Identifying Neuronal Populations Underlying Social Buffering

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Abstract

Social interactions are vital to human health, in part, due to social buffering. Social buffering is the phenomenon by which fear and anxiety are reduced by the presence of an affiliative conspecific. While studies have demonstrated the behavioral effects of social buffering, there is less known about how these effects are mediated within the brain. There is evidence that the infralimbic cortex (IL) cortex is important in processing social information, and previous work in the Donaldson lab has demonstrated that optogenetic reactivation of cells previously active during novel social interaction (socially-labeled cells) in the IL was sufficient to decrease fear and anxiety behaviors in mice. To confirm activation of the IL in response to a conspecific's presence, this study examined c-Fos expression in the IL in response to novel or familiar social interaction. Analysis of tissue revealed significant differences in cell counts between the social conditions and the controls, but not between the novel and familiar social conditions. In addition to the IL, the lateral septum (LS) has been implicated in social cognition and anxiety. Thus, this study also examined the behavioral effects of optogenetically activating socially-labeled cells in the LS of mice. Little behavioral differences were exhibited on the real-time place preference task, elevated plus maze, open field test, and social interaction task which may suggest that the LS is not involved in social buffering. However, due to limited cell labeling, further research is warranted. Together, this study further elucidates important neuroanatomical regions involved in social processing and social buffering.

Introduction

Social interactions are integral to our lives as humans and play a large role in our well-being. Strong social support has been associated with numerous health benefits. For example, patients who reported strong social networks were less likely to be re-hospitalized after heart failure than patients with weaker social networks. (Rodrígrez et al., 2006). In addition to increased recovery from illness, positive social interactions also have a protective effect in physical health. For example, individuals with high social participation were found to have lower risk for acute myocardial infarction (Ali et al., 2006). Among older populations suffering from diabetes, individuals with more social connections had lower risk of mortality (Zhang et al., 2007).

Beyond these physical health correlations, social support is also vital to mental health. For instance, veterans who reported having high social support were significantly less likely to develop post-traumatic stress disorder (Ozbay et al., 2007). Additionally, individuals who lived in areas with greater potential for social support, such as neighborhoods with high per capita residence, were less likely to develop mental illnesses (Stockdale et al., 2007). Conversely, research shows that a lack of social connections can increase the risk for developing a stress-related disorder, such as depression (Rutledge et al., 2008).

These salutary effects have also been demonstrated in a number of animal models. Placed in a novel environment, goat kids exhibited lower stress responses in the presence of the mother goat (Liddell, 1949). In guinea pigs, it was found that the presence of the mother, or even a novel female, was sufficient to decrease stress responses in male guinea pigs exposed to a novel environment (Hennessy et al., 2000). The number and duration of freezing responses in an open field test using rats was reduced when another rat when present (Davitz & Mason, 1955).

Additionally, the presence of a conspecific has been found to increase time spent in the open arms of an elevated plus maze, increase exploration of a novel environment, as well as decrease freezing time after a foot shock in mice (Colnaghi et al., 2016).

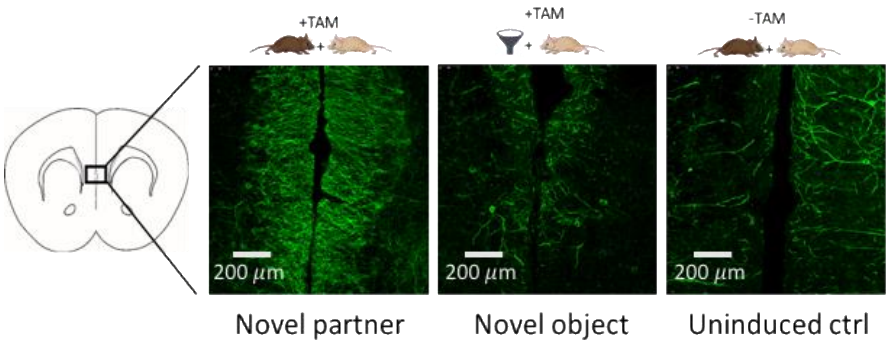
These studies provide evidence that the presence of a conspecific can reduce fear and anxiety; a phenomenon known as social buffering. While the positive effects of social buffering are well-documented, the neural mechanisms underlying the phenomenon are less understood. Fortunately, because this behavior is highly conserved, as demonstrated above, we can use rodent models to study these neural mechanisms. Therefore, the present research employs mice to expand our understanding of mechanisms that modulate the behavioral effects of social buffering.

Identifying the specific neuronal populations involved in social buffering is a preliminary step toward identifying targeted interventions to improve human health. Previous research has indicated that the medial prefrontal cortex (mPFC) demonstrates increased activity in response to social interaction (Lee et al., 2016). Several other studies have focused on the importance of the infralimbic cortex (IL) in processing social information, especially as it pertains to stress and social interaction (Farrell, Holland, Shansky, & Brenhouse, 2016; Minami, Shimizu, & Mitani, 2017; Novick et al., 2015; Ovtscharoff & Braun, 2001).

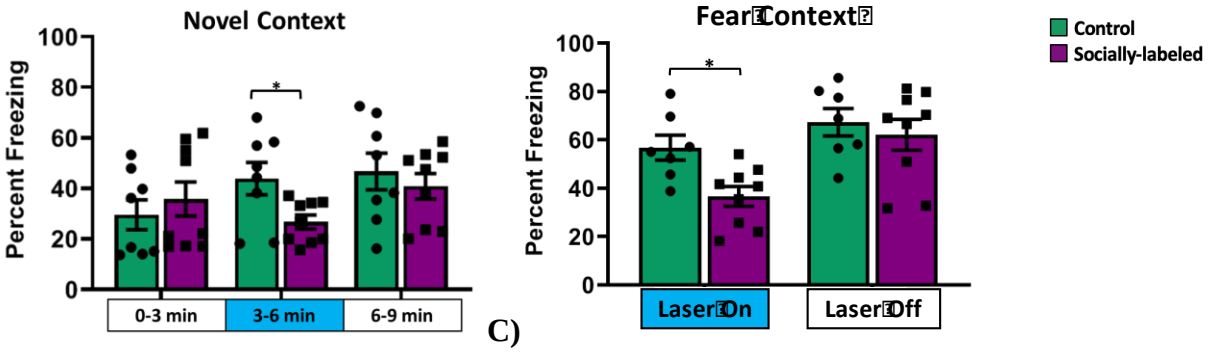
Based upon such findings, this project takes advantage of the cell activity marker, c-Fos, an early immediate gene that becomes active in response to novel stimuli, to characterize cell activity in the IL. Since c-Fos expression peaks an hour after activation, the brains of animals can be stained for c-Fos sacrificed one hour after a particular behavior and the cells active during the behavior can then be visualized (Barros et al., 2015). In this project, male mice were presented with a novel ovariectomized (OVX) female, a familiar OVX female, or were not presented with

a social stimulus. One hour following this presentation, animals were sacrificed and their cerebral tissue was stained for c-Fos. By examining the cell activation, as indicated by c-Fos staining in the IL, this study will confirm that the IL is activated in response to social stimuli.

