Examining the Impact of Neonatal Pain and Infection on Dopaminergic Function During Learning in Long Evans Rats

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jax:
here's to being cursed forever on a twin-sized mattress

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Abstract

Admission to the Neonatal Intensive Care Unit may potentially result in early life trauma. The effects of early life stressors are associated with possible disruptions to the dopaminergic system, resulting in changes to adulthood aversion. Gomes and Barr (2020) simulated the NICU experience via pain and infection exposure on rodents; experimental groups were divided into exposure to pain, to infection, combined conditions, and a non-exposed control. Aversive behavior was measured in adulthood via Conditioned Place Aversion tasks, and brain samples were collected. In the current study, we stained for tyrosine hydroxylase to measure dopaminergic change in relation to early life trauma exposure. Due to their association with dopamine expression, the ventral tegmental area and substantia nigra were targeted for immunohistochemical analysis. In the substantia nigra, there were no significant changes in tyrosine hydroxylase expression between groups; however, tissue damage limited analysis of the ventral tegmental area. These results suggest tyrosine hydroxylase expression in the substantia nigra has no impact on early life pain and infection and their association with a change in aversive behaviors during adulthood.

Introduction

Infants born preterm are more likely to be cared for in the Neonatal Intensive Care Unit (NICU) in order to be under 24/7 supervision by a team of experts (Medina et al., 2018); however, despite the necessity of this specialized care, preterm infants placed in the NICU experience a multitude of necessary yet painful procedures, as well as exposure to infection, which can result in early life traumatic experiences (Zhao et al., 2022). Preterm infants may undergo over 300 pain-inducing procedures (Perry et al., 2018), while 5.4% are exposed to Escherichia coli (E Coli) (Chmielarczyk et al., 2014)), a bacteria associated with fatal bloodstream infections (Bergin et al., 2016). Though the short-term and long-term effects of these two individual traumas have been investigated, there is little literature investigating the interaction of pain and infection combined, and the possible consequences these stressors have on the long-term chemical makeup of preterm infants' brains.

In an effort to bridge this gap, researchers Gomes and Barr (2020) conducted an experiment with the intention of exploring the long-term cognitive, emotional, and behavioral effects pain and infection have on the brains of NICU preterm babies (Gomes & Barr, 2020). A rat model was utilized in order to simulate the NICU experience. According to previous studies, the brains of rats on Postnatal Day 0-3 are comparable to that of premature infants, allowing for the results to be relatively translatable when considering human responses (Pressler & Auvin, 2013; Semple et al., 2013). Gomes and Barr created four groups comprised of newborn Long Evans rats; one experimental group was exposed to Carrageenan (in an effort to create inflammatory, acute pain (Lopez-Cano et al., 2018)), one group was exposed to E Coli, and one group was exposed to both E Coli and Carrageenan, alongside a control group only exposed to

saline. Rats were exposed to E Coli on Postnatal Day 2 (PN2) in order to simulate early-onset infection in premature human infants; similarly, rats were exposed to a Carrageenan injection on PN3 in order to mimic possible inflammatory injuries and procedures premature babies are subjected to while in the NICU.

Post-exposure, Gomes and Barr (2020) were interested in exploring possible long-term emotional and cognitive outcomes of pain, infection, and the two conditions combined. In addition to modeling pain and infection, researchers explored the ways in which early life trauma impacts emotional outcomes and learning tasks, both in adolescence and in adulthood. With the intention of examining these effects after NICU simulation, researchers measured possible changes in aversive behavior; aversion is an indicator of negative emotional conditioning which can then be utilized as an assessment of emotional learning with and without manipulation (Waters, Henry & Neumann, 2009). Gomes and Barr (2020) conducted Conditioned Place Aversion tasks on all four groups in order to measure changes in aversive behavior. When considering the results, it was found that the rats exposed to both pain and infection displayed more aversive behavior in adulthood when compared to rats exposed to only one trauma (pain or infection) as well as rats in the control who were exposed to neither trauma.

