Supplementary Material for Johnson et al. (2021) Assessment of Mapping the Brain, a Novel Research and Neurotechnology Based Approach for the Modern Neuroscience Classroom. J Undergrad Neurosci Educ 19(2):A226-A259.

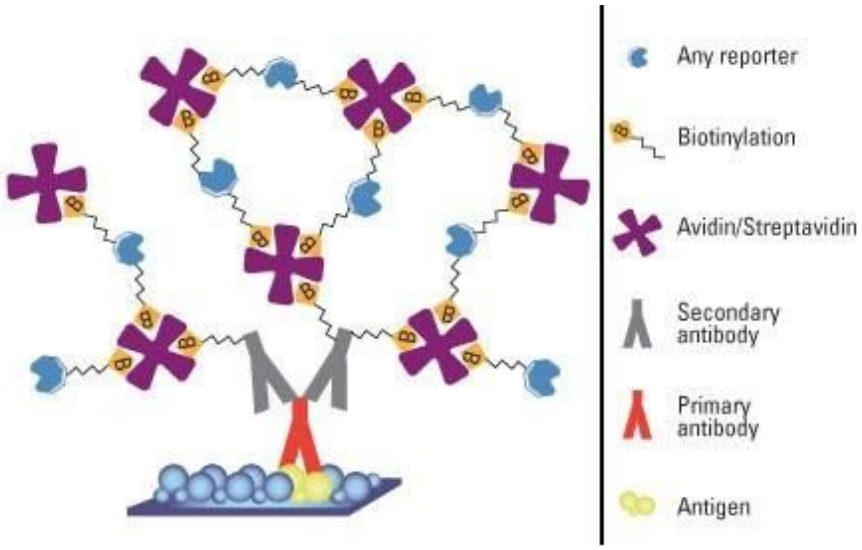
Laboratory Protocol 3

Mapping a subset of norepinephrine neuron projections (PART A)
&
Analysis of DREADD induced neuronal activation (PART B)

Background

Neuronal projections (PART A)

Previously we explored the expression of mCherry-hM3Dq and eGFP expression in norepinephrine (NE) neurons using immunohistochemistry and fluorescence microscopy. We also briefly investigated the projections (i.e. axons and axon terminals) of the eGFP expressing NE neurons in a few brain regions. Because the ultimate function of a neuron depends on its axonal targets, we can learn a lot about the potential function of neuronal subpopulations by studying their projection patterns. In today's lab we will explore the projection profile of the eGFP expressing subpopulation of NE neurons in more detail by using a colorimetric immunostain. Neuronal projections are small, fine structures that can be difficult to visualize with fluorescence microscopy especially without the aid of confocal microscopy. We will use the avidin-biotin complex (ABC) immunohistochemical method for projection staining to enhance our ability to detect neuronal projections across the entire brain. The ABC method takes advantage of the high binding affinity of biotin (Vitamin H) for avidin (see the diagram below). The biotin-avidin interaction is a strong non-covalent interaction that can occur with up to four biotins bound per complex. To detect eGFP projections in our tissue we will use a biotinylated secondary antibody in conjunction with a biotinylated enzyme (peroxidase) instead of a fluorescent secondary antibody like we did in Lab 2. The peroxidase will convert the colorless substrate 3,3'-diaminobenzidine (DAB) into a brown precipitate that will fill the neuronal projections. Since four biotins are bound per avidin complex, multiple enzymes are present at a single antigenic site, increasing sensitivity and signal intensity. The ABC detection method not only increases our ability to detect neuronal projections across the brain but also provides a permanent immunostain that can be imaged repeatedly without signal loss, unlike fluorescence where signal intensity diminishes quickly over time and with light exposure.



