Quantifying Differential Neuronal Activity After Prairie Vole Partner Loss

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Abstract

The loss of social attachments can pose a grave risk to both mental and physical health. The recovery from bereavement is an active process and requires substantial rearranging of one's life in order to obtain a new normal. Yet the molecular mechanisms underlying this adaptation process are largely still unknown. The prairie vole (*Microtus ochrogaster*) is a lab-amenable species that forms socially monogamous relationships with opposite-sex conspecifics that are akin to human romantic relationships called pair bonds, and exhibit distress upon partner separation in a time dependent manner. Together, these translational characteristics make prairie voles an ideal study species for examining the neuromolecular basis of adapting to partner loss. In this thesis, I explored how the brain adapts to loss on the neuronal level by quantifying levels of an activity dependent neuronal marker, PS6, after short-term (48hrs) and long-term (4wks) partner separation and subsequent re-introduction. I hypothesized that there will be higher levels of PS6 induction within the nucleus accumbens (NAc) of the short-term separated group as their pair bond should still remain largely intact, and therefore being with their partner is more rewarding, versus the long-term separated group that should demonstrate less PS6 induction as their pair bond should have dissipated and the reward association with their partner has weakened. Contrary to my hypothesis, we report significantly higher PS6 induction in the NAc in the long-term cohort than the short-term cohort. As a control, we don't see significant differences in the piriform olfactory areas. Thus, we conclude that after long term separation, and therefore long-term social deprivation, male prairie voles experienced more reward when seeing their partner, a familiar social stimulus, than after re-introduction at the short-term time point. This conclusion is supported by the fact that the prairie voles seem to have sustained partner preference and therefore differences in NAc activity could be attributed to social deprivation.

Key words: prairie vole, nucleus accumbens, grief, pair bond, separation

Introduction

Forming and maintaining social bonds are a central aspect of human social behavior. Social relationships have positive effects on a person's mental and physical well-being and can buffer against stress, depression and anxiety (Lieberwirth, Wang, 2016). Additionally, intimacy between individuals have been positively correlated with improved immune function and cardiovascular health (Young et al., 2011). Unfortunately, some of the most important social bonds made in our lifetimes do not last. The loss of social attachments can pose a grave risk to both mental and physical health. Disrupted social bonds can lead to medical morbidity, depression, anxiety, substance abuse, and complicated grief, a condition where an individual never fully recovers from the symptoms of grief (Sun et al., 2014).

Adapting To Loss Over Time

The loss of an important person in our lives can completely alter our day to day normal. Greif can be considered one of the hardest and most damaging life events to occur. The recovery from bereavement is an active process and requires substantial rearranging of one's life in order to obtain a new normal. It is evident for the majority of people experiencing grief that over time the pain of loss subsides as indicated by a lessening of intensity of symptoms brought on by depression and anxiety (Stroebe et al., 2007). Yet for some others, phycological bereavement, also referred to as complicated grief, occurs. Complicated grief is characterized by a stalling the grieving process and elongating symptoms such as anxiety and depression for months and even years after experiencing loss (Shear et al., 2005). The molecular mechanisms allowing such

adaptation, or conversely the stalling of such adaptation, to a new life after loss is still unknown. Knowing such molecular mechanisms will grant insight into how the brain adapts to loss and point to potential therapeutics to help individuals struggling with the grieving process.

The Prairie Vole As A Model Organism

Ameliorating the negative aspects of grief through therapeutic treatments is difficult because little is known about how the brain adapts to partner separation. Our lack of understanding is partially due to the fact that popular animal model organisms in lab settings, such as rats and mice, do not form socially monogamous relationships making the study of pair bonding, and therefore partner loss, difficult. Of the 3-5% of mammals that exhibit social monogamy (Carter, Getz, 1993), the prairie vole (*Microtus ochrogaster*) is a lab-amenable species that forms socially monogamous relationships with opposite-sex conspecifics that are akin to human romantic relationships called pair bonds. Upon forming a pair bond, prairie voles will exhibit a partner preference, selective aggression to stranger conspecifics, and perform bi-parental care of offspring, all behaviors that also characterize human monogamous relationships (Lieberwirth, Wang, 2016). Additionally, prairie voles show duration of separation dependent distress upon partner separation (Sun et al., 2014). It is possible that forming a pair bond, and losing a pair bond, may have the same underlying biological mechanisms reflected in neuronal activity and neuromolecular signatures.

Partner Separation

Partner separation in prairie voles can be used as a way to study the loss of social attachment in humans. Upon partner separation, both humans and prairie voles exhibit

disruptions to the oxytocin, vasopressin and corticotrophin-releasing hormone systems that are dependent on depression and anxiety due to the separation (Sun et al., 2014, Purba et al., 1996, Raadsheer et al., 1994). Using fMRI to measure brain activity, humans experiencing complicated grief have higher activity within the nucleus accumbens (NAc) when asked to imagine their previous partner than those that have successfully adapted to the loss of their partner (Gündel et al., 2003). In the male prairie vole, the loss of a female partner increased passive-stress coping mediated by corticotropin-releasing factor receptor 2 specifically in the NAc shell (Bosch et al., 2016), indicating an important role the NAc has in loss recovery in both human and voles. Additionally, most people will exhibit acute distress with depression, and anxiety symptoms within the first few months of loss that taper off over time (Stroebe et al., 2007). In prairie voles, following 4 weeks of separation from their partner, anxiety and depression like behaviors occurred, that were reduced following long-term separation as well as a dissolution of their pair bond. This was discovered using an elevated plus maze and forced swim test (Sun et al., 2014). Therefore, there is significant translational relevance between prairie voles and humans due to similar behavior and neurobiology in response to partner separation. Thus, I used the prairie vole as my model species for characterizing brain-wide activity levels following short- and long-term partner separation.

