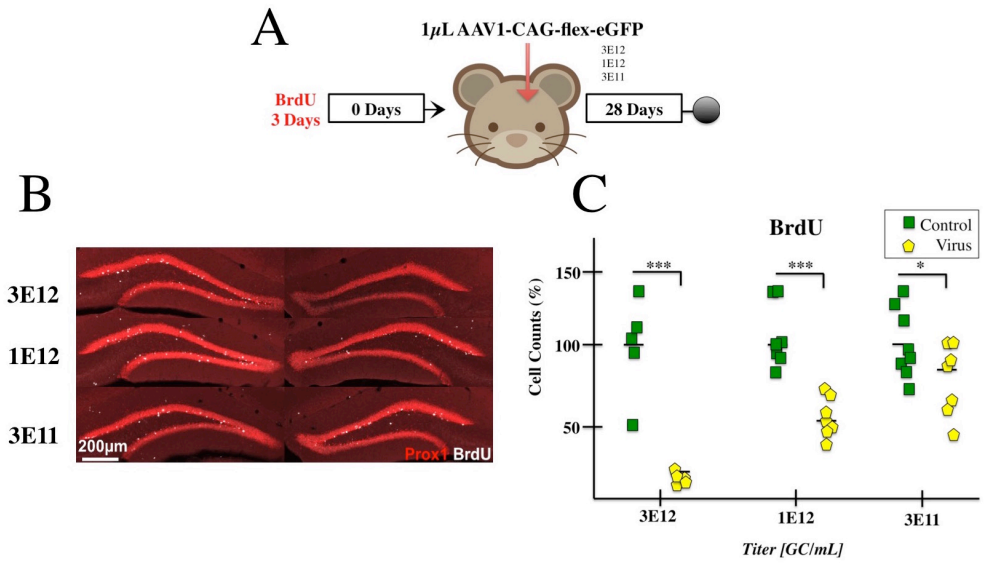

Figure 5. rAAV-Induced Ablation of Adult Neurogenesis is Dose-Dependent. A) Experimental schematic to determine if rAAV injected in progressively higher titers affected populations of proliferating cells in the hippocampus. B) Confocal images taken of the hippocampus stained for BrdU+ cells (white). C) Mice injected with higher titers of rAAV demonstrated a significant decrease in BrdU+ cells, while mice injected with lower titers of rAAV demonstrated a smaller elimination of BrdU+ cells.

Mice injected with 1uL 3E12 GC/mL rAAV showed a nearly complete ablation of BrdU+ cells (-84.3% ± 6.7%, p <0.0001). Mice injected with 1μL 1E12 GC/mL rAAV exhibited a partial elimination of BrdU+ cells (-52.1% ± 6.7%, p <0.0001), and mice injected with 1μL 3E11 GC/mL rAAV exhibited a reduced reduction of adult neurogenesis (-23.4% ± 7.2%, p= 0.012; **Figure 5C**). This data demonstrated that increased viral titers leads to a more pronounced elimination of adult neurogenesis in the hippocampus.

rAAV Toxicity is Cell-Specific

Next, we sought to determine which cells were most vulnerable to rAAV. After BrdU labeling, mice were unilaterally injected with 1μL 3E12 GC/mL rAAV and sacrificed at 2 days, 7 days, or 28 days after stereotactic injections of rAAV. Brain tissue was then stained for the expression of transcription factors SOX2, eomesodermin (Tbr2+), and doublecortin (DCX), which serve as different histological markers associated with different stages of neuronal development during adult neurogenesis.