Additionally, previous work in the Donaldson lab has shown that optogenetic re-activation of cells labeled in response to novel social interaction, or socially-labeled cells (**Figure 1A**), in the IL can reduce fear behavior in both novel (innate) and conditioned fear contexts, recapitulating the fear-reducing effect of the presence of a conspecific (**Figure 1B, 1C**).



A)



B) **C)**

Figure 1. Optogenetic reactivation of socially-labeled cells in the IL. A) Robust ChR2-EYFP expression in the IL after injection of tamoxifen (TAM) using ArcCreER^{T2} mice. Mice were injected with TAM, then exposed to a novel partner, novel object, or were injected with vehicle and exposed to a novel partner (uninduced ctrl). Optogenetic reactivation of socially-labeled neurons in mPFC decreases innate (B) and conditioned (C) fear expression**.** Control or socially-labeled neurons were re-activated in innate and conditioned fear contexts. Freezing was reduced following light application in socially-labeled mice (n = 7) but not in controls (n = 9).

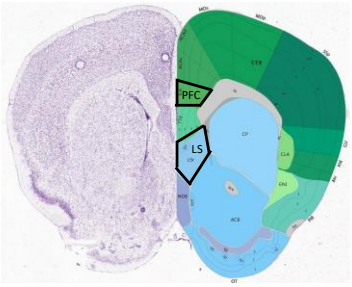


Figure 2. Diagram demonstrating location of the IL of medial prefrontal cortex (PFC) and lateral septum (LS) in coronal section of mouse brain. Adapted from Allen Brain Atlas.

Given this information, this study explores the possibility that functionally related brain regions also play a critical role in social buffering. The lateral septum (LS) (**Figure 2**) has been identified as an important region of the brain for processing social information, especially social recognition (Bredwold et. al, 2015; Bychowski, Mena, & Auger, 2013; Lukas, Toth, Veenema, Neumann, 2012). Additional studies implicate the LS in modulating fear and anxiety behaviors, suggesting that this region may be important for regulating both social information and fear and anxiety behavior (Garcia & Jaffard, 1996; Trent & Menard, 2011). So, in addition to confirming the importance of the IL in social processing, this thesis addresses the following question: Does optogenetic re-activation of socially-labeled cells in the LS also result in reduced fear and anxiety behaviors?

In order to answer this question, a transgenic mouse line, ArcCreER^{T2}, was used. This mouse line allows for the selective and permanent labeling of cells with channelrhodopsin (ChR2) during the presentation of novel stimuli (Denny et al., 2014). ChR2 is light sensitive, allowing select cells labeled with the channel to be optogenetically reactivated by blue light administered via a laser through implants in the LS during a variety of behavioral tasks. The channel is also tagged with a green fluorescent protein (GFP) that can later be visualized via immunohistochemistry. Previously, the Donaldson lab has demonstrated that there is robust labeling of cells active during social interaction in the IL, validating this model for social-specific cell labeling (**Figure 1A**).

To evaluate the behavioral effects of optogenetic activation in the LS, mice were observed while performing four different tasks. Anxiety behavior was measured using an elevated plus maze, a previously validated task to measure anxiety in rodents (Walf & Frye, 2007). The real-time place preference test -- a two-chambered apparatus in which optogenetic stimulation was given in one chamber, but not the other -- was used to assess if the stimulation itself was rewarding or aversive. Additionally, the open field task, shown to be useful for assessing fear behavior in mice by measuring wall-hugging behavior, or thigmotaxis, was utilized (Simon, Dupuis, & Costentin, 1994). Lastly, optogenetic stimulation was given to the mice in their home-cage with a cage-mate present to see if reactivation of socially-labeled cells facilitated further social interaction.

In short, this study examined two aspects of social processing. First, it sought to identify specific cells in the IL cortex of the mPFC activated in response to different types of social stimulation. Secondly, the study included the observation of the behavioral effects of optogenetically activating socially-labeled cells in the LS of the mPFC. Both types of data underscored the importance of the mPFC in processing social information and expanded upon our understanding of the effects of social buffering.

Methods

Animals

Male mice were used experimentally. Experimental animals were of genotype ArcCreER^{T2} (+) R26R-STOP-floxed ChR2-EYFP (+) given tamoxifen (4-OH-TAM) injections. Control animals were of genotype ArcCreER^{T2} (-) R26R-STOP-floxed ChR2-EYFP (+), ArcCreER^{T2} (+) wild-type given 4-OH-TAM injections, or ArcCreER^{T2} (+) R26R-STOP-floxed ChR2-EYFP (+) given vehicle injections. Mice were housed five per cage with cagemates of the same sex on a 12-hour light-dark cycle (06:00-18:00) at 22°C and ad libitum food and water were provided. Mice were in the age range of 55 to 95 days at the start of behavioral testing. All behavioral experiments took place during the light cycle. All animal procedures were approved by the University of Colorado's Institutional Animal Care and Use Committee.

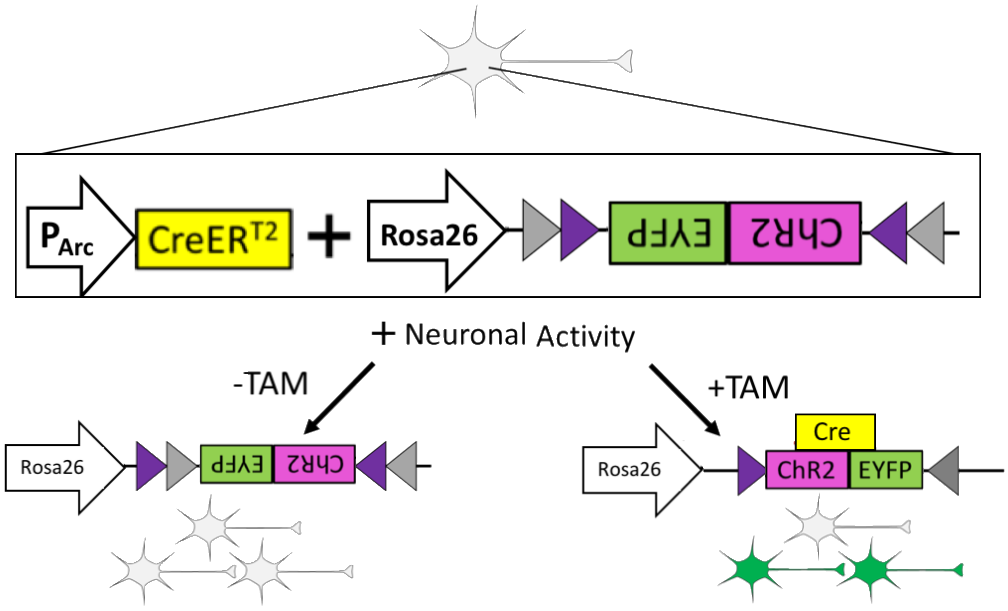


Figure 3. ArcCreER^{T2} mouse line with Arc promoter and stop-floxed channelrhodopsin (ChR2) tagged with a green fluorescent protein (EYFP). Without 4-OH-TAM in the system, Cre is bound to a heat shock protein with a modified estrogen receptor which prevents Cre from entering the nucleus. When 4-OH-TAM is administered, it binds to the estrogen receptor, changing the confirmation of the heat shock protein and allowing Cre to dissociate from the protein. The free Cre can enter the nucleus and Cre-mediated recombination results in the deletion of floxed sequences allowing channelrhodopsin, which is tagged with a fluorescent protein, to be expressed in Arc-expressing cells.