Activity Markers

When neurons respond to extracellular stimuli immediate early genes (IEGs) are upregulated within the brain and used as regulatory transcription factors to induce longer term responses. The IEG *c-fos* is a transcriptional activator and repressor that has peak protein abundance around 90 minutes after stimulus and is commonly used as a proxy for detection of

neuronal activity (Sheng, Greenberg, 1990). However, *c-fos* is not the only indicator of neuronal activity. Instead, I used the neuronal activity marker phosphorylated serine 6 (PS6). S6 is a structural component of a translating ribosome that becomes phosphorylated in active neurons (Knight et al., 2012). PS6 offers an advantage over *c-fos* because it can be used to query the transcriptional landscape of active neurons in future studies. The phosphorylated ribosomes and their corresponding mRNA can be selectively isolated using immunoprecipitation for further molecular classification (Knight et al., 2012). Therefore, I can use PS6 as a marker to differentiate active and non-active neurons following a defined stimulus as well as discover the molecular identity of individual cells for future studies.

I used the prairie vole to characterize how the brain responds to reintroduction of a previous partner after short-term (48 hours) and long-term (4 week) partner separation. I hypothesized that there will be higher levels of PS6 induction within the nucleus accumbens (NAc) of the short-term separated group as their pair bond should still remain largely intact, and therefore being with their partner is more rewarding, versus the long-term separated group that should demonstrate less PS6 induction within the NAc as their pair bond should have dissipated and the reward association with their partner has weakened.

If successful, these results will provide one explanation of how the brain adapts to pair bond loss on the cellular level. These results will also provide the foundation for investigating the neuronal activity and subsequent neurobiological changes that occur in brain regions connected to the NAc following partner separation to provide a further understanding of how the brain adapts to grief. The grief of losing a loved one or an important person in our lives can be devastating and destructive. These results will help us better understand the cellular mechanisms that contribute to adapting to loss and grief in order to develop therapeutics to aid in recovery.

Methods

Thesis Aims

This study was conducted in two parts. First, Julie Sadino used immunofluorescence to validate that PS6 was an appropriate neuronal activity marker by co-localization with the well-known immediate early gene and proxy for neuronal activity, *c-fos*. Second, I quantified PS6 positive neurons in the nucleus accumbens (NAc) and piriform olfactory area of male prairie voles. These brain regions are of interest because the NAc is known to play a crucial role in forming pair bonds in prairie voles (Walum, Young, 2018) and the olfactory area is important in social recognition (Wilson, 2008). I used the program ilastik for automized cell quantification to provide unbiased and consistent results and WholeBrain to segment and define specific brain regions using a defined brain map (Pollak et al., 2014).

Experimental Design

This experiment was conducted by Julie Sadino, Xander Bradeen and Gracie Sapp, under the direction of Dr. Zoe Donaldson. Opposite-sex prairie vole pairs (ST: N = 10, LT: N = 8) were placed in a cage together (2 in a cage) to cohabitate for 2 weeks. After two weeks a baseline partner preference test was done to ensure a pair bond was formed by confirming that the test animal spent the majority of its time with it partner compared to a novel individual. The pairs were then separated into clean cages for either two days (n=10) or 4 weeks

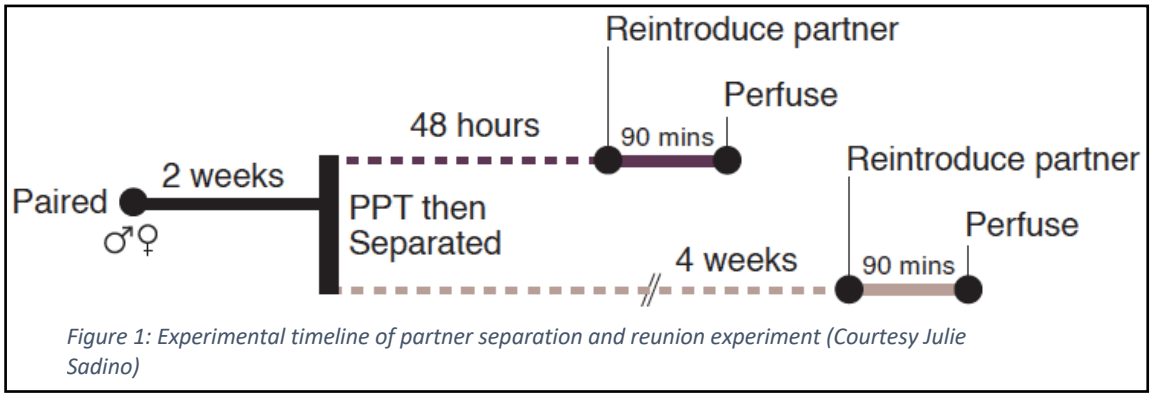


Figure 1: Experimental timeline of partner separation and reunion experiment (Courtesy Julie Sadino)

(n=8). After short-term or long-term separation, the pairs were reunited with their original partner for 90 minutes to allow for peak accumulation of PS6. Tissue was collected from male prairie voles via perfusion and anti-PS6 immunohistochemistry was conducted to identify cells active during partner reunion (Figure 1). PS6 positive cells were quantified within specific brain regions using the programs ilastik and WholeBrain**.**