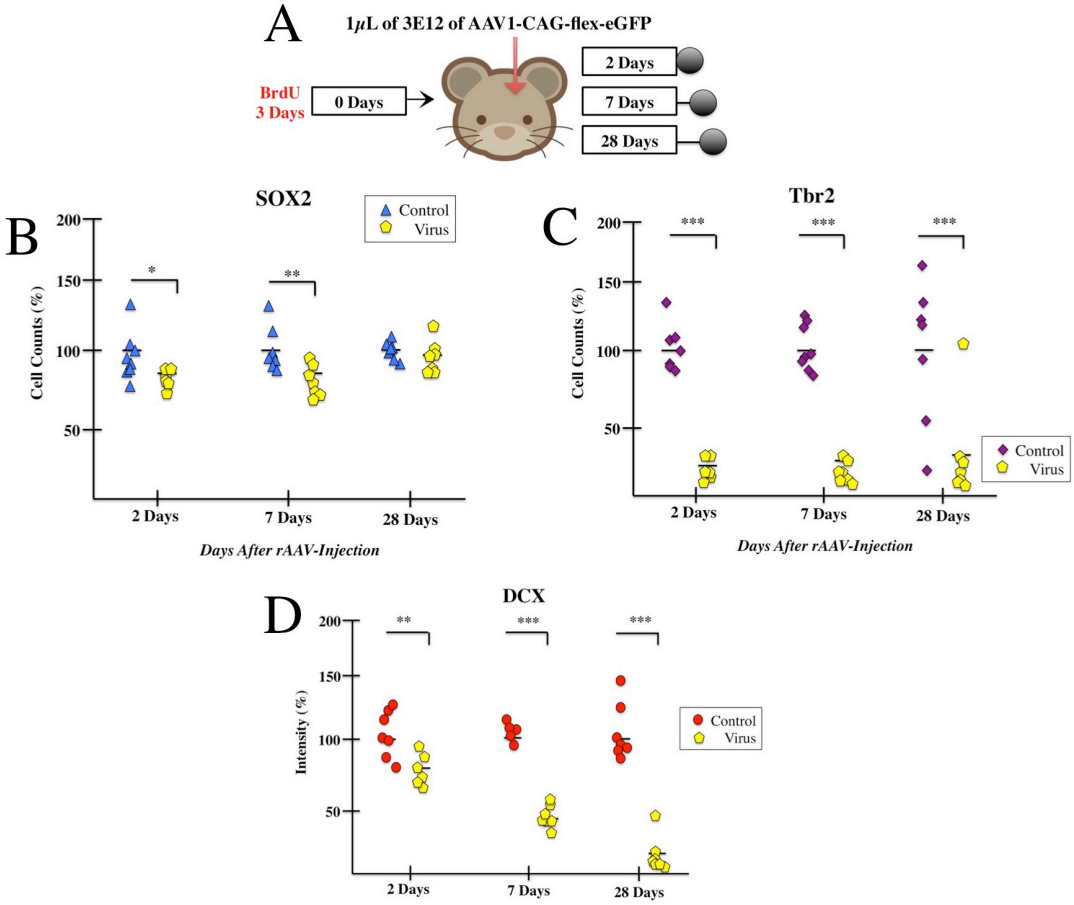

Figure 6. rAAV-Induced Ablation of Adult Neurogenesis is Cell-Specific. A) Experimental schematic to determine which neural precursor cells are most vulnerable to rAAV toxicity. B) SOX2 is expressed by undifferentiated embryonic stem cells, and SOX2 expression is largely protected from rAAV. C) Tbr2 is expressed during the intermediate developmental phase of NPCs, and Tbr2 expression is significantly reduced at 2, 7, and 28 days after exposure to rAAV. D) DCX is expressed during the late stages

of NPC development and during the early development of immature abDGCs. DCX expression is significantly reduced after exposure to rAAV at 2 days and steadily declines at 7 and 28 days after rAAV injection.