Social Introductions

(Performed by Ashley Cunningham). ArcCreER^{T2} (+) or (-)::R26R-STOP-floxed ChR2-EYFP heterozygous (+/-) and ArcCreER^{T2} (+) wild-type mice were presented with social stimuli in their home-cage. Social interactions were videotaped for the first 10 minutes. Animals were either presented with a novel OVX female or a familiar OVX female. A control group of animals received no social stimulus. The animals were allowed 1 hour to interact with the novel or familiar female, or reside in their home-cage with no social stimulus. Following this hour, the animals were sacrificed and perfused.

Optogenetic Ferrule Construction and Surgery

Ferrule construction. A 200-μm core with 0.37 numerical aperture (NA) multimode fiber was glued with epoxy and threaded through a 230-μm core ferrule. The ferrules were polished, and fibers were cut at 3.0 mm for implantation. Implants were tested for light output (greater than ~70% light recovery) and acceptable light cone production.

Surgery (performed by Ashley Cunningham). Male animals were anesthetized with isoflurane for the duration of the surgery. They were surgically implanted with fiber optic implants after P60. A midline incision was made in the scalp of each animal with sterile scissors allowing access to the skull from between eye level to the ears. Two small (~1mm) holes in the skull were drilled with a dentist's drill and a sterile 22-gauge drill bit. Two fiberoptic cannulas were stereotaxically implanted at a 10° angle through the holes in the skull using a custom arm placed on the surgical apparatus (AP +1.20 mm DV -2.54 mm ML +/-1.09). Two small screws were also inserted in small holes located on the base of the left and right hemispheres to stabilize the dental cement surrounding implants. The surgical implants were then fixed to the skull with dental cement. The skin on each mouse was closed around the implant with VetBond tissue

adhesive. A small amount of viscous lidocaine ointment was then applied to the area of the incision. Animals recovered for a minimum of 10 days before experimental behavioral testing.

TAM injections (performed by Ashley Cunningham). Animals were separated the Wednesday prior to 4-hydroxy-tamoxifen (4-OH-TAM) injections on Friday. ArcCreER^{T2} mice were injected with 0.15ug (in 0.15mL of 10% EtOH in corn oil) of 4-OH-TAM to label the Arc+ cells activated in the brain during partner introduction. As stated, two control groups were used: 1) ArcCreER^{T2}+ R26R-STOP-floxed ChR2-YFP animals that were injected with vehicle (10% EtOH in corn oil) and 2) ArcCreER^{T2}- R26R-STOP-floxed ChR2-YFP or wild-type animals that were injected with 4-OH-TAM. Five hours after injection, an ovariectomized female, was introduced to the cage, and social interaction was videotaped for the first ten minutes. Animals were then placed in a dark room with their partner for 3 days.

Optogenetic Behavioral Tasks

Elevated Plus Maze. Animals were split across two days. Animals were habituated the three days prior to experimental day. During habituation animals were scruffed and the fiberoptic cable with ferrule sleeve was attached to the ferrule implants in their heads. They were allowed to roam freely in a traditional cage bottom. On experimental day, animals were allowed to acclimate in the behavior room for one hour prior to the experiment. Ferrules were cleaned with VWR clean wipes and 2-Isopropanol. Index Matching Gel (Thor labs, G608N3) was injected in the ferrule sleeves between animal runs to prevent air bubbles between the laser and fiber optic. The laser was tested every 3 animals to make sure the output was correct. Animals were run on the elevated plus maze for 9 minutes while attached to a bifurcated fiberoptic patch cable. The optogenetic laser was on for the middle 3-minute epoch using 10-12 mW at 10Hz stimulation 5ms pulses. Clodox was used to clean the maze between animals. The Cleversys Top scan

program was used to track and record animals' movements during behavior.

Real Time Place Preference. Animals were split across two days. On experiment day, animals were allowed to acclimate in the behavior room for one hour prior to the experiment. Ferrules were cleaned with VWR clean wipes and 2-Isopropanol. Index Matching Gel (Thor labs, G608N3) was injected in the ferrule sleeves between animal runs to prevent air bubbles between the laser and fiber optic. The laser was tested every 3 animals to make sure the output was correct. Animals were placed in the Real Time Place Preference box for 20 minutes alone. They were all initially placed on the side in which the laser was off. The laser on and laser off sides were alternated to account for preference between the two sides of the cage. On the laser on side, light stimulation was given using 10-12 mW at 10Hz in 5ms pulses. The bedding was replaced between each animal and the chambers were cleaned with Clodox. The Cleversys Top scan program to was used to track and record animals during behavior.

Fear Conditioning (performed by Magda Woroniecka). Animals were split across two days. The fiber optic cable was not connected to the ferrules. On experiment day, animals were allowed to acclimate in the chemicals area next to the behavior room for one hour prior to experiment. Each mouse was individually transported into the room using Chinese Takeout containers. A laptop was connected to a webcam that was taped to the ceiling of the fear conditioning box. A head lamp set to red light was placed inside the box in order to allow some form of light so the camera could record the animal. 100µl of a lemon scent was pipetted onto a paper towel and placed under the metal grid in the fear conditioning box. This scent was replaced every 3 animals. The box was cleaned with 70% ethanol after every animal. The animals were placed into the fear conditioning box and, after a 3-minute habituation period, 2-second shocks

were given every minute for 3 minutes. After completing the 6-minute program, the animals were removed from the box, placed into the takeout container and returned to their home cage.

Open Field. A different experimenter than the person who performed fear conditioning, wearing different laboratory attire, performed open field behavior testing. Animals were split across two days. The animals were allowed to acclimate in the behavior room for one hour prior to the experiment. The room was divided by a curtain. One side of the room was lit with overhead lights and had the computer with CleverSys Top Scan program to track the animals (this was the side of the room in which the animals acclimated). A fan was also placed on the light side of the room pointing towards the door to rid the room of any residual lemon scent once the experiment had started. The open field box was placed on a table on the other side of the room. This side of the room was dark with two lamps lighting the open field box. The light intensity at the center of the open field box was measured prior to running the experiment. Index Matching Gel (Thor labs, G608N3) was injected in the ferrule sleeves between animal runs to prevent air bubbles between the laser and fiber optic. Ferrules were cleaned with VWR clean wipes and 2-Isopropanol. The laser light output was tested every three animals to make sure the output was correct. Additionally, an extension cord that could spin was attached to the laser so that the fiber optic cable would not get tangled and impede the animals' movements during behavior.