Animals

Sexually naive adult prairie voles (*Microtus ochrogaster*, N = 32: 16M, 16F) were bred in-house in a colony originating from a cross between voles obtained from colonies at Emory University and University of California Davis, both of which were established from wild animals collected in Illinois. Animals were weaned at 21 days and housed in same-sex groups of 2 – 4 animals in standard static rodent cages (7.5 x 11.75 x 5 in.) with ad-lib water, rabbit chow (5326-3 by PMI Lab Diet) supplemented with alfalfa cubes, sunflower seeds, cotton nestlets, and igloos for enrichment until initiation of the experiment. All voles were aged between post-natal day 60 and ~180 at the start of the experiment. Throughout the experiment, animals were housed in smaller static rodent cages (11.0 in. x 8.0 in. x 6.5 in.) with ad-lib water, rabbit chow (5326-3 by PMI Lab Diet), and cotton nestlets. They were kept at 23–26°C with a 10:14 dark: light cycle to facilitate breeding. All procedures were performed in accordance with standard ethical guidelines (National Institutes of Health Guide for the Care and Use of Laboratory Animals) and approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

Tubal Ligations

All females were tubally ligated to avoid any confounds of pregnancy, but to keep the ovaries intact as to not impact hormonal function (Souza et al., 2019). Females were anaesthetized using 2% Isoflurane with O_2 gas and depth of anesthetize was monitored by toe pinch throughout surgery. Prior to surgery, animals were weighed and given subcutaneous injections of the analgesic Meloxicam SR (4.0 mg/kg, Zoopharm) and saline (1 mL). The lumbar region was then shaved of fur and disinfected with iodine prior to making a horizontal cut through the skin. An internal cut was made near each ovary and each fallopian tube was cauterizered until separation. The internal cuts were sutured using vicryl-coated sutures (size 4-0, Fischer Scientific) and the external cut was closed with surgical staples. Animals were monitored once a day, for three days and staples were removed one week post-surgery. Females were given 2 weeks to heal before pairing with a male.

Pairing & Cohabitation

Non-sibling pairs were determined by proximity in age and voles were moved from their home cages into smaller static rodent cages (11.0 in. x 8.0 in. x 6.5 in.) with *ad libitum* water and rabbit chow (5326-3 by PMI Lab Diet), a cotton nestlet, and an igloo. Females were not induced and introductions were not recorded. Partners cohabitated undisturbed (except for weekly cage changing) for 2 weeks prior to a partner preference test to establish that a pair bond formed.

Partner Preference Test (PPT)

PPT was carried out as described by TH Ahren (Ahern, Modi, Burkett, & Young, 2009) to assess selective partner affiliation prior to partner separation and were performed between the hours of 9:00 and 13:00 each testing day. Each 3 hour test was recorded by overhead cameras

(Panasonic WVCP304) that film two boxes simultaneously. The test animal is placed in the middle chamber for 10 minutes with the cage dividers still in place. The test begins when the cage dividers are removed and the test animal is allowed free range of all three chambers for the duration of the test. The movement of the test animal was tracked post-hoc using Topscan High-Throughput software (v3.0, Cleversys Inc.) and frame by frame behavioral data was analyzed using a custom Python script to calculate the average distance between the test animal and tethered animals when in the same chamber, time spent huddling with each tethered animal, and total distance traveled. This data was then used to generate a number of behavior metrics including the partner preference score (Partner Huddle/Partner + Novel Huddle).

Separation & Reunion

Immediately following PPT, each pair was separated into fresh small static rodent cages (11.0 in. x 8.0 in. x 6.5 in.) with *ad libitum* water and rabbit chow (5326-3 by PMI Lab Diet), a cotton nestlet, and an igloo. Animals were placed far enough away from each other to eliminate visual and olfactory cues of their partner. Each animal in the pair remained in their individual cages undisturbed for either 48 hours (short-term separation) or 4 weeks (long-term separation) except for weekly cage changes. After the appropriate separation duration, pairs were reunited by placing the female partner in the male's cage for 90 minutes prior to perfusions. Reunions were not recorded.

Perfusions

Intracardial perfusions were done as described by ZV Johnson (Johnson et al., 2016). Briefly, animals were anesthetized with a lethal dose of xyalizine and ketamine (0.4 mg/kg) until

unresponsive to toe and tail pinches. These procedures are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medicine Association and the AVMA Guidelines on Euthanasia. Then, a 22 gauge needle was inserted into the right ventricle, the left atrium was cut, blood was drained with ice cold 1X PBS, and the tissue was fixed with cold 4% paraformaldehyde. The brain was then removed from the skull and post-fixed in 4% PFA for 24 hours at 4C before being transferred to 30% sucrose for 3 days prior to tissue slicing.

PS6 Staining

Brains were sliced at 50 microns on a frozen microtome and stored in 0.05% sodium azide/1X PBS until tissue staining. Slices were separated into a 12-well plate with a maximum of 8 slices per well and rinsed three times in 1X PBS (phosphate-buffered saline, prepared in house). Tissue was permbalized and blocked with 0.3% Triton-X/1X PBS (Fischer BioReagents) with 10% Normal Donkey Serum (NDS) (Jackson ImmunoResearch) for 2 hours at room temperature prior to incubating in the primary PS6 antibody (Invitrogen) at 1:500 in 0.3% TWEEN/5% NDS solution for 48 hours at 4C. Slices were then prepped for DAB staining by rinsing three times in 1X PBS, incubating in a 1:500 dilution of Biotinylated Donkey anti-rabbit in 0.3% TWEEN/1X PBS (Fisher BioReagents) for 3 hours at room temperature, rinsing in 1X PBS again, and incubating in a 1:1000 dilution of Streptavidin conjugated to horseradish peroxidase (HRP) (Abcam) for one hour at room temperature. Then the slices were once again rinsed, treated with the DAB (3,3' Diaminobenzidine) systems (Fischer Scientific) for one minute, rinsed in DI water twice, and then mounted on Superfrost Plus glass microscope slides (Thermofischer) with Moweol (prepared in house) and sealed with clear nail polish (Electron Microscopy Sciences).