Mice sacrificed 2 days post-injection demonstrated a minor reduction in SOX2 expression in the SGZ of the hippocampus (-18.9% ± 6.5%, p= 0.026). Mice sacrificed 1 week after rAAV injection also demonstrated a small reduction in SOX2 expression (-21.9% ± 6.1%, p= 0.006), but mice sacrificed 4 weeks post-injection had negligible reduction in SOX2 expression (-2.5% ± 6.5%, n.s). Within 2 days of rAAV injection, expression of Tbr2+ was significantly reduced (treatment -75.5% ± 6.6%, p<0.001; interaction) and did not recover by 4 weeks post-injection (2 days: -80.2% ± 11.8%, p<0.0001) Mice sacrificed 1 week and 4 weeks after rAAV injection also demonstrated a significant loss of Tbr2+ cells (1 week: -76.9% ± 11%, p<0.0001; 4 weeks: -69.4% ± 11.8%, p<0.0001). DCX expression shows a progressive decline until complete loss at 4 weeks post-injection (2 days: -27.7% ± 8.0%, p=0.0077; 1 week: -58.7% ± 7.5%, p<0.0001; 4 weeks: -92.0% ± 8.0%, p<0.0001; **Figure 6**). The minor reduction of SOX2+ cells following rAAV injection indicates the population of NSCs is only partially vulnerable to rAAV toxicity. However, the progressive loss of DCX+ expression is consistent with the loss of the Tbr2+ neural progenitor pool, which cannot replenish and further produce into abDGCs. This data demonstrates that rAAV toxicity primarily affects adult neurogenesis by targeting intermediate NPCs and immature abDGCs in the hippocampus.

rAAV Toxicity is Time-Sensitive

After demonstrating rAAV-induced ablation of adult neurogenesis was cell-specific, we looked to determine how quickly rAAV affected adult neurogenesis after

injection. Following BrdU injections, mice were injected bilaterally with 1μL 3E12 of AAV1-CAG-flex-eGFP into one dorsal DG and 1μL of saline into the contralateral DG as a control. Mice were then sacrificed 12 and 18 hours after bilateral hippocampal injections, and brain tissue was stained for BrdU, Tbr2, and Caspace 3 expression.