For the open field task, the ferrule sleeves were first attached to the animals' ferrules, then the animals were placed in the open field box for 6 minutes. The sides of the open field box were covered with poster paper. In the middle of the box was a petri dish containing a clean cotton swab. The laser was set up to turn on after two minutes had passed using 10-12 mW at 10Hz stimulation in 5ms pulses. The laser was on for two minutes, then turned off for the

remainder of the 6-minute period. Then, the cotton swab was removed, dipped in a liquid lemon scent, and returned to the petri dish. The animals were then given another 6 minutes in the box with the scent. Again, the laser turned on after two minutes had passed, stay on for two minutes, and then turn off for the remainder of the 6-minute period. After each animal, the open field box was cleaned with Clodox. The CleverSys program was used to track and record the animals during behavior.

Home-cage interaction. The behavior was run in the colony room where the animals were housed so no acclimation time was required. The animals performed behavior in their home cages which were placed on the floor of the room. Two animals were run at a time in separate cages. A manila folder was placed between the cages to separate them. The ovariectomized females that were also housed in the cages with the experimental animals were left in the cages for the duration of the experiment as they were already familiar with the experimental animals and therefore presented no new stimuli. A camera was set up on a tripod above the cages and was used to record the behavior videos. A cart was set up near the cages. The laser was set up on the cart and was attached to a fiberoptic attached to a commutator which was attached to the bifurcated fiberoptic patch cable. Another fiberoptic cable (that was not attached to laser and served as a control) was taped to the cart as well. Index Matching Gel (Thor labs, G608N3) was injected in the ferrule sleeves between animal runs to prevent air bubbles between the laser and fiber optic. Ferrules were cleaned with VWR clean wipes and 2-Isopropanol. The laser light output was tested every three animals to make sure the output was correct.

As stated, for the home-cage interaction, two animals were run at a time. The animals were paired based on genotypic group (control, vehicle, or experimental). In each pair, one was assigned as a control (attached to the ferrule sleeve that was not attached to the laser) and the

other was designated to be the experimental animal (attached to the split ferrule sleeves that were attached to laser). First, the control animal was attached to its ferrule sleeve, not attached to the laser, and placed back in its home cage. Then the experimental animal was attached to its ferrule sleeves, attached to the laser, and placed in its respective cage. The laser was then turned on which was set to use 10-12 mW at 10Hz stimulation 5ms pulses. After the laser had been turned on, both animals were allowed 10 minutes in their home cage. Animals were recorded during this period. After 10 minutes, the laser was turned off, the animals were removed from their cages, freed from the ferrule sleeves, and returned to their home cages. One hour after the behavior was completed, the animals were sacrificed and perfused. Each pair of animals was run 20 minutes apart. The side of the room in which the experimental animals' cages were placed was counterbalanced.

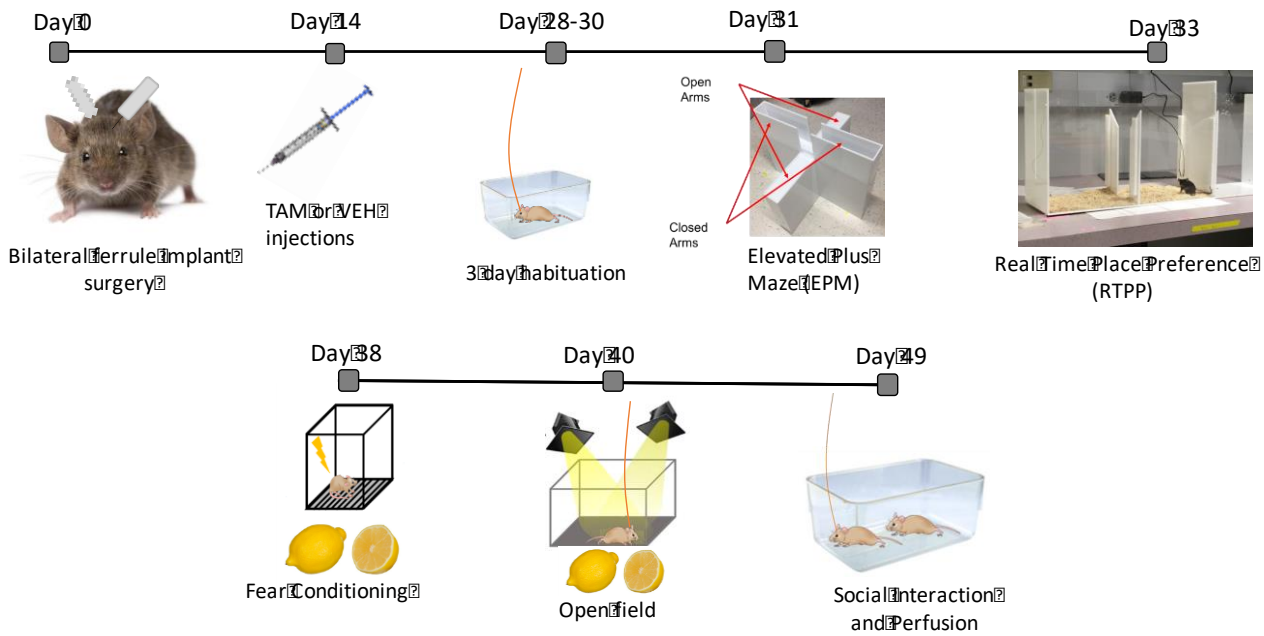


Figure 4. Timeline of optogenetic project.

Perfusions, Immunohistochemistry, and Imaging

Perfusions (performed by Ashley Cunningham). Animals were anesthetized with a lethal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Perfusions proceeded when mice no

longer responded to tail and toe pinches. Animals were perfused with 0.1M phosphate buffer saline (1X PBS) followed by 4% paraformaldehyde in 1X PBS. Perfused brains were removed and placed in 1ml of 4% paraformaldehyde for 24 hours in 4°C. After this 24-hour period, the brains were transferred to 5ml of 30% sucrose and allowed to sink in 4°C prior to sectioning.

Tissue sectioning and immunohistochemistry. Perfused brains were mounted onto a microtome (Leica JungSM2000R) using OCT (Fisher Healthcare) and frozen with dry ice. Coronal sections of 30μm sections were cut from each brain and stored in 24-well plates containing 1ml of 1X PBS and 0.05% sodium azide in 4°C. For immunohistochemistry, tissue was first washed in 4 ml of 1X PBS 3 times for 10 minutes each at room temperature. The tissue was blocked in 0.3% PBST/10% NDS for 2 hours at room temperature then incubated in the primary antibody (1:1000 anti c-Fos Rabbit polyclonal IgG (SySy) in 0.3% PBST/3% NDS) for 3.5 days at 4°C. Then tissue was again washed in 4 ml of 1X PBS 3 times for 10 minutes each at room temperature. Subsequently, the tissue was blocked in 0.3% PBST/2% NDS for 1 hour at room temperature. Then it was incubated in the secondary antibody (1:500 Cy3 conjugated Donkey anti-rabbit IgG (Jackson) and 1:500 Cy2 conjugated Donkey anti-chicken IgG (Jackson ImmunoResearch) in 0.25% Tween) for 2 hours at room temperature. Tissue underwent a final wash in 1X PBS 3 times for 10 minutes each at room temperature. Tissue sections were mounted immediately following the last wash.

