HHS Public Access

Author manuscript

J Vis Exp. Author manuscript; available in PMC 2020 July 16.

Assaying circuit specific regulation of adult neural precursor cells

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Abstract

Adult neurogenesis is a dynamic process by which newly activated neural stem cells (NSCs) in the subgranular zone (SGZ) of the dentate gyrus (DG) generate new neurons, which integrate into an existing neural circuit and contribute to specific hippocampal functions. Importantly, adult neurogenesis is highly susceptible to environmental stimuli, which allows for activity-dependent regulation of various cognitive functions. A vast range of neural circuits from various brain regions orchestrates these complex cognitive functions. It is therefore important to understand how specific neural circuits regulate adult neurogenesis. Here, we describe a protocol to manipulate neural circuit activity using Designer Receptor Exclusively Activated by Designer Drugs (DREADD) technology that regulate NSCs and newborn progeny in rodents. This comprehensive protocol includes stereotaxic injection of viral particles, chemogenetic stimulation of specific neural circuits, thymidine analog administration, tissue processing, immunofluorescence labeling, confocal imaging, and imaging analysis of various stages of neural precursor cells. This protocol provides detailed instructions on antigen retrieval techniques used to visualize NSCs and their progeny and describes a simple, yet effective way to modulate brain circuits using Clozapine N-Oxide or CNO-containing drinking water and DREADDs-expressing viruses. The strength of this protocol lies in its adaptability to study a diverse range of neural circuits that influence adult neurogenesis derived from NSCs.

SHORT ABSTRACT:

The goal of this protocol is to describe an approach for analyzing behavior of adult neural stem/progenitor cells in response to chemogenetic manipulation of a specific local neural circuit.

Keywords

Adult Neurogenesis; DREADD; Neural Stem Cells; Hippocampus; Dentate Gyrus; Immunofluorescence

DISCLOSURES: The authors have nothing to disclose.

INTRODUCTION:

Adult neurogenesis is a biological process by which new neurons are born in an adult and integrated into the existing neural networks[1]. In humans, this process occurs in the dentate gyrus (DG) of the hippocampus, where about 1400 new cells are born each day[2]. These cells reside in the inner part of the DG, which harbors a neurogenic niche, termed the subgranular zone (SGZ). Here, hippocampal adult neural stem cells (NSCs) undergo a complex developmental process to become fully functional neurons that contribute to the regulation of specific brain functions, including learning and memory, mood regulation, and stress response[3-6]. To influence behaviors, adult NSCs are highly regulated by various external stimuli in an activity dependent manner by responding to an array of local and distal chemical cues. These chemical cues include neurotransmitters and neuromodulators and act in a circuit specific manner from various brain regions. Importantly, circuit wide convergence of these chemical cues on NSCs allow for unique and precise regulation of stem cell activation, differentiation, and fate decisions.

One of the most effective ways to interrogate circuit regulation of adult NSCs *in vivo* is by pairing immunofluorescence analysis with circuit wide manipulations. Immunofluorescence analysis of adult NSCs is a commonly utilized technique, where antibodies against specific molecular markers are used to indicate the developmental stage of adult NSCs. These markers include: nestin as a radial glia cell and early neural progenitor marker, tbr2 as an intermediate progenitor marker, and dcx as a neuroblast and immature neuron marker[7]. Additionally, by administering thymidine analogs such as BrdU, CidU, Idu, and Edu, cell populations undergoing S phase can be individually labeled and visualized [8-10]. By combining these two approaches, a wide range of questions can be investigated ranging from how proliferation is regulated at specific developmental stages, to how various cues affect NSC differentiation and neurogenesis.

Several options exist to effectively manipulate neural circuits including electrical stimulation, optogenetics, and chemogenetics, each with their own advantages and disadvantages. Electrical stimulation involves an extensive surgery where electrodes are implanted to a specific brain region which are later used to transmit electrical signals to modulate a targeted brain region. However, this approach lacks both cellular and circuit specificity. Optogenetics involves the delivery of viral particles that encodes a light activated receptor which is stimulated by a laser emitted through an implanted optical fiber, but requires extensive manipulations, large cost, and complex surgeries [11]. Chemogenetics involves the delivery of viral particles that encode a designer receptor exclusively activated by designer drugs or (DREADDS), which is subsequently activated by a specific and biologically inert ligand known as Clozapine N-Oxide or CNO[12]. The advantage of utilizing DREADDS to manipulate local neural circuits that regulate adult NSCs lies in the ease and various routes of CNO administration. This allows for a less time-consuming approach with reduced animal handling, that is easily adaptable for long term studies to modulate neural circuits.

The approach described in this protocol is a comprehensive collection of various protocols required to successfully interrogate circuit regulation of adult hippocampal neurogenesis that

combines both immunofluorescence techniques and circuit manipulations using chemogenetics. The method described in the following protocol is appropriate for stimulating or inhibiting one or multiple circuits simultaneously *in vivo* to determine their regulatory function on adult neurogenesis. This approach is best used if the question does not need a high degree of temporal resolution. Questions requiring precise temporal control of stimulation/inhibition at a certain frequency, can be better addressed using optogenetics. [13, 14]. The approach described here is easily adapted for long term studies with minimal animal handling especially where stress is a major concern.

PROTOCOL:

Note: All procedures including animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina Chapel Hill.

1. Stereotaxic Injection of viral particles

1.1 Determine the neural circuits in question. This will determine the virus and the mouse line utilized for the following procedure. For this example, contralateral mossy cell projections were stimulated an its effects on adult neurogenesis were analyzed. For this example viral particles encoding AAV5-hSyn-DIO-hM3Dq-mCherry were delivered to the DG of 5ht2A-Cre mice[15].

1.2 Administer meloxicam (5mg/kg, subcutaneous) at least 30 minutes pre-operatively to provide pre-emptive analgesia to 8-week-old male heterozygous 5ht2A-Cre mouse.

1.3 Anesthetize mouse using a 4% isoflurane oxygen mixture until its breathing slows down and is unconscious using an isoflurane chamber. Toe pinch the mouse to assure it is not responsive.

1.4 Place the mouse in the stereotax on a small animal heating pad for thermal regulation and apply eye lubricant to each eye. Reduce isoflurane to 1.5% once the animal is inside the stereotax. **Note:** Ear bar placement during this stage is important. Assure the head is level after ear bar placement. More detailed instructions can be found here [16].

1.5 Place hair removal product (see materials list) on the head and let it sit for up to one minute maximum. Remove the hair by wiping the head with ethanol wipes. If the hair is not completely removed repeat this process until top of the head is hairless.