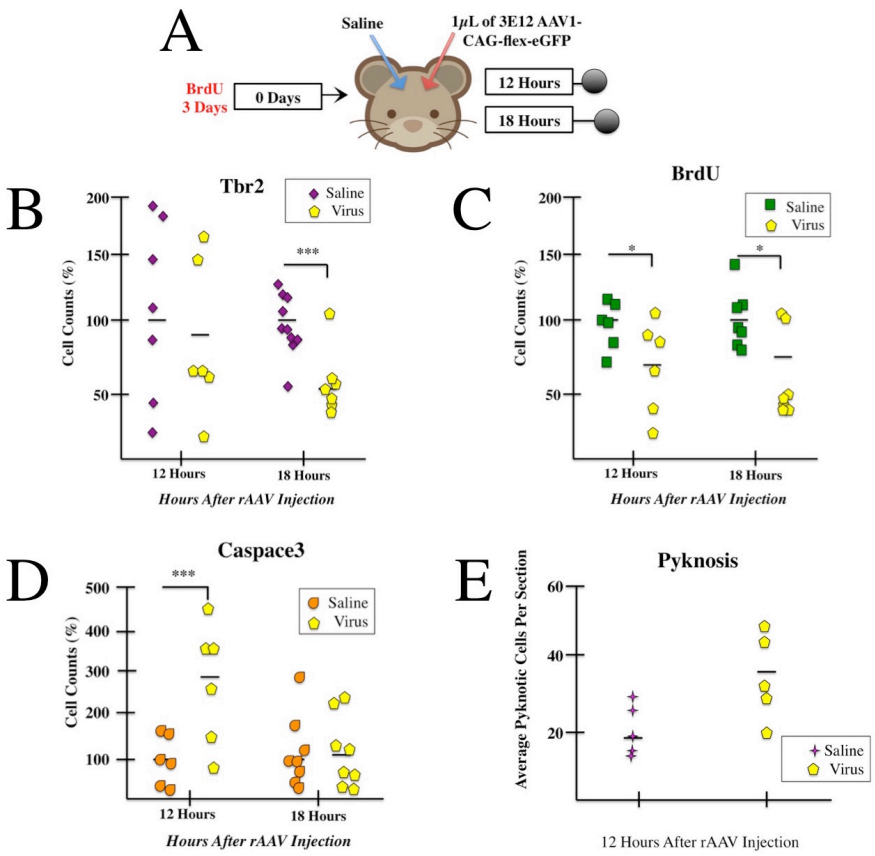

Figure 7. rAAV-Induced Ablation of Adult Neurogenesis is Time Sensitive. A) Experimental schematic to determine timeframe of rAAV-induced cell loss. B) Tbr2 expression is not affected within 12 hours of rAAV injection, but is significantly reduced by 18 hours. C) BrdU expression is affected as early as 12 hours after rAAV injection. D) Caspace 3 is expressed by cells undergoing cellular apoptosis, and loss of cells was preceded by an increase in Caspace 3 expression by 12 hours after rAAV injection. E) Pyknosis is the irreversible process by which chromatin is condensed during apoptosis, and condensed chromatin can be used as marker for apoptosis. Mice injected with rAAV showed an increase in pyknotic cells compared to contralateral controls.

The number of Tbr2+ cells was only significantly decreased 18 hours post injection (-28.3% ± 6.961, p=0.001; **Figure 8B**). DGs of mice injected with rAAV

demonstrated a moderate reduction in BrdU+ cell expression 12 and 18 hours post rAAV injection compared to the contralateral DG control treated with saline (-27.9% ± 7.5%, p=0.003; **Figure 8C**). Cell loss was preceded by an increased number of Caspase-3+ apoptotic cells relative to saline injected contralateral controls at 12 hours (12h: +188.6% ± 29.8%, p <0.0001, **Figure 8D**). Mice injected with rAAV also demonstrated an increased in pyknotic+ cells in the DG relative to saline injected contralateral controls (Δ = +14.7 ± 6.0 cells/section, p=0.0581, **Figure 8E**). Furthermore, there was a significant increase in cells co-labeled with BrdU and pyknotic cells (Δ = +2.3 ±0.7 cells/section, p=0.019). This data shows that rAAV toxicity can affect populations of abDGCs and intermediate NPCs within 12-18 hours. Our experiments also demonstrate that injections of rAAV make abDGCs and NPCs more likely to trigger apoptosis.

rAAV Toxicity Not Mediated by Viral Capsid or cGAS-Sting Pathway

After demonstrating adult neurogenesis was affected by rAAV toxicity as early as 12-18 hours, we looked to to determine the mechanism by which rAAV initiated the ablation of adult neurogenesis. We first examined the cGAS-Sting pathway, a component of the innate immune system that detects foreign DNA and triggers cellular apoptosis.

However, unilateral injections of rAAV in Sting knockout mice continued to significantly reduce BrdU and Tbr2 expression (BrdU: -90.2% ± 14.2, p<0.001; Tbr2: -88.7% ± 12.9%, p=0.0002; **Figure 8A**).