Imaging and cell counting. Sections were mounted onto Superfrost Plus glass slides (Thermo Fisher) and allowed to dry. They were then treated with 200ul of ProLong Diamond Antifade Mountant (Invitrogen), covered with cover slips (Globe Scientific), and stored at room temperature for 24 hours. After this 24-hour period, the slides were sealed with nail polish (Electron Microscopy Sciences). The mounted tissue sections for animals implanted with ferrules

were imaged in 3-micron Z-stacks using a Yokogawa CV1000 Confocal Scanner System (University of Colorado Light Microscopy Core Facility). Single plane images of tissue used for cell counting were taken on the Keyence or VS120 Olympus microscopes. All images were stitched using the computer program ImageJ. For each animal, sections in the mPFC that contained the infralimbic cortex were chosen for cell counting. The Allen Brain Atlas was used as a reference. Cells labeled by cFos were manually counted in the infralimbic cortex for each animal using ImageJ. Cell counts were averaged across the left and right hemispheres.

Statistical Analysis

All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS). Differences between groups were determined using an independent samples t-test and differences within groups was determined using a paired samples t-test. Variance among more than two groups were calculated using a repeated measures analysis of variance (ANOVA). Statistical significance was defined using ** $p < 0.01$, * $p < .05$. Data outside of a calculated range were excluded from data analysis as outliers. This range was calculated by first multiplying the interquartile range (IQR) by 1.5. The upper bound of the range was calculated by adding the IQRx1.5 to the third quartile. The lower bound of the range was calculated by subtracting the IQRx1.5 from the first quartile. Data that were not normally distributed after outliers were removed were transformed until normal.

Results

Social Interaction Cell Activation

Cell counting of c-Fos positive cells in the IL. c-Fos positive cells were manually counted in the IL of animals. The mean cell count represents the average of the cell counts of the left and right hemispheres. There were significant differences in c-Fos positive cells in the IL between controls and the novel and familiar social contexts (**Figure 5B**). There were no differences in cell count found between the novel and familiar social conditions (**Figure 5B**).

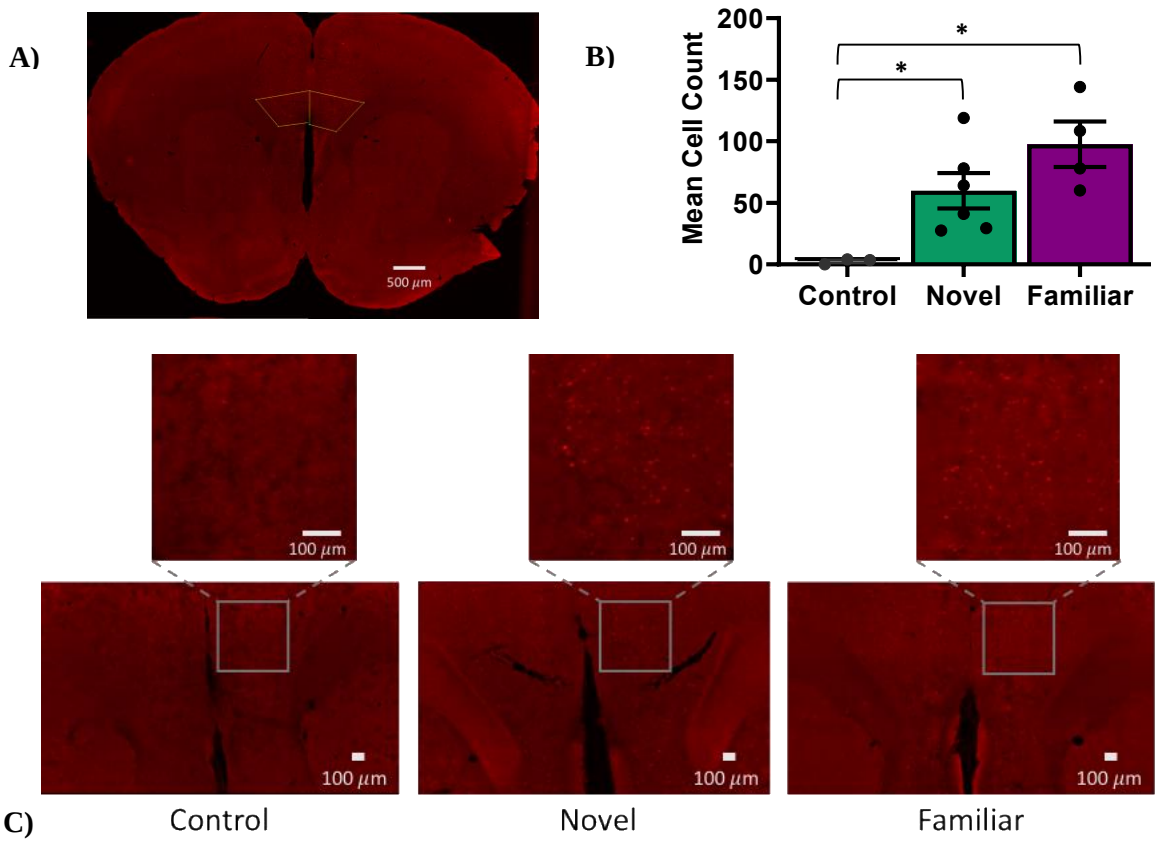


Figure 5. **Mean cell counts in the infralimbic cortex of mice presented with no social stimulus, a novel female, or a familiar female**. A) Representative image of selected cell count area containing the IL. B) There were significant differences between control and novel female (p = .029) and control and familiar female groups (p = .007). There were no significant differences between the novel and familiar social conditions (control n = 3, novel female n = 6, familiar female n = 4). C) Representative images of c-Fos cell staining in the IL of animals that received no social stimulus (control) or interacted with a novel or familiar OVX female.

Optogenetic Cell Labeling Induction

Cell labeling in the lateral septum. Confocal images of the LS region of the animals were taken. Animals were exposed to a novel social partner either after a 4-OH-TAM injection, to induce cell labeling, or vehicle injection. As expected, there were no cells labeled when animals were not given 4-OH-TAM (**Figure 6**). There was limited cell-labeling in response to TAM-mediated induction of socially-labeled cells as compared to uninduced controls (**Figure 6**).

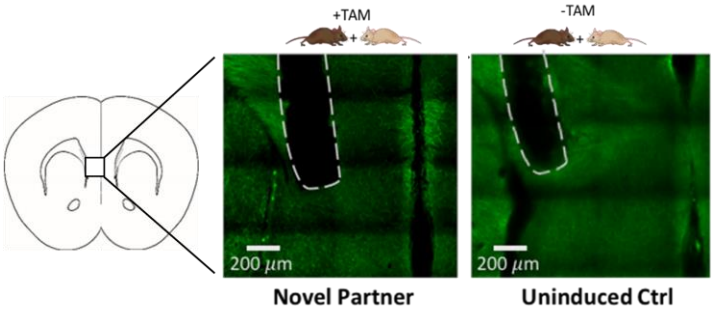


Figure 6. Limited cell-labeling in the lateral septum in TAM-induced CreER^{T2} mice in response to novel social interaction. Dotted lines indicate implant site.