Image Acquisition & Data Analysis

Images were captured using an Olympus IX83 Fluorescence Microscope with cellSens software (v3.1) at the University of Colorado Boulder STEMTECH facility. To ensure the full identification of PS6 positive neurons throughout the brain, each brain slice was taken as a 15 step (45 microns total) z-stack image with a 10X objective with the best focus as the middle step. Z-stacks were then collapsed using the cellSens software (v3.1) into a single plane using EFI projection and exported as TIFFs. PS6 positive neurons were quantified using the machine learning based program ilastik (v1.3.3). ilastik cell counts were then registered to specific brain regions using WholeBrain (v1.1). WholeBrain overlays a specified section of the mouse brain atlas (openbrainmap.org) onto each brain slice to identify which brain regions the PS6+ cells are in. The PS6 counts were quantified and pooled in both left and right hemispheres across all sections where the region is present in the atlas.

Statistical Analysis

Using the coding program R (3.5.2), PS6+ neurons in the entire brain, NAc and olfactory areas across the two time points, short-term and long-term, were analyzed using an independent-samples, two-tailed t-test.

Results

Cell quantification has been a useful technique in the fields of biology and neuroscience for decades, from measuring brain activity to cell proliferation. Providing valuable insight for scientists, there is a drive to streamline this process to analyze larger amounts of data in shorter amounts of time. Manual cell counting can be time consuming and create artificial bias altering the interpretation of data. The push to automate cell counting would allow for the processing of large amounts of data with little to no inconsistencies between datasets. Though sound in theory, automated cell quantification is deceptively difficult. My Mentor, Julie Sadino, and I worked to establish a high throughput workflow from imaging to automated quantification of cells in the vole brain (Figure 2).

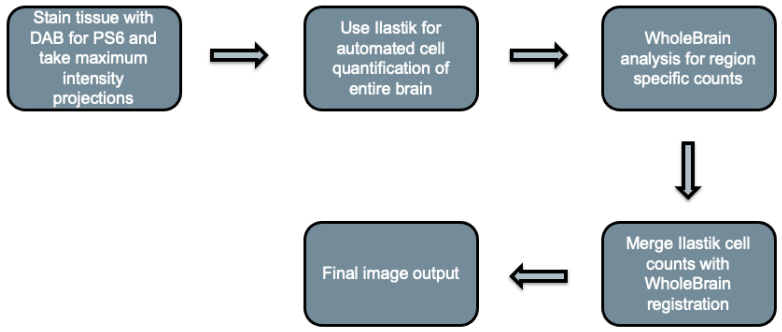


Figure 2: Pipeline for processing images

Instead of using the more popular *c-fos* as our activity dependent neuronal marker we opted for using Phosphorylated Serine 6 (PS6) as PS6 allows for future studies that are not possible using *c-fos* (detailed in Future Directions).

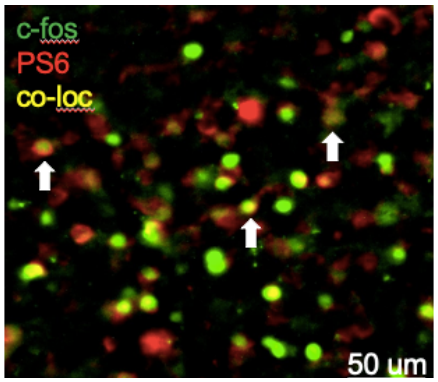


Figure 3: Co-localization of c-fos (green) and PS6 (red). Arrows point to areas of significant co-localization (yellow)

In order to ensure that PS6 would be a reliable proxy for neuronal activity, we co-localized PS6 and *c-fos* induction after haloperidol administration. A high degree of co-localization (yellow) between *c-fos* (green) and PS6 (red) provided us with the confidence that PS6 was accurately and reliably labeling active cells following a stimulus (Figure 3). As such,

the tissue from our experiment was stained for PS6 to quantify active cells in response to partner reunion at both separation time points.

Automated cell quantification requires a consistent set of thresholds and parameters in order to ensure accurate cell quantification. However, this can be difficult to achieve due to differences between imaging datasets. Popular programs such as ImageJ exacerbate the issues of finding a consistent set of analysis parameters due to the rather crude options available for identifying cells. The program ilastik approaches cell quantification in a different manner by using machine learning to classify background and foreground objects (i.e. cells) (Figure 4). To train the program to identify the differences between background and cells in multiple conditions, I used sample images from each animal where one was good quality, one was bad quality and one had heavily aggregated cells. Once there was adequate painting to differentiate the background vs cells, I then set a threshold to segment cells and label each with a different "identity" (color) (Figure 5). After completing the training, thresholding, and cell identification on the sample images those parameters were then applied to full images in a batch processing of approximately 30 images for each animal. The resulting output had information