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Neuronal activation and immediate early gene expression (PART B)

Remember, through the application of a **recombinase based intersectional genetic approach**, our mice are designed to express mCherry-hM3Dq in a genetically defined subset of NE neurons while all other NE neurons express only eGFP. This engineered receptor is designed to respond only to the otherwise inert compound CNO and enables us to study the affects of "turning on" a specific subset of central NE neurons. Today you will be working with brain tissue from two animals that express mCherry-hM3Dq in a genetically defined subset of NE neurons. One animal was treated with CNO just before brain tissue collection and the other was treated with a vehicle control. We will map and compare neuronal activation patterns in these animals by exploring the expression of the immediate early gene, ***c-fos***. Our goal is two-fold, to provide evidence for CNO activation of the mCherry-hM3Dq expressing NE neurons and to discover other neuronal populations that may have been activated as a result of "turning on" the mCherry-hM3Dq expressing NE subpopulation. But why is *c-fos* a reasonable indicator of neuronal activation? "Activated neurons" that receive significant synaptic inputs will experience depolarization and calcium influx. In many cells, calcium entry not only depolarizes the cell but also acts as a second messenger that will trigger intracellular signaling cascades. In activated neurons, calcium-dependent kinase cascades will activate transcription factors (such as *c-fos*) to trigger rapid induction of specific genes. Thus, by using immunohistochemical techniques to detect *c-fos* expression researchers can make a map of neuronal activation patterns in response to specific behaviors or stimulations. In our case, we will explore the neuronal activation pattern across the brain in response to activating *En1*-derived NE neurons (i.e. the mCherry-hM3Dq expressing subpopulation).

Materials:

- 200 µL aliquots of H_2O_2
- 600 µL aliquots of NGS
- Platform shaker
- Brain sections
- Sharpies
- Paint Brushes
- Eppendorph tubes and tube racks
- Bench top mats
- 15 and 50 mL conical tubes
- PBS and PBST squirt bottles
- Graduate cylinder for rinsing runoff
- 12 well plates
- Stock PBS and PBST
- Chicken anti-GFP (AB13970; AbCam; 1.5µL aliquots)
- Rabbit anti-cFos (sc-52; Santa Cruz; 6 µL aliquots)

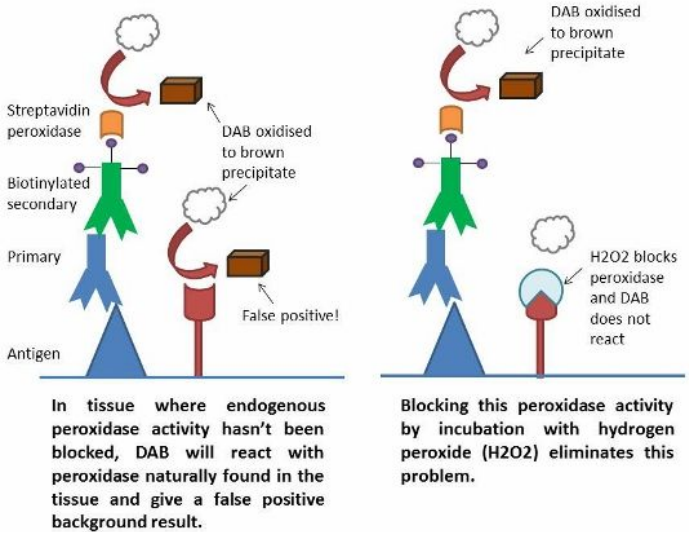
Day1

Purpose: Start colorimetric immunohistochemistry (quench endogenous peroxidase activity and primary antibody incubation)

1. Thaw your tissue sections. Wash them 3x for 5 minutes with PBS in the net wells on the platform orbital shaker.
    a. Prepare your 0.3% hydrogen peroxide solution for step 2 while you wait on your washes. The stock hydrogen peroxide solution you have been given is 30%. You will need 4mLs of the 0.3% hydrogen peroxide solution for each net well.

2. Quench endogenous peroxidase in 0.3% hydrogen peroxide in PBS for 30 minutes **in the net wells** on the platform orbital shaker.

This quenching step is crucial in preventing a high background signal. Peroxidases are present in brain tissue, and will non-specifically oxidize the chemical substrate that we are using for our colorimetric reaction, 3, 3'-diaminobenzidine (DAB). Pre-treatment with saturating amounts of hydrogen peroxide will irreversibly inactivate peroxidases present in the tissue and prevent DAB oxidation.