Optogenetic Behavioral Tasks

Elevated plus maze behavior. Anxiety-like behavior was measured by assessing the duration the mice spent on the open arms, which are more anxiety-producing, as well as the duration of time mice spent dipping their heads over the side of the open arms. Time spent on the open arms was calculated as the time spent on the open arms divided by the total duration of the task. Open arm head dip duration was calculated similarly. There were no significant differences in time spent on the open arms of the maze over time or during the middle 2-minute epoch in which the optogenetic stimulation was given between the control and socially-labeled groups (**Figure 7A**). Additionally, there were no significant differences in head dip behavior over time or during the middle 2-minute epoch on the open arms between groups (**Figure 7B**). Animals were split into two groups and completed the behavior on consecutive days, however, there were no significant differences in behavior across days.

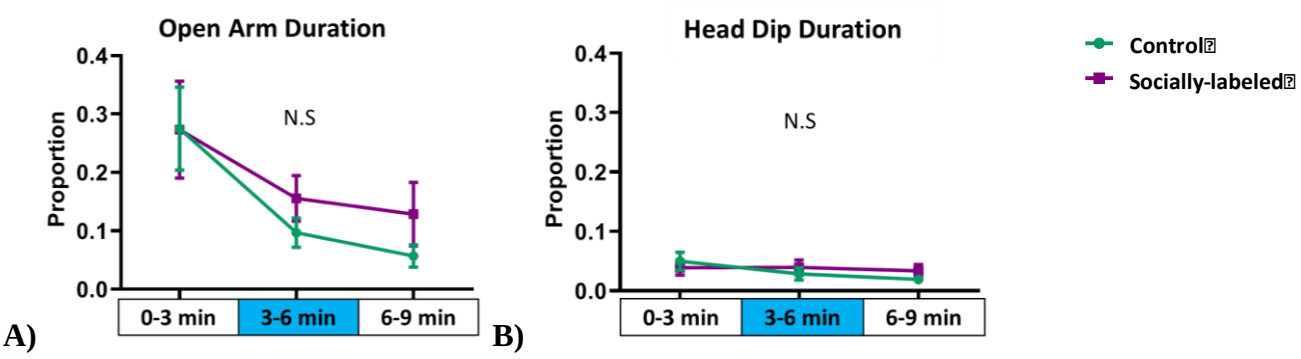


Figure 7. No differences in anxiety-like behavior observed on elevated plus maze in response to optogenetic stimulation. Optogenetic reactivation of socially-labeled cells in the LS did not affect (A) time spent in the open arms (control n = 10, socially-labeled n = 8) or (B) time spent head-dipping (control n = 11, socially-labeled n = 9). The second 3-minute epoch (blue) represents the time-point at which optogenetic stimulation was given.

Real time place preference task. The potentially rewarding or aversive effects of optogenetic stimulation were assessed by the percent duration the mice spent in the side of the apparatus in which optogenetic stimulation was given (laser on). The percent duration was calculated as the duration the animals spent in the laser on side divided by the total duration of the task. Locomotor ability during optogenetic stimulation was also assessed by the distance traveled by the animals in the laser on compartment. The percent distance traveled was calculated as distance traveled by the animal in laser on compartment divided by the distance traveled in both compartments. There were no significant differences in time spent in the compartment in which optogenetic stimulation was given (laser on) between the control and socially-labeled groups either over time (**Figure 8A)** or in total (**Figure 8B)**. There were also no significant differences in distance traveled in the laser on compartment between groups; again, either over time (**Figure 8C**) or in total (**Figure 8D**) There were no significant effects of the side of the box in which the laser was turned on, which was counterbalanced during the experiment.

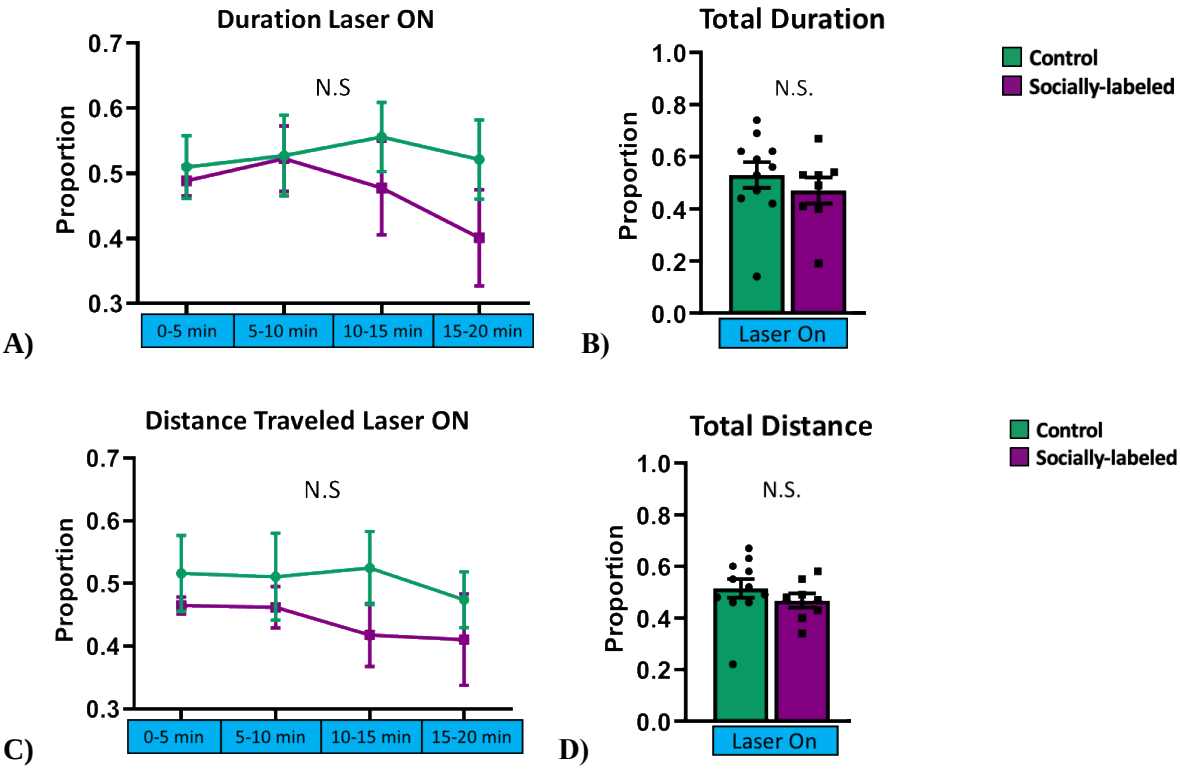


Figure 8. Optogenetic stimulation did not affect the duration spent or distance traveled in the laser on compartment. There were no significant differences in duration spent in the laser on compartment over time (A) or in total (B) or in distance traveled in the laser on compartment over time (C) or in total (D). (control n = 11, socially-labeled n = 8).