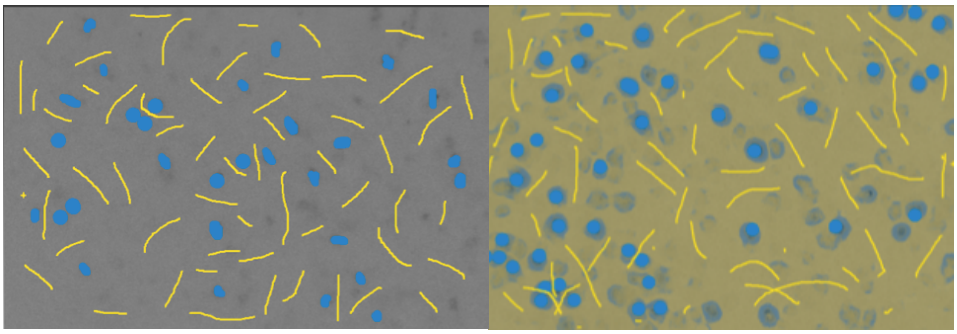


Figure 4: Machine learning to train ilastik to differentiate between background (yellow) and cells (blue)

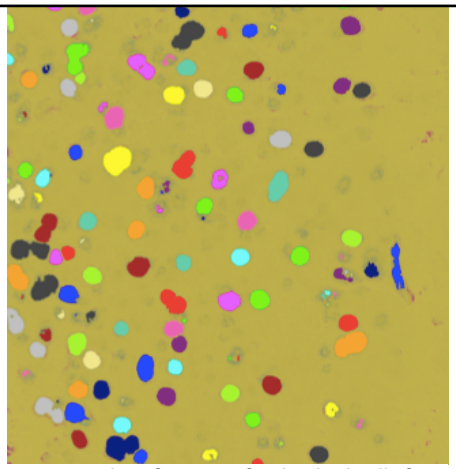


Figure 5: Identification of individual cells for quantification. Background (yellow) Cells (multicolor)

containing cell counts, cell intensity, size in pixels, and the location of each cell in the image. I first wanted to know if there were any brain-wide activity differences between time points. To answer this, we used the cell counts from the entire brain from each male animal involved in the separation and reintroduction study. Contrary to what was originally hypothesized, long-term animals demonstrated significantly higher brain-wide PS6 induction (ST: N = 10, LT: N = 8, p = 0.004126 **) (Figure 6). However, these results do not contain any information about what specific brain region each cell count originated from. In order to determine the cell count in the NAc for each animal the ilastik cell counts were registered to specific brain regions in the next step of analysis using WholeBrain.

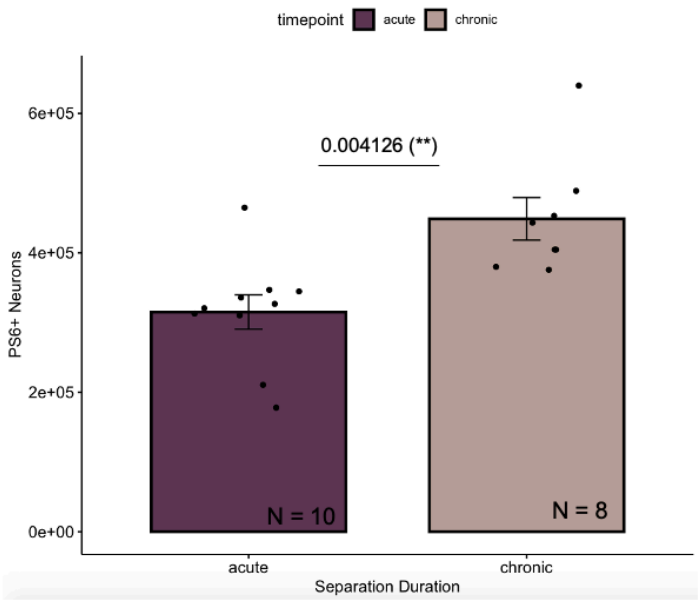


Figure 6: Average PS6+ neurons in entire brain between short-term acute (2 days) and long-term chronic (4 weeks) separated male prairie voles. Purple indicates average PS6+ neurons after acute (2 day) separation (N = 10). Tan indicates average PS6+ neuron count after chronic (4 week) separation (N = 8). Independent t-test reveals a statically significant difference between acute and chronic PS6+ neuron induction (ST: N = 10, LT: N= 8, p = 0.004126).

Next, we used WholeBrain to delineate ilastik cell counts to specific brain regions. First, we matched adjacent brain slices between animals with the appropriate Bregma coordinate position using both the Paxinos Mouse Brain Atlas (MBA) and the Allen Open Brain Map (OBM) by identifying key anatomical regions (Figure 7). In this study we registered and analyzed one slice from each animal (N = 18, n = 1) with MBA coordinate 0.74 mm and OBM coordinate 0.75 mm. This coordinate was chosen as it had adjacent slices in every animal and has easy to identify anterior commissure and subsequent NAc shell and core. Since voles are non-model organisms, they do not have the many additional resources that mice do so the program

WholeBrain allows us to conform the OBM atlas to fit the size of a vole brain (Figure 8). When I began registering the mouse brain atlas on to the vole brain using WholeBrain there were noticeable species differences. The orange map on the left of Figure 8 overlays the un-warped mouse brain atlas on to our vole target image whereas the purple map on the right shows the atlas warped to the vole brain. Though similar anatomically, the mouse and vole brain are of striking different sizes and have different placements of key anatomical features (Figure 8). In order to ensure accurate fitting of the mouse atlas onto the target vole brain we made sure to fit the corpus callosum (CC), anterior commissure (AC) and subsequently the NAc area, olfactory areas (OLF), and outline of the brain with perfect precision (Figure 8). Once the registration of the OBM onto the vole brain was complete we then merged the ilastik cell counts with the registered brain to accurately obtain cell counts in specific brain regions (Figure 9). Cells within each different brain region have their own color making visual inspection of the mapping easy.