1.6 Disinfect the hairless area with a povidone-iodine solution (see materials list) at least 3 times.

1.7 Place topical lidocaine solution on the hairless skin of the head and wait for a minute. Remove topical lidocaine from head and make a small incision on the head from the start of the eyes to the start of the ears about 2 mm using a surgical scalpel. **Note:** Toe pinch the mouse to assure that it is completely sedated before making any incisions.

1.8 Retract the scalp and clean the connective tissue on top of the head using sterilized cotton swabs until bregma is easily identifiable.

1.9 Locate bregma and adjust the head so that bregma and lambda are both at the same plane by placing the drill at both coordinates and assuring they align. Additionally, align the left and right hemisphere by placing the drill to the left/right of a region between bregma and lambda. **Note:** This is a very important step, improperly aligned head placement will disrupt stereotaxic coordinates.

1.10 Drill at the following coordinates from bregma using a .5 mm drill bit: anterior posterior axis (AP) −2.00 mm, medial lateral axis (ML) +1.50 mm. Make a .5mm to 1mm in diameter drill hole. **Note:** Modify this step with coordinates specific to the circuit in question. This example targets a unilateral dentate gyrus containing mossy cells and determines their effect on adult neural stem cells on the contralateral side.

1.11 Switch drill to 5 μL Hamilton syringe and 26 - 33 gauge needle. Zero at bregma and then inject at the following coordinates by placing the needle in the drill hole at anterior posterior axis −2.00 mm, medial lateral axis - 1.50 mm, dorsal ventral axis −2.3 mm.

1.12 Infuse 500 nl of virus to appropriate hemisphere at 50-100 nl/s using an infusion pump (see materials) and wait at least 5 minutes post injection before slowly removing needle. **Note:** These coordinates may require adjustment based on the rodent's age and size. Certain viral serotypes have different diffusion patterns, it is best to perform pilot experiments to test viral spread before a complete experiment.

1.13 Clean scalp and skin around incision using saline, then seal incision using vet bond while holding skin together with tweezers. Perform all post-operative procedures such as monitoring during recovery on a heat pad until mouse is active, applying analgesic on the wound, and administering painkillers for two days. **Note:** Many experiments require 2 - 4 week wait time after viral infusion for proper viral expression.

2. CNO Administration

Drinking Water

2.1 Prepare stock CNO solution by dissolving 10 mg CNO in 100 μl of dimethyl sulfoxide (DMSO) and vortexing. **Note**: If solution does not dissolve completely, increase the volume of DMSO, but too much DMSO may make the water bitter. Do not exceed .1% DMSO in CNO water solution or more than 200 μl of DMSO for a 200 ml solution. CNO stock solution can be stored at −20 $^{\circ}$C for up to two weeks.

2.2 Add between 10 μl to 50 μl of 10 mg/100 μl CNO stock solution to every 200 ml of water for a final concentration of 1 mg/200 ml to 5 mg/200 ml. Prepare CNO water mixture fresh every day. Some groups have supplemented up to 1% saccharin in the water to mask bitterness if mice are refraining from drinking. **Note:** The circuit investigated showed a different response based on the extent of activation. In general 1 mg CNO/200 ml is sufficient to stimulate most circuits [17].

2.3 Place CNO solution in foil covered or light protected containers when administering to animals over a period of 4 days. CNO is light sensitive reduce exposure to light during the entire process.

2.4 Measure and record consumed CNO water solution every day when preparing fresh CNO solution. On average, an adult mouse will consume about 4 ml of CNO water mixture. Additionally, record mouse weight daily to assure they are drinking.

2.5 Assure that proper controls are utilized for each experiment. Include both a CNO and DREADD control. An example of an experimental setup would include: 1) vehicle+ AAV with viral reporter no DREADD, 2) CNO + AAV with viral reporter no DREADD, 3) CNO + AAV DREADD. **Note:** All groups include DMSO in the solution. DMSO controls were not included since animals are receiving less than .1% DMSO which hasn't been shown have adverse effects on adult neural stem cells in mice. If there are concerns regarding DMSO use in drinking water, add an additional no DMSO and saline control.

3. Thymidine Analog labeling

3.1. Label proliferating cells, on tissue collection day by performing a series of thymidine analog, 5-Ethynyl-2'-deoxyuridine (Edu) intraperitoneal injections, 4 per animal, every 2 hours. **Note:** This protocol utilizes Edu. However, there are several thymidine analogs that can efficiently label proliferating cell populations including Brdu, Idu, and Cidu[8].

3.2 Weigh Edu and dissolve in veterinary grade 0.9 % sodium chloride injection solution at 4 mg/ml by vortexing and placing on a rotor for 15 minutes. **Note**: Thymidine analogs are toxic and light sensitive. Follow Material Safety Data Sheets (MSDS) when handling and cover solution from light exposure using aluminum foil.

3.3 Administer Edu intraperitoneally at 40 mg/kg or .1 ml/10 grams of body weight of the 4 mg/ml Edu solution 4 times every 2 hour. **Note:** Doses above 50mg/kg reach near saturation levels and will not increase the amount of labeled proliferating cells significantly [18]. It is crucial that all animals receive the same amount of Edu injections since improper labeling can skew results.

3.4 Prepare animal for tissue harvesting and perfusion 2 hours after last Edu injection to harvest tissue.

4. Tissue preparation and processing

4.1 Anesthetize rodent using an isoflurane chamber until breathing is significantly reduced and toe pinch to assure it is completely sedated.

4.2 Secure rodent to surface using needles and make a small incision exposing the heart. Insert needle to the left aorta and cut the right ventricle for a transcardial perfusion with phosphate buffered saline solution until liver tissue is cleared using a flow rate of (1-4 ml/min).

4.3 Switch perfusion solutions to 4% paraformaldehyde (PFA) and perfuse around 15 - 20 ml to fix brain tissue. **Note:** More detailed instructions on the perfusion process can be found here[19]. Animal tremors will be observed when done properly.

4.3 Remove the head using a pair of large scissors and then perform a series of incisions to liberate the brain from the skull. Store brain tissue at 4 ○C in 4 % PFA overnight to continue fixing.

4.4 Remove brains from 4 % PFA solution and place in a 10 % sucrose phosphate buffer solution to cryoprotect tissue for 24 hours at 4 ○C. Then transfer tissue to a 30 % sucrose solution for another 24 hours at 4 ○C before sectioning. Brain tissue will sink in sucrose when ready for microtome sectioning. Brain tissue can be stored long term in 30 % sucrose.

4.5 Section brain tissue coronally in 40 μm sections using a microtome and serially store each section in rows of 6 in a 48 well plate filled with antifreeze solution (ethylene-glycol 150 ml/sucrose 150 g/ fill to 500 ml 0.1 M PB).