Open field fear behavior. Fear behavior on the open field task was measured by time spent in the center of the box as well as the distance traveled within the center. The percent duration was calculated as the time spent in the center of the box divided by the total time allowed for the task. Behavior was evaluated both before and after the fear conditioned scent was administered. The duration animals spent in the center of the open field box changed significantly over time within groups before ($p = .028$) and after ($p = .037$) scent administration. However, there were no significant differences in duration spent in the center of the open field over time in controls as compared to the experimental animals either before or after scent administration (**Figure 9**). The middle 2-minute epoch, during which optogenetic stimulation

was given, was also assessed independently, both before and after scent administration. The control group spent significantly less time in the center of the box after scent administration than before (p = .037) while the experimental group did not show significant behavioral differences before and after scent administration (**Figure 10)**. There were no significant differences between groups.

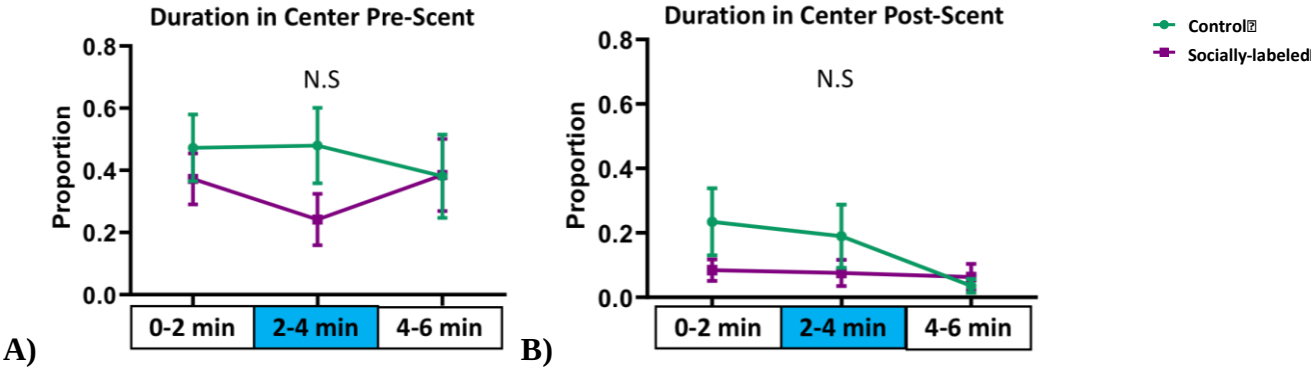


Figure 9. **Percent duration animals spent in the center of the open field.** There were no significant differences in the time animals spent in the center of box between the control and socially-labeled groups either before (A) or after (B) the fear-conditioned scent was administered (control n = 10, socially-labeled n = 7).

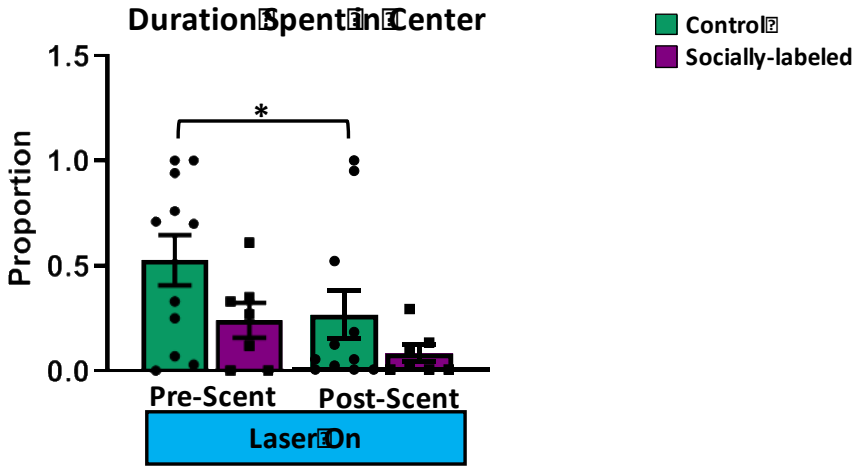


Figure 10. **Percent duration spent in center of open field box during middle 2-minute epoch in which optogenetic stimulation was given**. There was no significant difference between groups. The control group spent significantly less time in the center of the box after scent administration (p = .037). The socially-labeled group showed no within group behavioral differences (control n = 11, socially-labeled n = 7).

The distance animals traveled in the center of the open field box changed significantly over time within groups before (p = .002) and after (p = .007) scent administration. However, there were no significant differences in the distance traveled in the center of the open field box before or after scent administration between the controls and experimental animals either over time or during the middle 2-minute epoch during which optogenetic stimulation was given (**Figures 11, 12**). Furthermore, animals were split into two groups and completed the behavior on consecutive days, however, there were no significant differences in behaviors across days for duration spent or distanced traveled in the center of the open field box.

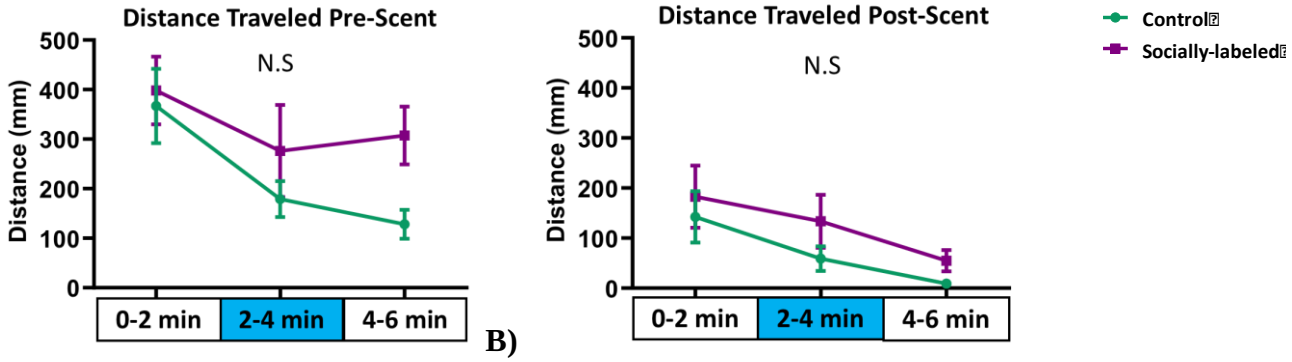


Figure 11. **Distance traveled in the center of the open field.** There were no significant differences in the distanced traveled in the center of box between the control and socially-labeled groups either before (A) or after (B) the fear-conditioned scent was administered (control n = 8, socially-labeled n = 8).

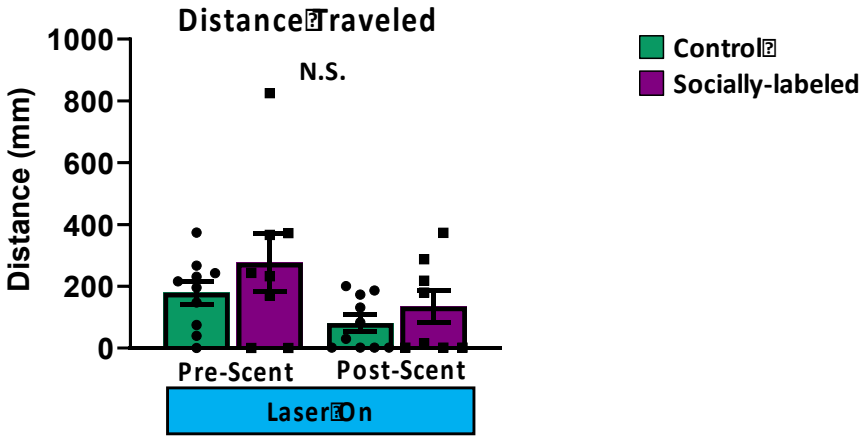


Figure 12. **Distance traveled in the center of the open field during middle 2-minute epoch during which optogenetic stimulation was given**. There were no differences between or within groups before or after scent administration (control n = 8, socially-labeled n = 8).