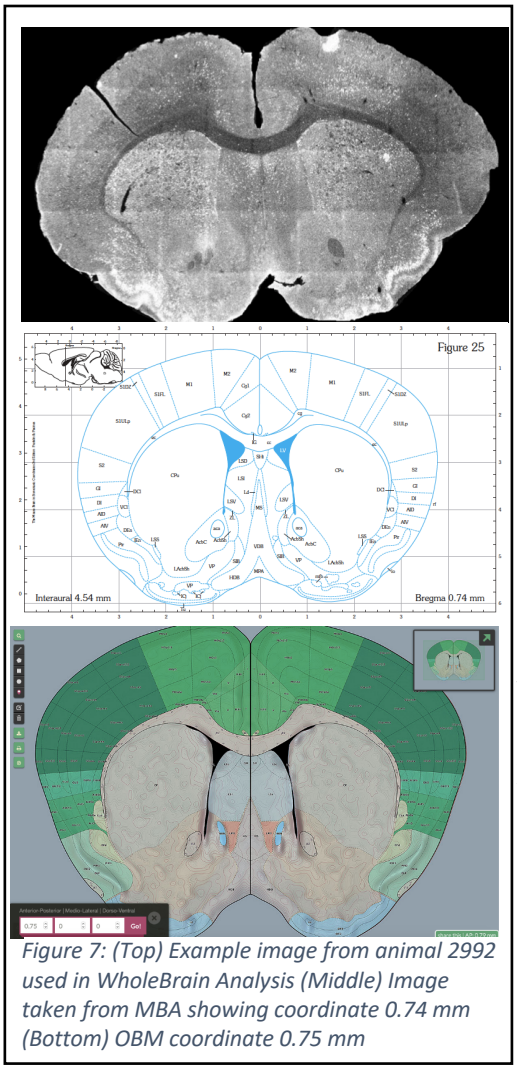


Figure 7: (Top) Example image from animal 2992 used in WholeBrain Analysis (Middle) Image taken from MBA showing coordinate 0.74 mm (Bottom) OBM coordinate 0.75 mm

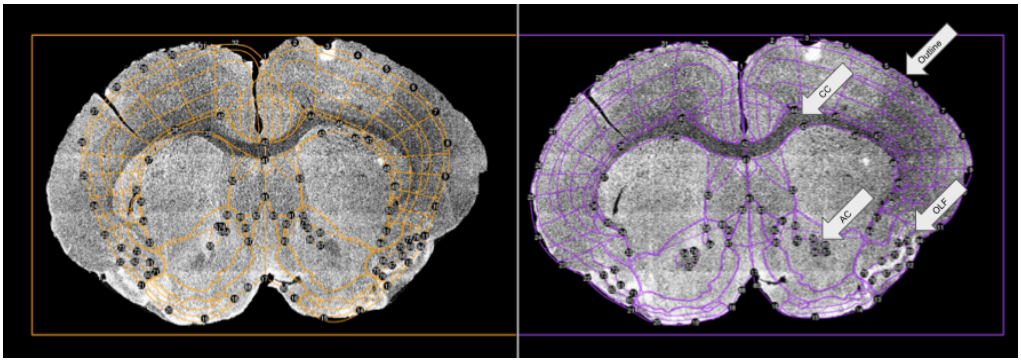
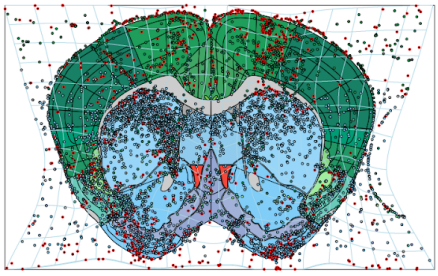


Figure 8: Open Brain Map (orange) overlayed onto vole brain target image (grey) (left). Right image shows Open Brain Map conformed to vole brain using anatomical regions for perfect fit

Together, ilastik and WholeBrain allow us to accurately obtain cell counts from regions of interest while eliminating inconsistencies or bias with guessing where the brain regions are and what amount of cell counts are there in these regions. Another output WholeBrain provides is a dot plot to further visualize Figure 9 (Figure 10). This plot provides us with lateralized cell counts (right) in specific brain regions (left).



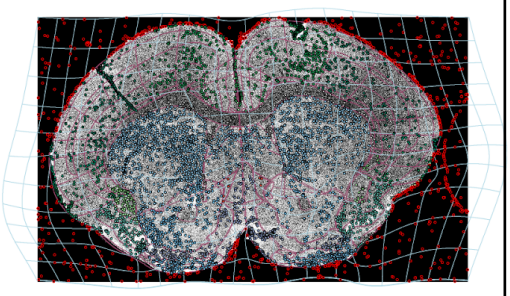


Figure 9: WholeBrain analysis with registered brain regions and ilastik cell counts combined