4.6 Collect sections beginning at the start of the dentate gyrus, about −1.20 mm from bregma, and ending after plate is complete with the first section being the most anterior and the last section being the most posterior. Consult a mouse brain atlas to accurately identify the DG and starting tissue collection coordinates.

5. Immunohistochemistry

Basic Protocol (floating)

5.1 Transfer sections from antifreeze solution to Tris-Buffered Saline (TBS) in serial order in a 48 well plate.

5.1.1 Wash sections twice in TBS-triton (.05 % TBS-triton) for 5 minutes by aspirating the solution each time while shaking or on a rocker at slow speeds.

5.1.2 Permeabilize sections using permeabilization solution (.5% TBS-triton) for 20-30 minutes while shaking at slow speeds.

5.1.5 Make blocking buffer by adding .33 μl of donkey serum in to 10 ml of TBS-triton. Make fresh and use within 3 days.

5.1.6 Aspirate permeabilization solution and incubate sections in blocking buffer for 30 minutes to 1 hour at room temperature.

5.1.7 Make primary antibody solution in blocking buffer and add to each well. 500 μl/well is sufficient for all tissue to be completely submerged. Incubate overnight at room temperature on a rocker or shaker with.

5.1.8 Aspirate solution and rinse in TBS-triton 3 times for 10 minutes each to remove traces of primary antibody from tissue.

5.1.9 Incubate in fluorophore conjugated secondary antibody against primary antibodies prepared in blocking buffer solution for 2 hours at room temperature on a rocker.

5.1.10 Wash sections 3 times for 5 minutes each in TBS-triton then incubate sections in DAPI solution (300 μM solution at 1:100) diluted in PBS for 15 minutes.

5.1.11 Wash sections 3 times in PBS and mount sections maintaining serial order from anterior to posterior on a plus charged slide. Let tissue dry at room temperature until moisture is visibly gone, usually about 2 −5 minutes, before coversliping with mounting media.

Antigen retrieval (If staining nestin perform this step before thymidine analog step, skip for Tbr2 or DCX staining)

5.2 Place tissue sections in PBS and mount 5 −8 sections on plus charged slides maintaining serial order from anterior to posterior. Let tissue sections dry at room temperature to completely adhere to slides. Tissue should be visibly absent of moisture which takes about 2 −5 minutes.

5.2.1 Prepare citrate buffer boiling solution in a container, usually a 1000 µl pipette tip box. Citrate buffer solution is comprised of 9ml of citric acid stock [.1 M] + 41 ml of tri-sodium citrate stock [.1 M] + 450 ml of ddH20.

5.2.2 Heat citrate buffer in a microwave (1000 watts) for 5 minutes until solution is boiling. While solution is heating, place mounted sections in 20 slide glass slide holder. After the five minutes, carefully set the slide holder with the sections in to the pipette box.

5.2.3 Set microwave power to 50% and cook time for 7 minutes. Start a timer for 7 minutes and watch microwave during these 7 minutes stopping the microwave when solution starts to boil and continuing after boiling stops. **Note:** The goal of this step is to keep water temperature right below boiling temperature for 7 minutes. Stop after the timer runs out even if the cook time on the microwave has not finished. More detailed instructions can be found here[20].

5.2.4 Take warm box with citrate buffer and tissue slides and place it in an ice bucket to cool. Cover to prevent ice or other materials from entering the solution. Wait for about 30 minutes or until solution is cool to the touch.

5.2.5 Proceed to thymidine analog staining step 5.3.2 if using thymidine analog.

Thymidine Analog Staining

5.3 Place tissue sections in PBS and mount 5 −8 sections on plus charged slides maintaining serial order from anterior to posterior and same orientation. Let tissue sections dry to completely adhere to slides and then draw a border using a hydrophobic pen or PAP pen.

5.3.1 Permeabilize with permeabilization solution (.5 % TBS-Trition) for 20 - 30 minutes. Then wash sections 2 times using TBS-triton for 5 minutes each. Permeabilization aids intracellular antibody penetration. **Note:** Permeabilization time can be adjusted depending on tissue thickness and antibody efficiency. Alternatively, one can increase detergent concentration to increase permeabilization potency. However, care should be taken to not permeabilize for too long since tissue fragility increases the longer it is permeabilized.

5.3.2 Prepare Edu reaction solution by adding 1mg CuSO4.5H20 in 4 ml of .1M Tris pH 8.5. Then add fluorescently conjugated Azide (i.e. Alexa488-Azide) at 1:40 from a 600 µM stock

solution made in PBS. Lastly mix 10 mg/ml L-Na+ ascorbate right before applying solution to tissue sections. The final concentration of Alexa488-Azide 15 μM in 1 ml of Edu reaction solution.

5.3.3 Incubate sections in Edu Azide solution for 30 min - 1hour and then wash 3 times in TBS-triton for 5 minutes each. Cover slides in aluminum foil to protect from light after this step. **Note:** At this stage check if the Edu reaction worked by using a fluorescent microscope. Edu labeled cells will fluoresce under an epifluorescence microscope.

5.3.4 Block using blocking buffer raised in the same animals as the secondary antibody, (for this example donkey serum was used) for 30 min - 1 hour and then wash 2 times in TBS-triton for 5 minutes each.

5.3.5 Prepare primary antibody during the blocking step, (i.e chicken anti-nestin, Aves, at 1:200) solution by mixing primary antibodies in blocking buffer solution. 250 μl per slide is sufficient to assure that tissue is completely submerged in solution.

5.3.6 Apply primary antibody solution over night at room temperature after blocking washes. Modify this step depending on the primary antibody used. **Note:** If antibodies have high back ground or non-specific binding, incubating at 4 $^{\circ}$C instead of room temperature may improve results. Antibodies with poor tissue penetration can be left to incubate for two days if needed.

5.3.7 Wash three times using TBS-triton for 5 minutes. Then apply fluorophore conjugated secondary antibodies (i.e. Alexa 647 anti-chicken at 1:200) prepared in blocking buffer solution for two hours.

5.3.8 Wash sections 3 times in TBS-triton for 5 minutes then apply DAPI 300 μM solution at 1:100 in PBS for 15 minutes.

5.3.9 Wash 3 times in PBS for 5 minutes and remove pap pen circle from around tissue using a cotton swab or a delicate task wipe. Let sections dry and then apply mounting media and cover slip. Let mounting media dry before imaging slides.