Home cage interactions. Behavior was recorded and later scored manually by a blinded researcher. During scoring, the researcher recorded the number and duration of social interactions. Social interaction was defined as the test animal's nose being within 1 cm of the social partner. There were no significant differences in the duration of social interaction initiated by the test mouse during optogenetic stimulation (**Figure 13**).

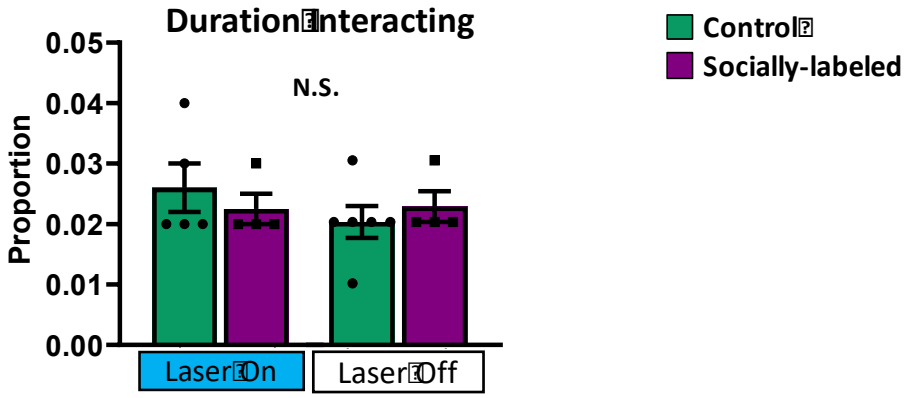


Figure 13. Optogenetic stimulation did not result in increased social interaction with a cage-mate in home-cage conditions. The duration of interaction did not differ significantly in the laser on or laser off conditions either within or between groups (control laser on n = 5, control laser off n = 6, socially-labeled laser on n = 4, socially-labeled laser off n = 4).

Discussion

Since there is evidence that the mPFC is important for social processing, with studies suggesting that the IL is of particular importance, the first part of this thesis examined cell activation in the IL cortex of mice exposed to different social conditions: exposure to a novel or familiar OVX female. There were significant differences in cell counts found between the control group and social conditions, but no differences found between the novel and familiar social interactions. This suggests that the IL cortex may be important for attending to social information, but may not differentiate between novel and familiar social stimuli. Nonetheless, these findings confirm the importance of the IL cortex in processing social information.

The second part of this thesis addressed the question: Does optogenetic re-activation of socially-labeled cells in the LS also result in reduced fear and anxiety behaviors? In general, there were no significant effects of optogenetic activation on behavior during the various behavioral tasks. In the open field task, control animals spent significantly less time in the center of the box after scent administration. However, there were no behavioral differences between control and experimental groups making it difficult to conclude an effect of optogenetic stimulation on fear behavior. As previous research in the Donaldson lab found behavioral effects of activating cells in the IL, this may suggest that the IL -- but not the LS -- is involved in social buffering. However, there are other explanations for the apparent lack of reduction in fear and anxiety. To begin with, there was limited labeling of cells in the LS in response to novel social interaction. Due to this limited labeling, the activation of these cells may have been insufficient to produce behavioral changes. Another possibility is that ArcCreER^{T2} mouse line is effective for labeling cells in the IL, but not the LS, using the labeling conditions employed here.

Given the information this research study provides, there are many future research avenues. Mice that underwent social introductions for c-Fos cell analysis were also of genotype ArcCreER^{T2}, which allowed for cells active during the presentation of a novel stimuli to be labeled with channel rhodopsin which is tagged by GFP. These animals were exposed to two separate sets of conditions. As well as undergoing an exposure to a novel, a familiar, or no social stimulus, which allowed for c-Fos analysis, these animals also underwent an initial social exposure during which cells were labeled with GFP. These initial introductions were either to a novel object or a novel female. Thus the tissue assessed was stained for both GFP, representing cells active during the initial introduction, and c-Fos, cells active during the secondary introduction. Although the c-Fos cell counts did not differ between mice exposed to the novel versus familiar social conditions, a co-localization analysis could tell us whether or not the cell populations that processed simply novel stimuli differed from the cell populations that processed socially novel stimuli. Such follow-up might reveal, for example, that significant overlap exists between cell populations active in the novel and familiar social conditions and/or that the cell populations active in response to general novel stimuli (i.e. exposure to a novel object) are distinct from those active in response to social stimuli.

There are also many research opportunities for further optogenetic studies. The pilot study of optogenetic activation in the IL was conducted using a unilateral implant; therefore, this study hoped to expand on those findings using bilateral implants in the same region After visualizing the cortical tissue, it was discovered that the implant sites were more caudal than intended with many of the implants hitting the LS. Thus, it would be interesting to further explore the possibility that the IL is a region involved in the behavioral effects of social

buffering. Another avenue of research would be to investigate the downstream targets of the IL. This would further our understanding of the neuronal circuits involved in social buffering.

Interestingly, this study assessed c-Fos cell activation and behavior in male cohorts only. Female cohorts underwent the same initial novel or familiar OVX female social introductions. Moreover, a female cohort also completed behavioral tasks while receiving optogenetic stimulation. Thus, the same assessments for c-Fos activation and behavioral effects of optogenetic re-activation in the female cohort might reveal differences not evident in the male cohort. Future work could also address female-female interactions as this study was limited to male-female social interactions. This could tell us whether different cell populations are responsible for processing social information between the male and female sexes. It could also reveal whether combining different sexes makes a difference in the behavioral effects resulting from optogenetic activation.

Since the behavioral effects of social buffering are well documented, but the underlying neural mechanisms are not, this project aimed to elucidate neuronal populations underlying social buffering. Results confirmed that the IL is a region involved in processing social stimuli and suggested the potential value of future efforts to address co-localization of cells active in response to different social conditions. The brain regions that might be involved in mediating the behavioral effects of optogenetic stimulation were also studied. Despite potential shortcomings in the techniques utilized, the elucidated role of the mPFC in processing social information and the posited behavioral effects of social buffering provide the groundwork for future studies.

Ultimately, this information is relevant to improving human health. Identifying cell populations underlying social buffering can extend our understanding at the neurological level of how social isolation poses a risk for stress-related disorders. This may help us understand how to

better treat and/or prevent these disorders. For instance, it might enable us to enhance the protection afforded by social support through targeted treatments that ultimately reduce the incidence of mental health issues.

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