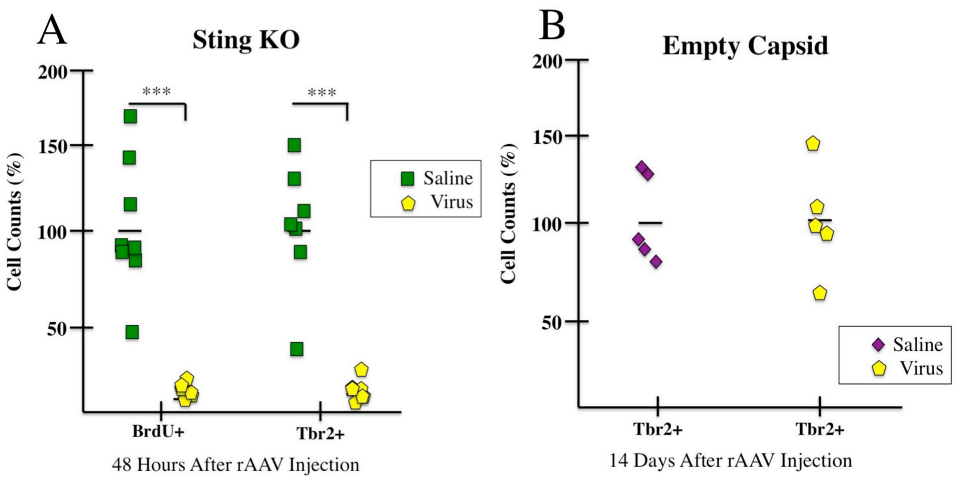

Figure 8. rAAV-Toxicity Not Mediated by Viral Capsids or the Sting Pathway. STING plays an integral role in the innate immune system that detects foreign DNA and triggers inflammation that can lead to apoptosis. A) BrdU and Tbr2 expression is significantly reduced in Sting KO mice 48 hours after rAAV injection. B) Injections of 1 uL of empty rAAV8 capsid viral particles did not result in a loss of Tbr2+ intermediate neural progenitor cells 14 days after rAAV injection.

We then looked to determine if the viral capsid of rAAV was responsible for the ablation of adult neurogenesis. Mice were administered unilateral hippocampal injections of 1μL of an equivalent number of empty AAV1 viral capsids. At 2 weeks post-injection, Tbr2 expression was nearly identical compared to the contralateral control side (+6.8% ± 24.7%, ns; **Figure 8B**).

Next, we looked to evaluate the expression of glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1 to determine if the rAAV-induced ablation of adult neurogenesis was triggered by inflammation caused by astrocytes or microglia.

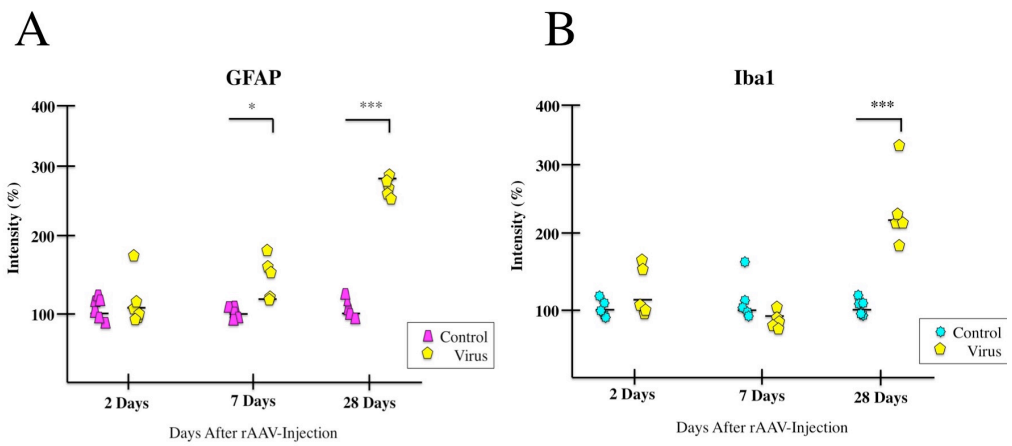

Figure 9. rAAV-Toxicity is Not Mediated by Inflammatory Immune Response. A) GFAP is highly expressed by astrocytes during the immune response, however GFAP expression is unaffected 2 days post-injection, moderately increased after 7 days, and significantly increased 28 days after rAAV injection. B) Iba1 is highly expressed by macrophages and microglia as part of the immune response and is used as a marker for inflammation. However, Iba1 expression alsp does not significantly increase until 28 days after rAAV injection.