6. Image collection

6.1 Blind experimental groups from control groups by covering slide labels and image the same side DG using a confocal microscope (see materials and methods) with a 40x oil magnification optical lens at 1 μm step size or a 20× 2x zoom at 1 μm. **Note:** The 40x with oil will give increased resolution but take longer than the 20× 2x zoom.

6.2 Set objective lens to 40X and then click on the locate tab on the upper left corner in the confocal software (see materials and methods) and set the desired DG section in the middle of the field of view.

6.3 Locate a DG and switch to the acquisition tab in the upper left corner then check the following boxes: Z-stack, tile-scan, position.

6.4 Set channel settings between 600-750 gain, 1% −15% laser intensity, and 1 – 10 offset. Don't exceed 20 % laser intensity for any of the channels. Assure that no pixels are oversaturated when setting gain and intensity. **Note:** This range will vary depending on the efficiency of the equipment utilized and staining efficiency.

6.5 Set the tiles to 7 horizontal by 3 vertical in the tile scan window and press scan overview image using the same settings as the ones currently being used. For example use 7 horizontal by 3 vertical and the 20x objective with 2 times zoom.

6.6 Assure that the DG is completely within expected image after overview scan. If not, adjust the view of the DG until it is entirely in the overview image since this is a representative image of what you will be obtaining.

6.7 Set imaging depth by scrolling through different Z-stacks using the fine focus knob. Assure that the entire DG is within the start and end points. Assuming 1 μm step size, each image should be around 40 steps.

6.8 Set scanning speed to 9 with bi-directional scanning and no averaging in the acquisition mode window. Then add position in the position window. By repeating the following steps, several DG's can be set to image at once. **Note:** Increasing scan speed lowers image quality but reduces overall imaging time. If image quality is too low, reduce scan speed or increase averaging.

6.9 Click start experiment button when ready. Scan 5 DG's per animal along the anterior to posterior axis. For example: If the left DG is imaged for section one, image the next most posterior left DG for section two.

6.10 Stitch images together using the stich feature under the process tab in the confocal software. Alternatively, FIJI (Image J) can be used to stich images as well.

6.11 Save stitched images for quantification.

7. Image analysis

7.1 Open each section using FIJI as both a maximum projection and as a composite image with the channels merged in distinct colors to easily visualize colocalization.

7.2 Measure the volume of the dentate gyrus for each section of the maximum projection image using the polygon selection tool (third box from the left) and record for each section of each animal. **Note:** This will be the area of the dentate gyrus utilized to calculate the density.

7.3 Record the number of cells in the dentate gyrus from the composite image that have colocalizing primary antibody (i.e. nestin) and the thymidine analog Edu, by using the FIJI plugin cell counter found under plugins -> analyze -> cell counter -> cell counter. Additionally, record the total number of Edu positive and nestin positive cells with a radial process. **Note:** For the case of nestin, it is very important to pay attention to morphology. If quantifying neural stem cells, assure only cells with a radial process are quantified. When

quantifying sections assure that the same criteria is applied when visualizing cells outside of a focal plane. (See the following for more detailed instructions on stereological quantification.[21])

7.4 Enter cell counts in to a spreadsheet software to compile all pieces of data for analysis later.

7.5 Calculate the density of colocalized cells for each section by dividing the total number of colocalized cells by the total volume for each section in each animal. For example to obtain stem cell density = (sum of nestin+/edu+ cells in one animal/ sum of DG volume in one animal) The volume of each section is the (area x total Z-step increments assuming each step was 1 μm). **Note:** For this example, total steps should be close to 40, since tissue was sectioned at 40 μm. Assure that each animal is a data point since the goal of this approach is to estimate total amount of colocalized cells in one hemisphere of a hippocampus.

7.6 Perform additional necessary calculations for the question one is trying to address. In this example we calculated the overall number of proliferating cells, total stem cell population, and percent of proliferating stem cells after stimulating contralateral mossy cells.

REPRESENTATIVE RESULTS:

Following the experimental procedures described above, we were able to determine the effects of stimulating contralateral mossy cell projections on the neurogenic niche within the hippocampus (Figure 1A, Figure 1B). By utilizing a cre dependent Gq coupled stimulating DREADD virus paired with a mossy cell labeling 5-HT2A Cre– line, we were able to selectively activate excitatory projections from mossy cells on to the contralateral dentate gyrus and determined that strong mossy cell stimulation promoted stem cell quiescence (Figure 1C). We verified accurate viral delivery before the analysis of tissue. (Figure 2A, Figure 2B) Additionally, we verified activation of mossy cells via c-fos immunohistochemistry experiments (data not shown). In the case of improper viral injection, exclude animal from further analysis. An improper injection is one that fails to target the desired coordinates, has most of the expression outside the desired region, or has little to no viral delivery. For this experiment mossy cells in the hilus of the DG were the intended target, and if injections were outside of the hilus, they were excluded. By using a thymidine analog, edu, and antigen retrieval for the nestin staining outlined in section 5.2 and 5.3, we were able to successfully label proliferating neural stem cells (Figure 3A). Additionally, by omitting the antigen retrieval step, step 5.2, we were able to label TBR2 positive neural progenitor and neuroblast, and DCX positive neuroblast and immature neurons (Figure 3A). We demonstrate an example of the area quantified and used to calculate density and provide an example of mounted tissue on a slide (Figure 3B, Figure 4A). Moreover, both successful and sub-par experiments are provided as references for experimental approaches (Figure 4B). Lastly, there are several different quantifications that can be obtained from a successful experiment (Figure 5A-D) [15]. The quantifications include the density of proliferating neural stem cells (Nestin+/Edu+ /Volume) , the percent of proliferating neural stem cells (Nestin +/Edu+ /total nestin), total proliferating cells (Edu+/Volume) and total stem cell pool (Nestin +/Volume). Upon contralateral stimulation of a mossy cells a decrease in neural stem cell

proliferation is observed. Similar quantifications can be obtained for neural progenitor and immature neurons by using the appropriate antibody.

DISCUSSION:

The goal of this protocol is to assess how manipulating specific neural circuits regulates adult hippocampal neurogenesis *in vivo* using a series of immunohistochemistry techniques. Assaying activity dependent regulation of adult neurogenesis mediated by specific neural circuits is a valuable technique with great potential for modifications to study a diverse range of neural circuits. The success of these types of experiments depends on multiple factors including accurate viral delivery, proper viral selection for the desired manipulation, proper delivery of a thymidine analog, animal age, immunostaining efficiency, successful transcardial perfusions, and unbiased quantification of images. For example, inaccurate viral delivery may cause off target effects that result in a phenotype unrelated to the circuit in question. Additionally, low quality immunofluorescence techniques may hide the true number of present cells and therefore produce a phenotype that is not biologically relevant. Another very important factor to control is the age of the mice when performing experiments, considering that adult neurogenesis is age dependent[22]. Lastly, it is important that each section be unbiasedly scored. To reduce bias, take a methodical approach and assure that the person scoring is proficient at identifying the stages of adult NSC development using morphological information. Additionally, blind both control and treatment groups and reveal their identities after image quantifications. As an additional measure to reduce bias, two separate individuals can quantify the same data set to validate observed results.