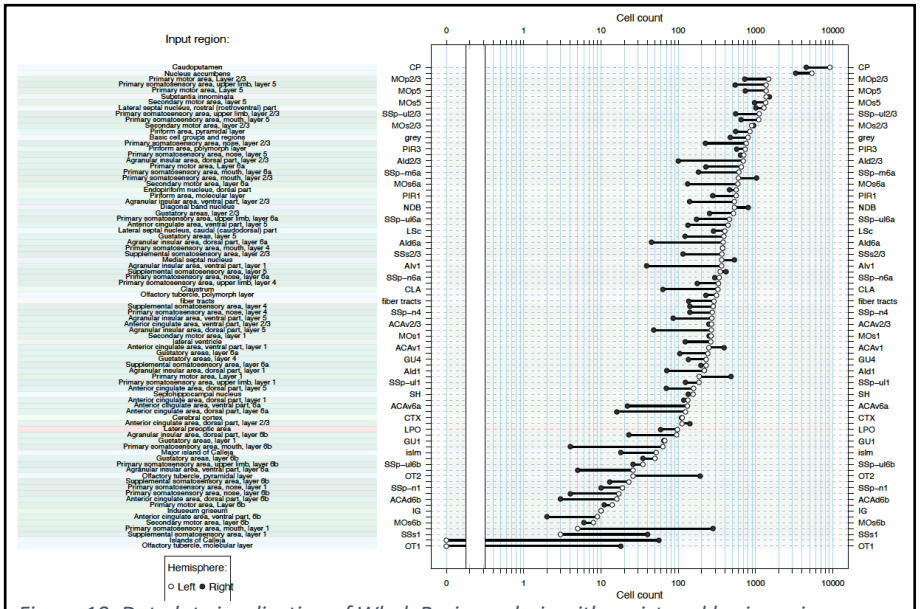


Figure 10: Dot plot visualization of WholeBrain analysis with registered brain regions and ilastik cell counts

We then examined the NAc specific cell counts between the 2 time points by adding both the left and right hemisphere NAc counts together. For NAc cell counts we hypothesized that the short-term (acute) group would demonstrate higher PS6 induction following reunion than the long-term (chronic) group due to the pair bond being maintained after only a short separation period. Much to our surprise, our results indicate that the long-term separation group demonstrated statistically higher PS6 induction than the short-term experimental group (ST: N = 9, LT: N = 8, n = 1, p = 0.01949 *) (Figure 11).

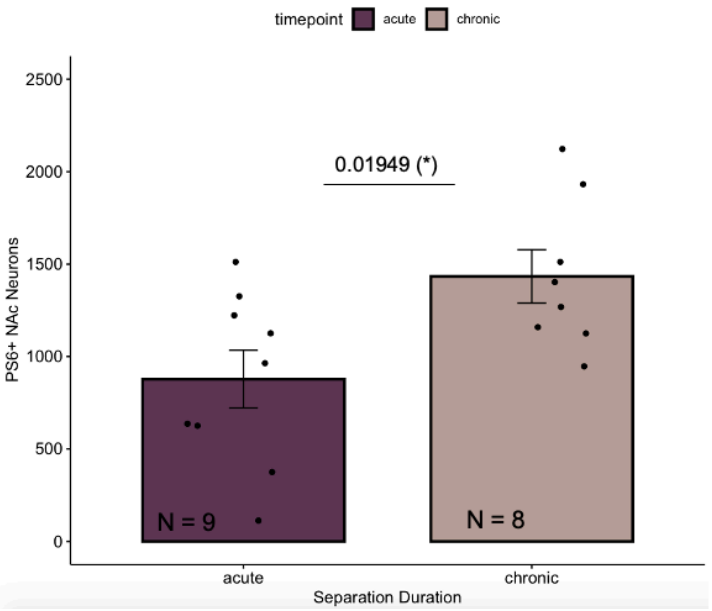


Figure 11: Average PS6+ neurons in the nucleus accumbens between acute (2 days) and chronic (4 weeks) separated male prairie voles. Purple indicates average PS6+ NAc neurons after acute (2 day) separation (N = 9, n = 1). Tan indicates average PS6+ NAc neuron count after chronic (4 week) separation (N = 8, n = 1). Independent t-test reveals a statically significant difference between acute and chronic PS6+ NAc neuron induction (ST: N = 9, LT: N = 8, n = 1, p = 0.01949).

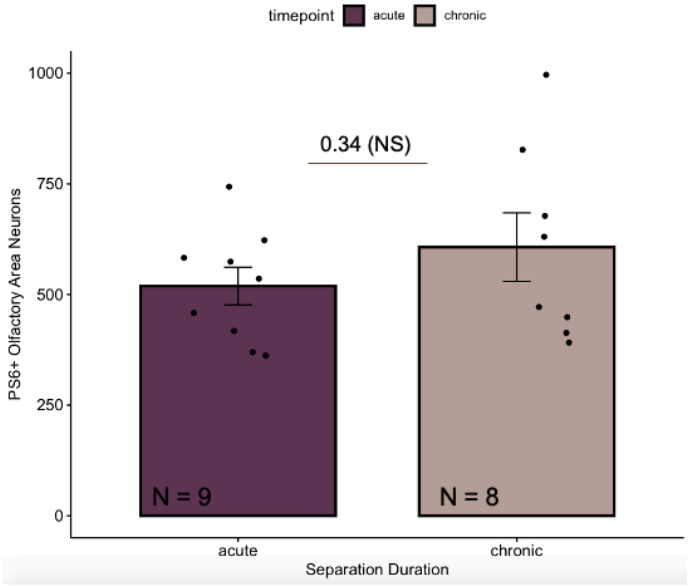


Figure 12: Average PS6+ neurons in the olfactory area between acute (2 days) and chronic (4 weeks) separated male prairie voles. Purple indicates average PS6+ Olfactory area neurons after acute (2 day) separation (N = 9, n = 1). Tan indicates average PS6+ Olfactory area neuron count after chronic (4 week) separation (N = 8, n = 1). Independent t-test reveals no statically significant difference between acute and chronic PS6+ olfactory area neuron induction (ST: N = 9, LT: N = 8, n = 1, p = 0.34).