In tissue where endogenous peroxidase activity hasn't been blocked, DAB will react with peroxidase naturally found in the tissue and give a false positive background result.

Blocking this peroxidase activity by incubation with hydrogen peroxide (H2O2) eliminates this problem.

3. Rinse your sections in PBS, shake in the net wells on the platform orbital shaker for 5 min.

4. Rinse your sections **2X** in 0.1% PBST, in the net wells on the platform orbital shaker for 5 min.
    a. Prepare 6mLs of 5% NGS in 0.1% PBST for step 5 while you wait on your washes. Aliquot the NGS into eppendorf tubes to prepare them for your tissue sections.

5. Incubate in 5% normal goat serum (NGS) in 0.1% PBST for 30 minutes on the rotator in eppendorph tubes.
    a. Prepare the **<u>two</u>** antibody solutions for step 6 while you wait on your NGS incubation, one for mapping projections chicken anti-GFP and one for mapping neuronal activation rabbit anti-cFos.

6. Incubate with primary antibody in 0.1% PBST in eppendorph tubes on a rotator in the 4°C fridge.
    Solution 1: Chicken anti-GFP antibody for 1 forebrain sample that has NOT been treated with CNO
    a. Prepare a total volume of 20mLs
    b. Chicken anti-GFP antibody (1:20,000)
    c. 1% NGS

    Solution 2: rabbit anti-cFos antibody for 1FB and 1HB sample from a CNO treated animal and 1HB from a vehicle (VEH) treated control animal.
    a. Prepare a total volume of 5mLs
    b. Rabbit anti-cFos antibody (1:1000 or 1:5000-be sure to record what you choose!)
    c. 1% NGS

    Your samples will rotate at 4°C for 48 hours then be placed on their side until we start Day 2 next week.

Day2

Purpose: Secondary antibody incubation, tissue section mounting and clearing

1. Rinse your sections 3X in 0.1% PBST for 10 minute in net wells on the orbital platform shaker.
    a. Prepare **<u>two</u>** secondary antibody solutions for step 2 while you wait on your washes, one for mapping projections (goat anti-chicken) and one for mapping neuronal activation (goat anti-rabbit).

2. Incubate with biotinylated secondary antibody in 0.1% PBST for 30minutes in eppendorph tubes on the rotator **Solution 1: Biotinylated goat anti-chicken** for 1 forebrain sample that has NOT been treated with CNO and was incubated with primary chicken anti-GFP (to visualize a subset of NE neuron projections).
    a. Prepare 2 mLs total volume
    b. Biotinylated goat anti-chicken (1:500)
    c. 5% NGS

    Solution 2: Biotinylated goat anti-rabbit for 1FB and 1HB sample from a CNO animal and 1HB from a VEH control animal and was incubated with the primary rabbit anti-cFos (to map neuronal activation).
    a. Prepare 5 mLs total volume
    b. Biotinylated goat anti-rabbit (1:500)
    c. 5% NGS

3. Rinse your sections 3X in PBS, shaking in net wells on the platform orbital shaker for 10 minute intervals.
    a. **During these washes**, prepare the Vectastain AB reagent for step 4 and allow it to stand for ~30 minutes before use (i.e. the duration of your washes).

4. **Vectastain Elite ABC Kit**: Add 2 drops of REAGENT A to 5ml of PBST mix, then add 2 drops of REAGENT B mix immediately and vortex. **Allow to stand for 30 minutes before use**. REAGENT A contains avidin that has been modified to minimize non-specific binding and REAGENT B contains biotinylated peroxidase H with enhanced enzyme activity.

5. In Eppendorph tubes on the rotator, incubate sections in the AB mixture for 30 minutes at room temperature.

6. Rinse sections 3X in 0.1% PBST, in net wells on the platform orbital shaker for 10 minutes intervals
    a. Prepare your DAB solution for step 7 during your **last 10 min wash**. **DAB is a hazardous chemical.** We will need to properly dispose of anything that comes into contact with the DAB mixture, so set these materials aside after working with them. Be careful when handling DAB use gloves, eye protection and lab coats.