There are several limitations associated with this approach to study circuit activity dependent regulation of adult NSCs and newborn progeny. The first limitation is that this approach does not provide information about the specific cell types within a circuit that mediate the overall effect on NSCs from manipulating the circuit in question. This means that although there might be a phenotypic effect on adult NSCs, the effect may be acting through one or several intermediate cell types. An efficient way to address this concern is to pair these studies with electrophysiology to pin down the intermediaries. An additional limitation of this protocol is the need to have either a specific CRE mouse (5-HTR2A) line or a viral construct (AAV5-camKII-hM3d-mcherry) that can target the desired circuit. If an effective cell specific CRE mouse line is not readily available for a question of interest, the ability to study this circuit becomes increasingly difficult. However, many cell types in the brain have CRE specific mouse lines. A lesser limitation in this protocol is related to CNO as an effective inert ligand. Recently, studies demonstrated that CNO, the inert chemical used to activate DREADDs, metabolizes to clozapine, which may cause behavioral phenotypes [23]. However, an efficient way to address is to include proper controls in each experiment. An example of proper controls includes both a CNO and DREADD control, where CNO is administered in combination with a control reporter virus (AAV5-DIO-mCherry), and a saline only control where no CNO is administered to a reporter virus group. By including these controls, the effects of only CNO can be isolated. Alternatively, a secondary inert ligand known as C21, has been recently demonstrated to have similar efficacy and potency with no demonstrated behavioral effects [24]. Lastly, a final limitation of

this protocol is controlling the amount of CNO that each animal consumes during the experiment. Different animals drink CNO-containing water at varying degrees and may therefore have a range of effects on adult neurogenesis. In general, a mouse tends to drink about 4 ml of CNO water in a 24-hour period. This means that at the concentration of 1 mg/200 ml animals receive a total of .02 mg of CNO per day which is comparable to the amount of a single CNO dose injected intraperitoneally. If timely coupled administration of CNO is a concern, switching to intraperitoneal injections may be a better alternative.

The advantage of using this protocol is the degree of specificity achieved when modulating adult neurogenesis. Past neurogenesis studies have utilized systemically administered pharmacological agonist or antagonist to modulate circuit components. These non-specific manipulations may produce phenotypic differences but provide little insight about mechanisms involved in adult neural stem cell regulation. Additionally, this protocol can be easily modified to investigate various circuit wide effects on adult neurogenesis. For example, by switching to an inhibitory DREADD, or by targeting one or multiple brain regions at once, one can ask an array of questions to understand circuit specific regulation of adult neurogenesis. Another advantage of using this protocol over previous approaches is that the use of a nestin antibody, eliminates transgenic animal breeding of fluorescently encoded neural stem cell reporters such as nestin:GFP, increasing efficiency and reducing time per experiment[8]. Furthermore, this technique limits rodent handling when administering CNO, which reduces rodent stress during experiments. It's important to mitigate stress when studying stress-sensitive process. Lastly, this approach is easily amendable to include a behavioral assay. For example, if one were interested in asking if the contralateral mossy cell circuit that modulates NSCs, also plays a role in spatial learning or stress resilience.

The main technical difficulty when using this approach is accurate viral delivery. Becoming a proficient rodent surgeon takes practice and can take significant troubleshooting. It is therefore advisable to perform a series of pilot experiments to test viral titer, labeling efficiency, and viral spread. We've found that certain serotypes have different spreading patterns and that the AAV2 serotype spreads less than AAV5 or AAV8. Additionally, it's best to have a trusted viral packaging provider for each of these experiments. By performing pilot surgeries, many of these concerns can be addressed and one can save time. It is also recommended that one test different CNO concentrations to stimulate or inhibit the desired circuits. In general, 1 mg/kg will sufficiently activate tested circuits, but certain cell types may require more or less CNO. It is important to note that the dose of CNO administration can differentially affect certain circuits specifically when looking at something like mossy cells[15].

Alternative applications for this protocol include simultaneous behavioral testing, modulation of alternative circuits, and additional analysis of neurogenesis features. To perform behavioral testing, one could follow the protocol described and after administering CNO, perform a specific behavioral task, like a novel location assay, or a spatial navigation task. The benefit of this approach is that a single experiment would yield both behavioral and circuit specific information that could lead to a circuit specific behavioral phenotype. To modulate alternative circuits, one can use a combination of different cre lines and viral

vectors. For example, if one were interested in understanding how inhibiting dopaminergic neurons from the Ventral Tegmental Area or VTA modulates adult neurogenesis, one could use a tyrosine hydroxylase Cre mouse line and inject a Cre dependent hM4D (inhibitory) DREADD virus in to the VTA to determine dopaminergic specific regulation of adult neurogenesis. The possibilities to target alternative brain regions using this approach are vast and can be strategically used to interrogate compelling neural circuits. Lastly, this approach allows one to investigate additional stages of adult neurogenesis. If for example one wanted to understand how stimulating mossy cells affects arborization or dendritic length of immature neurons, one would follow a similar protocol but perform alternative analysis such as sholl analysis.

In summary, this protocol provides a detailed step-by-step process to assay circuit activity dependent regulation of adult NSCs and neurogenesis via DREADD technology. The strength of this protocol lies in its ability to be easily modified to address a vast range of questions regarding circuit specific adult neural stem cell regulation. With the advancement of CRISPR technology, it is now easier to generate cell specific CRE mouse lines to pair with sophisticated viral constructs to address increasingly complex questions expanding the applicability of this protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS:

L.Q. was supported by the National Institute Of Mental Health of the National Institutes of Health under Diversity Supplement R01MH111773 as well as a T32 training grant T32NS007431-20. J.S. was supported from grants from NIH (MH111773, AG058160, and NS104530).