Lastly, as a way to determine if the difference in NAc cell counts could be attributed to differences in social recognition, we examined PS6 induction within the piriform olfactory areas between the experimental cohorts. Data points for each animal were obtained by adding together both hemispheres of the piriform layer 1, piriform layer 2 and piriform layer 3 (Figure 12). The

piriform olfactory area between both time points demonstrated a non-significant difference in PS6 induction (ST: N = 9, LT: N = 8, n = 1, p = 0.34 NS).

Discussion

Together, this study indicates that after 4 weeks of separation male prairie voles demonstrate overall higher PS6 induction within the entire brain and specifically in the nucleus accumbens but not in the piriform olfactory area.

A possible explanation for these conclusions is that higher counts of PS6+ NAc neurons in the long-term separated group could be attributed to higher social reward after extended social deprivation. In mice, extended social isolation leads to increased levels of social interaction compared to non-separated controls indicating an interest in, and perhaps rewarding aspect of, social interaction after periods of deprivation (Brain, 1975). Therefore, it's possible that a similar rewarding phenotype is seen in our voles when they're given the opportunity to engage in social interactions. Interestingly, the differences in NAc activity don't appear dependent on changes in social recognition of the partner because activity within the olfactory areas is consistent between time points. Maintained partner recognition even after extended separation is supported by previous behavioral data from the Donaldson lab where even after 4 weeks of separation a pair bond was maintained as measured by % partner huddle over acute (ST) and chronic (LT) separation timepoints (Figure 13). Together, this data supports that the differences

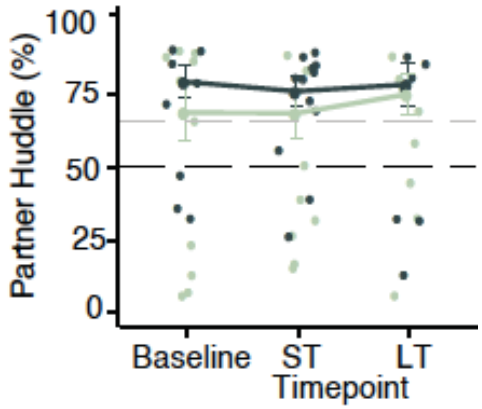


Figure 13: Partner huddle expressed as a percentage across baseline, acute (ST), and chronic (LT) separation timepoints. % partner huddle remained relatively consistent between each timepoint indicating a still intact pair bond

in NAc activity could be a function of social isolation rather than a response specific to the partner as the pair bond, and therefore partner recognition, appears to be intact even after extended separation.

Limitations in this study should be considered when examining the data and warrant future experiments. First, due to time constrains, we were only able to quantify one adjacent slice in every animal so we were unable to look at cell counts throughout the entire NAc and olfactory regions. Looking at cell counts throughout entire brain regions will provide us with the confidence that the results reported are not an artifact of the specific slice we quantified. Furthermore, it is unclear whether the increased NAc activity seen in the chronic animals could be attributed to general social deprivation or if it specific to partner deprivation. To address this, we would introduce a novel after separation instead of the previous partner. If we see similar levels of NAc activity in both the novel and partner conditions it could indicate that this response is a function of general social isolation instead of specifically partner separation. If the levels are different, with more NAc activity in the partner condition, due to the partner being a reward thereby eliciting activity in this region, then it can be reasonably concluded that this is a partner elicited response and not a function of social deprivation.

Future Directions

Moving forward with this study we would first like to finish our analysis of the whole NAc area in each animal. Next, we will expand our analysis to specific inputs and outputs of the NAc: medial prefrontal cortex, ventral tegmental area, paraventricular nucleus, and ventral hippocampal CA1 region. A key part of the complex social neural network is the reward seeking mesolimbic dopamine system. The nucleus accumbens—in cooperation with the prefrontal

cortex, ventral pallidum, ventral tegmental area, and lateral septum—constitute a conserved neural system that integrates reward with social cues (Johnson, Young, 2015). Therefore it would be of interest to explore other areas to expand upon our current conclusion. Along with this experiment we would like to expand this study to test female voles as well as include novel opposite sex animals. These controls are needed for two reasons. First, there are known sex differences between male and female voles during pair bonding and following separation. For example, previous experiments from the Donaldson lab demonstrated that separation in female voles induced a stronger anxiety behavior when compared to male voles. Second, novel opposite sex animals could determine if the differences between acute and chronic animals is due to social novelty or is specific to a partner.

Lastly, this project sits within a larger project aiming to uncover the genetic signatures of loss. PS6 was chosen over *c-fos* because it allows for selective immunoprecipitation of active ribosomes and their corresponding mRNA to allow for sequencing (Knight et al., 2012). In the future, we aim to perform RNA sequence on neurons active during partner reunion from the short- and long-term timepoints to determine if over the course of time there are any differing genetic markers that would be indicative of adapting to loss. My results demonstrate increased activity after chronic separation and with neural activation comes a physical change within the neuron warranting future studies into the exploration of potential genetic differences. These results will grant insight to the molecular mechanisms underlying adaptation to loss and allow for potential therapeutics to aid in loss recovery. Furthermore the molecular mechanisms will allow us to identify why symptoms of grief are stalled in individuals with phycological bereavement.

Every year millions of people experience loss and the grief that comes with that loss is damaging and life-altering. Greif itself is poorly understood within the sciences therefore impeding the potential development of therapeutics to aid in recovery. In this study we aimed to begin the exploration of neuronal mechanisms fostering adaptation to loss by examining PS6 induction and subsequent brain activity within the prairie vole. This study, and future studies, will expand upon the knowledge base of grief getting us one step closer to helping people handle one of the hardest life experiences.

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