7. To 15 ml of distilled water in a conical tube, add 6 drops of buffer stock and vortex. Add 12 drops of DAB stock solution and vortex. Add 6 drops of hydrogen peroxide solution and vortex.

8. **Let the instructor know before moving to this step and read all of the instructions before you start**. Using a ***new*** 12 well plate, fill the top row of wells with your DAB solution and fill the rest with dH2O. Prepare another washed 12 well plate by filling it with dH2O for washing. Make sure all the liquid is drained from your tissue sections. **Set a timer** (start it right when your tissue goes into the DAB solution). Move your net wells containing tissue into the DAB solution. Immediately begin shaking the sections **until suitable staining develops**. Immediately move to water rinse after suitable staining has developed (step 9).
    a. The immunostaining for GFP will likely take ~6min but have your instructor help you.
    b. **Record the time** needed to develop the cFos and GFP stain.
    c. If no stain develops after 15 minutes, then move to the next step.

9. Quickly (3-5 seconds) move the sections from well to well in the trays of water. After moving to the 2nd plate, increase the wash time to 5 minute intervals. DO NOT dump out the DAB 12-well plate. Put the lid on and wrap it in parafilm. Be careful not to spill. We will dispose of this later.

Tissue Section Mounting

10. As in the fluorescence lab, divide your sections anatomically in a 6 well plate filled with PBS. Mount the sections in the same fashion as before, labeling the slides appropriately **in pencil** (subsequent ethanol washes will run ink into your sections).

11. Dehydrating and clearing sections. **This must be done in hood**. **Xylenes are hazardous**. Soak the sections in the prepared baths according to the chart below. Be sure that paper towels are present to adsorb any xylene spillage. Anything that comes into contact with xylenes must remain under the hood and disposed of inside of a biohazard bag.

Solution	Repetitions	Time
50% Ethanol	1	5 minutes
70% Ethanol	1	5 minutes
95% Ethanol	1	5 minutes
100% Ethanol	3	5 minutes
Xylenes	3	5 minutes

Tissues, by nature, contain lots of water. The mounting medium we are using, Permount, is synthetic resin dissolved in toluene that is designed to create long lasting slides. Water is incompatible with Permount and needs to be completely removed in order for the media to adhere to the sections. The ethanol baths serve to extract any water from the tissue (dehydration). The xylene baths serve to clear the sections of Ethanol (clearing).

Take care to not expose the slide to the air once you have begun the baths. The sections will dry extremely quickly and may damage the tissue.

12. Using approximately 3 drops to of Permount to mount the cover slip as you did in the previous lab. Allow the slides to dry under the hood.

Day3

Purpose:

(1) Our first goal is to map neuronal projections from a subset of NE neurons (i.e. non-locus coeruleus neurons) across the forebrain. In our *En1^{Cre};Dbh^{Flpo};RC::hM3Dq* animals, NE neurons that have a history of *En1* expression, such as the locus coeruleus, will express mCherry-hM3Dq while all other NE neurons will express eGFP. The projection profile of the locus coeruleus has been studied extensively over decades of research while the rest of the NE system has received less extensive attention. Thus, through our analysis of eGFP projections, we have a unique tool to learn a lot about the connectome of non-locus coeruleus NE neurons (i.e. NE neurons that do NOT have a history of *En1* expression). Where do non-locus coeruleus NE neurons project? Do we find projections in unexpected regions, like the cortex or hippocampus where the locus coeruleus is believed to be the sole provider of NE input?

(2) Our second goal is to map neuronal activation patterns across the brain through analysis of IEG expression (*c-fos*).