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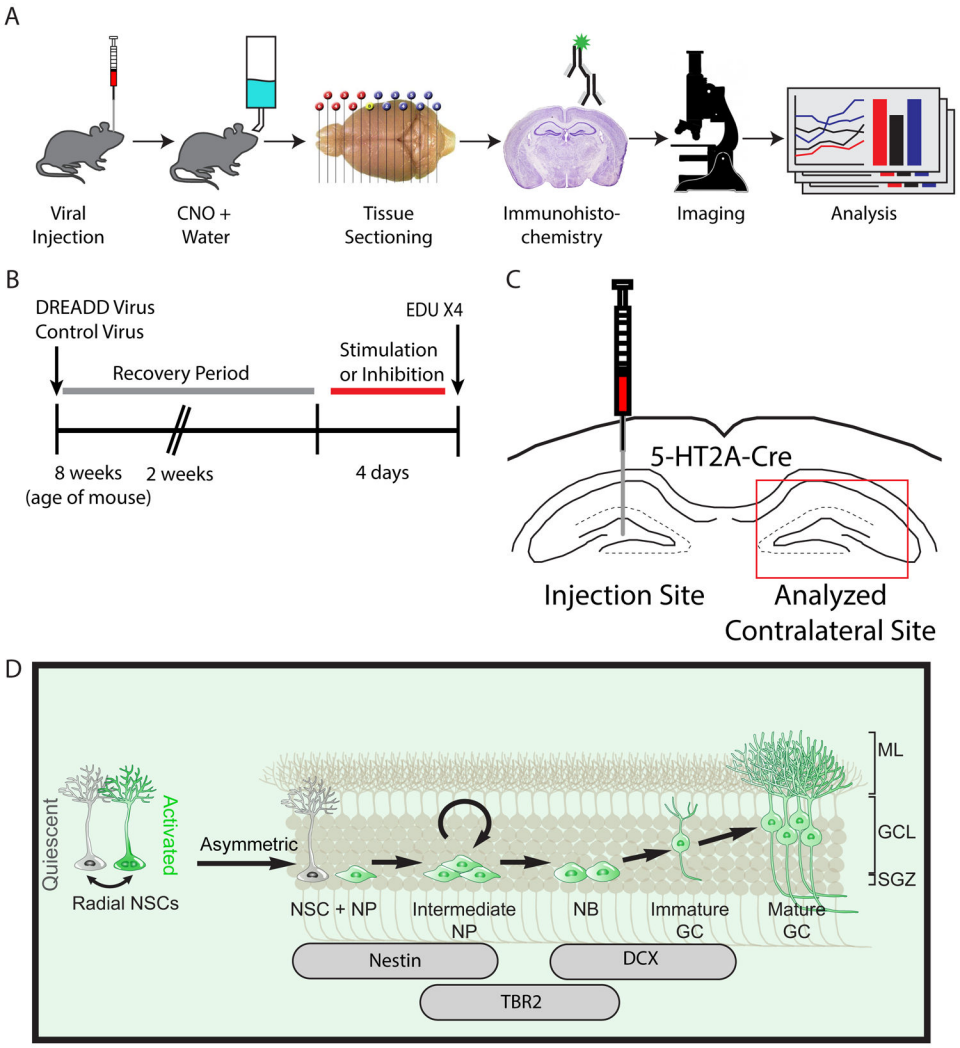

Figure 1: Experimental approach to assay circuit regulation of adult neural stem cells:
(A) Schematic representing the different steps outlined in the protocol. **(B)** Timeline of experimental approach used to stimulate mossy cells in rodents. **(C)** Injection schematic targeting contralateral mossy cells for stimulation. **(D)** Schematic of developmental lineage of adult neural stem cells in the SGZ with corresponding antibodies used at different developmental stages.

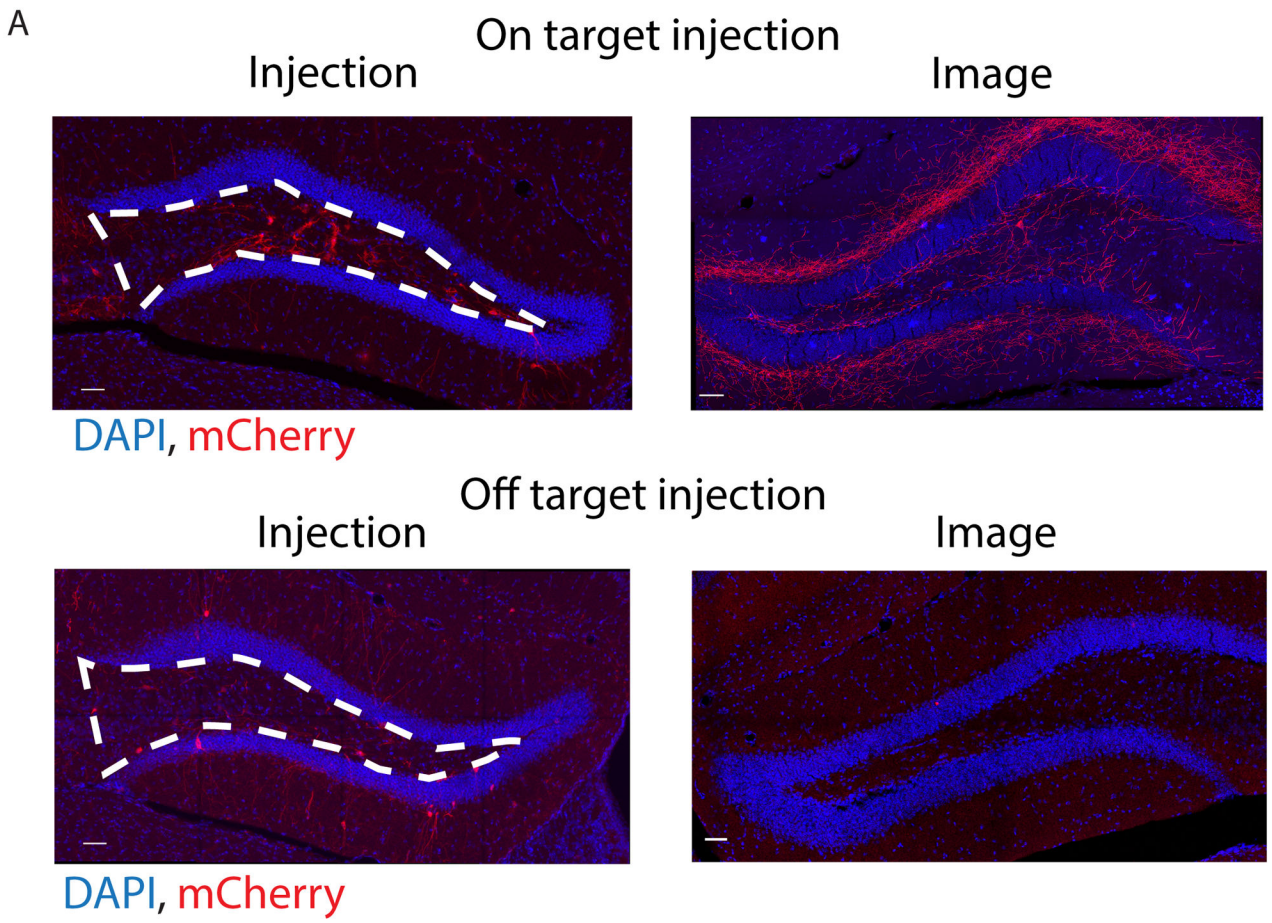

Figure 2: Demonstration of effective viral delivery:

(A) Immunofluorescence image of accurate and inaccurate viral delivery. Viral particles are labeled with mCherry and cell nuclei are labeled with DAPI. Image side of accurate viral delivery demonstrates clear mossy fiber projections from the contralateral injection side. These fibers are absent in the case of improper viral injection. Scale bar is 50 μm.