GFAP intensity was similar to contralateral controls at 2 days post rAAV injection (2 days: +21.6% ± 8.7%, p=0.066). GFAP intensity only slightly increased after 7 days (+25.2% ± 8.1%, p=0.018) before significantly increasing 4 weeks after rAAV injections (+165.5% ± 8.7%, p<0.0001; **Figure 9A**). Similar to GFAP expression, Iba1 expression after 2 days is similar to contralateral controls (+18.6% ± 10.3%, p=0.243), and is not significantly increased until 28 days after rAAV injection (1 week: +9.0% ± 9.7%, p=0.739; 4 weeks: +132.4% ± 10.34%, p<0.0001; **Figure 9B**). Our previous findings revealed that abDGCs and intermediate NPCs are affected after only 12-18 hours, and this data demonstrates that rAAV toxicity is not likely mediated by astrocyte- or microglial-activated immune responses.

Chapter 4. Discussion

A New Outlook on rAAV

To observe how immature abDGCs affected hippocampal circuits critical to pattern separation and pattern completion, we chose to label these newborn neurons with rAAV. rAAV has become increasingly popular as viral vector for various fields of research due to its ability to infect diving and non-dividing cells, perceived low immunogenicity, and wide-range of applications. However, our earliest data demonstrated that injections of rAAV affected proliferating cells in the DG, calling rAAV's perceived safety as a viral vector into question.

Recently, rAAV usage has been increasingly linked to genotoxicity and toxicity in different types of tissue. High doses of AAV administered intravenously in nonhuman primates resulted in degeneration of dorsal root ganglia sensory neurons, and DNA microinjections of rAAV ITRs into human embryonic stem cells induced rapid apoptosis (Hinderer et al. 2018, Hirsch 2011). Our experiments demonstrate that unilateral stereotactic rAAV injections to the DG of mice, at doses within or lower than previously used titers for in-vivo visualization of the hippocampus, significantly reduced adult neurogenesis. Our data shows that abDGCs and intermediate NPCs are the cells most affected by rAAV, and these cells are pushed towards apoptosis within 12 to 18 hours after rAAV injections. The progressive loss of DCX+ cells is likely linked to the loss of the intermediate progenitor pool that gives rise to immature abDGCs. We have demonstrated that rAAV-induced ablation of adult neurogenesis is dose-dependent, cell-specific and time-sensitive, and the use of rAAV should be carefully evaluated.

Future Directions

While its clear that adult neurogenesis plays a key role in producing neurons that regulate activity in the DG, the increased sensitivity of abDGCs to rAAV has led us to look towards other viral vectors. Our goals are to find a viral vector that allows functional 2-photon imaging within the DG without affecting neurogenesis or neuronal firing activity. AAV-retro is a variant of the rAAV viral vector that allows for the retrograde transfection of target cells via axonal projections. Utilizing AAVretro-Camk2a-JRGECO1a, our lab was able to successfully label mDGCs without affecting BrdU or Tbr2 expression. Although labeling with retroAAV is limited to mDGCs, retroAAV offers a promising alternative to rAAV for in-vivo imaging of the hippocampus that avoids ablation.

Currently, we are looking to determine if this toxicity is limited to rAAV, or if this toxicity is a characteristic shared by other viral vectors. Lentivirus is a single-stranded retrovirus derived from HIV that has been increasingly used as a viral vector due to its low cytotoxicity and long-term expression. Our initial data demonstrate reduced BrdU and Tbr2 expression, which indicates lentiviral vectors may also induce an ablation of hippocampal adult neurogenesis.

We have demonstrated that rAAV-induced ablation of neurogenesis is neither mediated by the cGAS-STING pathway nor rAAV capsid particles. To determine the mechanism by which rAAV eliminates abDGCs and NPCs, our lab has looked towards identifying the minimum rAAV components responsible for reducing abDGC populations. Currently, our lab is currently studying the effect of isolated rAAV genomic components, and early data indicate that rAAV ITRs are sufficient in preventing the

proliferation of NPCs in vitro. As rAAV continues to be used as a tool for gene therapy, our experiment has demonstrated that rAAV usage has potential risks that have not been fully evaluated and the usage of rAAV as a genetic tool must be fully evaluated.

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