An overarching goal of our collaborative, semester long project has been to characterize our newly generated *En1^{Cre};Dbh^{Flpo};RC::hM3Dq* mouse strain. Do these animals express the DREADD receptor where we expect (Lab 2) and can we activate these neurons upon application of CNO (current lab)? Here, we will compare *c-fos* expression across the brain in *En1^{Cre};Dbh^{Flpo};RC::hM3Dq* animals that were treated with CNO or a vehicle control. First, we will look for *c-fos* expression in the subset of *En1* NE neurons that we have showed express mCherry-hM3Dq (Lab 2) and then we will compare expression globally across the brain to identify interesting patterns of neuronal activation. Does *En1* NE neuron activation (i.e. activation of the locus coeruleus) consistently activate particular target regions of the brain?

1. Use your atlas! Start with exploring neuronal projections and work to fill out the table 1 below. Once you are finished with table 1 (eGFP projections) move on to table 2 (*c-fos* neuronal activation).

2. Use the 4x objective to give you a global perspective of the neuroanatomy of the section, but use the 10x and **20x** to scan for neuronal projections. **Fill out Table 1.**

3. Now, you can begin to explore neuronal activation patterns in your tissue. Our first goal is to determine if we see *c-fos* staining in the locus coeruleus of CNO treated animals. In CNO treated animals, mCherry-hM3Dq should activate locus coeruleus neurons and stimulate expression of cfos. Fill out the first row of table 2 to help you determine if our newly generated animal strain is working as it should.

4. Our second goal is to look for patterns of activation evoked by stimulation of the locus coeruleus. In other words, when we "turn on" locus coeruleus neurons with CNO, does this result in the activation of neurons in specific target regions of the brain? Here, you will have to explore the tissue. Where do you see *c-fos* staining? **Record your observations in the table 2 below and add rows if necessary**.

5. Take images of your results for your final lab report. Take as many pictures as you can to document your findings (ideally an image for each row in tables 1 and 2).

Table 1: Non-*En1*-NE neuronal Projections

Slide and Section #	Anatomical Nucleus	Neuronal Projection Expectations	Neuronal Projection Observations
	STMV and STLV (Bed Nucleus of the Stria Terminalis-BNST) **FIG 30**	Do you expect to see projections in the region from non-locus coeruleus neurons?	How dense are the NE projections in the region? How does the colorimetric staining of projections compare with the fluorescent staining in the region? Can you see more projections? Or less?
	PaDC, PaLM, PaMM, PaV, PaMP (Paraventricular Hypothalamic Nucleus) **FIG 38**		
	DI, AID, AIV (Insular Cortex) **FIG 16**		
	Cortex **FIG 5-70**	If you are unsure about the expectations here talk with your instructor	Remember this is a new tool for exploring projections for exploring the projections of a unique subset of NE neurons (non-locus coeruleus neurons), which means this is an opportunity for YOU to discover something new!!
	Hippocampus **FIG 40-63**	If you are unsure about the expectations here talk with your instructor	
	Brain region_____ **FIG_____**		Try to identify the anatomical region. Check with your instructor

	Find projections in a region not listed above.		

Table 2: cFos expression		
	CNO treated animal	**VEH control animal**
Locus coeruleus (FIG 75-79)	**Slide & section #: _____** **Observations:** What does the staining look like? Do you see *c-fos* positive neurons? Remember multiple sections likely contain LC neurons. Record notes for all of them.	**Slide & section #: _____** **Observations:**
Brain region _____ **FIG_____**		
Brain region _____ **FIG_____**		
Brain region _____ **FIG_____**		

Discussion Question

1. How do you feel about the two different methods for studying neuronal projections (fluorescence vs. colorimetric)? Did you prefer one method over the other? Why or why not?

2. Did you uncover any interesting projections in the cortex, hippocampus, or elsewhere? Why do you think projections to the region are interesting?

3. Do you see *c-fos* staining in the locus coeruleus of CNO and/or vehicle control animals? What do your results tell you about how well our new *En1^{Cre};Dbh^{Flpo};RC::hM3Dq* mouse strain is working?

4. If your *c-fos* staining did not work, describe at least one other way we could investigate neuronal activation patterns in response to DREADD activation in our mice. (*hint*: you will probably have to explore online or seek help from your instructor for this one!)