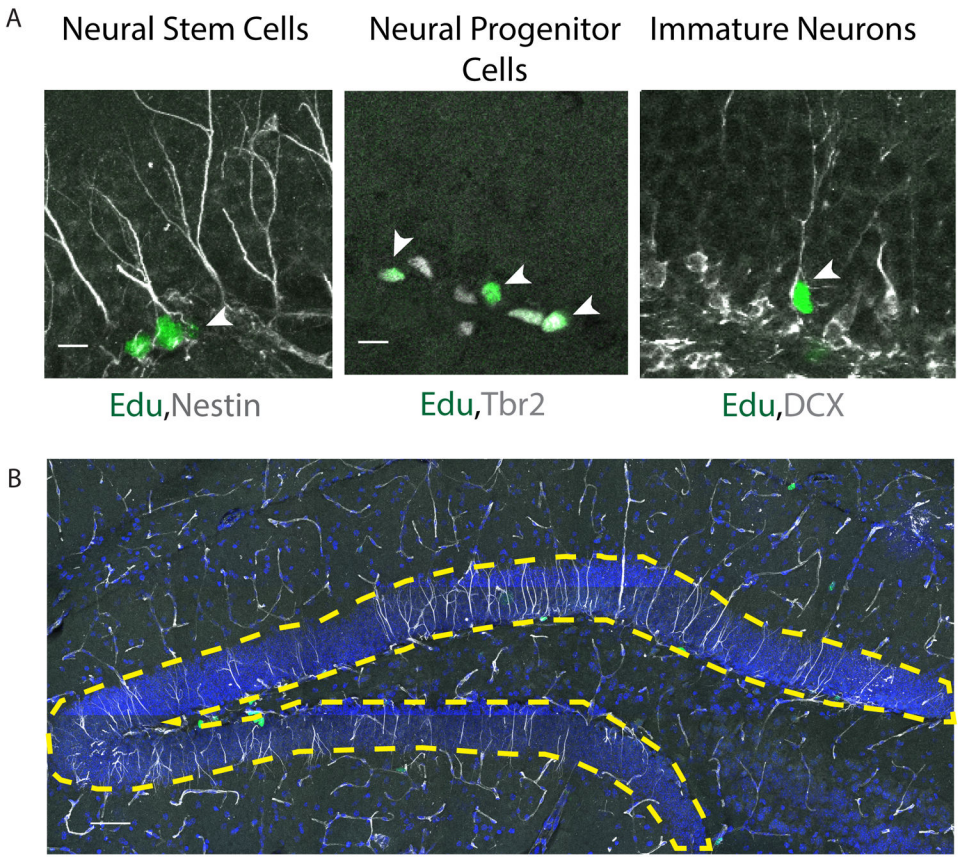

Figure 3: Analysis of proliferating neural stem cells and progeny:
(A) Immunohistochemistry images of thymidine analog edu colocalizing with specific cell stage markers nestin (neural stem cells and progenitors), Tbr2 (neural stem cells, neural progenitors), and DCX (neuroblast, immature neurons) indicated by white arrows. Scale bar is 10 μm. **(B)** Representative measurement of the area within the dentate gyrus used to calculate density. Scale bar is 50 μm.

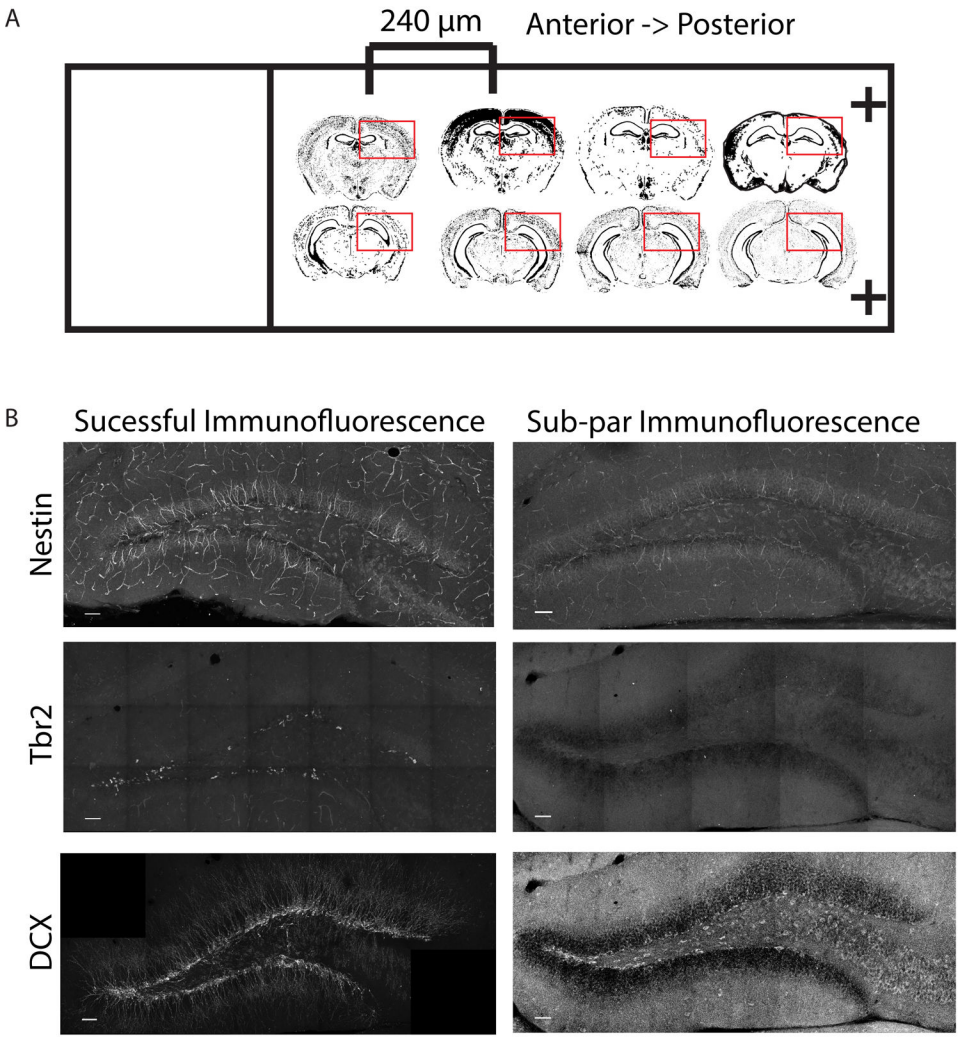

Figure 4: Demonstration of Immunofluorescence preparation:

(A) Schematic demonstrating stereological separation of mounted tissue sections from anterior to posterior axis. Red box denotes the side imaged. **(B)** Demonstration of successful and sub-par immunofluorescence experiments for neuronal lineage markers. Scale bar is 50 µm.

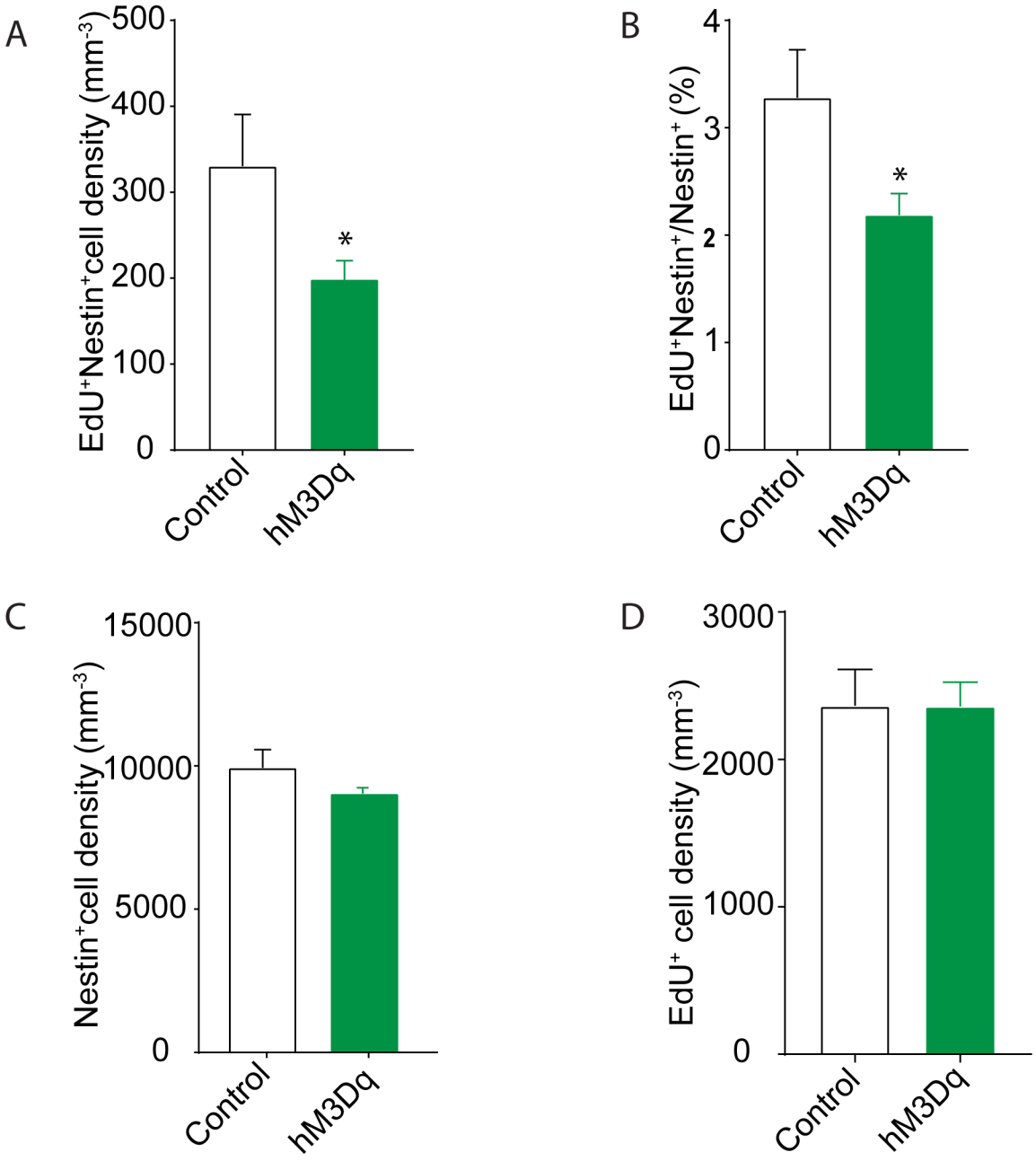

Figure 5: Contralateral activation of mossy cells decreases neural stem cell proliferation: **(A)** Immunohistochemistry quantifications of Nestin+/Edu+ cells in the dentate gyrus demonstrates a decrease in proliferation after stimulation of contralateral mossy cells. **(B)** Decrease in percent of proliferating neural stem cells in the group with activated contralateral mossy cells. **(C)** There was no significant change in neural stem cell density in either group. **(D)** There was no change in overall levels of proliferating cells in the dentate gyrus. (Figure adapted from[15]) Values represent means ± SEM. $p < 0.05$ (n = 3 control, 5 = hM3D group)

Table 1:

Solutions utilized for immunohistochemistry

Name	Recipe
Antifreeze Solution	ethylene-glycol 150 ml + sucrose 150 g + fill to 500 ml 0.1 M PB for 500 ml solution
Citrate Buffer	9 ml of citric acid stock + 41 ml of tri-sodium citrate buffer + 450 ml of ddH20
Citric acid stock	[0.1 M] Citric Acid 21 g/1 L ddH2O
Tri-sodium citrate stock	[0.1 M] Tri-sodium Citrate 29.4 g/1 L ddH20
Tris Buffered Saline -Triton (TBS - Triton)	.05 % 100-x Triton in TBS
Permeabilization Buffer	.5 % 100-x Triton in TBS
Blocking Buffer	.33 ml Donkey Serum in 10 ml TBS-Triton
Edu reaction solution 1	Make 1 mg CuSO4.5H20 in 4 ml of [0.1 M] Tris pH 8.5
Edu reaction solution 1 + 2	Add 1:40 of a 600 μM Alexa488-Azide solution and 10 mg/ ml of L-Na+ ascorbate to Edu solution 1