Conditioned Place Aversion tasks can measure a change in behavior after administration of a drug or stimuli (both positive or negative) (Prus, James & Rosecrans, 2009). The use of CPA tasks as a method of measurement is preferred, utilizing a negative stimulus to test and ultimately quantify location avoidance, which is then a valid indicator of levels of aversion (Gregory et al., 2013). Specifically, when considering the neurological impact of pain, CPA tasks are employed in order to measure the levels of aversive behavior associated with a negative painful stimulus

(Gao et al., 2020); this task is helpful in understanding and measuring behavioral changes as it is based on a learned relationship between both the pain and the environment in which the pain is administered (Wu et al., 2017).

Gomes and Barr's decision to measure the interaction of both pain and infection proves vital as behavior regarding aversion and preference may be altered both when subjects are exposed to pain (Victoria & Murphy, 2015), as well as when they experience infection (Abdulai-Saiku, Hegde, Vyas & Mitra, 2018). Premature infants' exposure to infection may have long-term consequences on neurological development, resulting in possible negative effects on both the neuroanatomical circuits as well as cognitive-emotional behavior (Dufford, Spann & Scheinost, 2021). Those exposed to infection have displayed an increase in concentrations of proinflammatory cytokines (Chau et al., 2012), indicating an increase in inflammatory activity. Further, there seems to be a correlation between neonatal exposure to E Coli and impaired motor skills in adulthood, as well as diminished white matter (Lieblein-Boff et al., 2013).

When considering pain's effect on neuroanatomical circuits, the effects are relatively expansive, and have consequences on both the affective and sensory systems; specifically, certain areas of the anterior cingulate cortex (ACC), the hippocampus, the prefrontal cortex (PFC), and the ventral tegmental area (VTA) are all implicated in this feedback (Tracey, 2008). These sites and their relationship to pain can be examined by a range of procedures and methods; many researchers use immunohistochemistry to stain rodent brains for the presence of cFos, a gene identified as a label for neuronal activity throughout the axis of the central nervous system (CNS) after external stimulation (Bullitt, 1990). Examining c-Fos can give insight into pain and neurochemical responses, as levels of expression increase when subjects are exposed to chronic

unpredicted stress, as well as painful stimuli (Li et al., 2016). Further, fMRI machines in human studies are also utilized in order to examine functional and structural changes (Wang et al., 2022), especially when considering subjects who cannot self-report, such as infants in the NICU. With this method, the machine measures blood-oxygen-level (BOLD) signals, which are changes in oxygenation within the blood, as well as blood flow within the brain; an increase in BOLD signals in NICU infants' brains are associated with the subjects who are exposed to more painful procedures (Fitzgerald, 2015), indicating a correlation between structural, dynamic changes in the brain and painful stimuli.

Dopamine (DA), one of the specific neurotransmitters associated with aversive behaviors, is typically associated with the reward circuitry of the brain (Juárez Olguín et al., 2016); on a physical level, abnormally low levels of DA are associated with motor issues and movement disorders (Ztaou et al., 2018) while behavioral symptoms include depression and psychotic episodes (Schwerdt et al., 2017). The DA pathways in the midbrain, commonly associated with these symptoms, are separated by location: the substantia nigra (SN) and the ventral tegmental area (VTA). These two locations are major sites for DA creation, as well as critical in the release of DA throughout the brain (Beier, 2022)**;** further, both the VTA and the SN are associated with not only motivation, reward, and movement systems, but also pain modulation (Li et al., 2019). However, the SN is primarily associated with the nigrostriatal pathway, which is generally linked to movement; further, previous studies indicate that the SN has an estimated amount of 75% of DA neurons while the VTA only has around 15% of DA neurons (Kwon & Jang, 2014). In comparison, the VTA is connected to the mesolimbic and mesocortical pathways (Settell et al.,

2017) and is specifically implicated in reward behaviors and reinforcement learning (Ishuji, 2019).

When considering these two similar, yet separate dopaminergic pathways, it is possible that the VTA is more implicated in aversive behavior; within the VTA, vesicular glutamate transporter 2 (VGlutT2) neurons are connected to aversion (Root, Estrin & Morales, 2018), as the activation of VTA VGlutT2 may supply glutamatergic input to the lateral habenula (LHb), a structure within the brain associated with motivation and cognitive functionality, generating aversive behaviors in addition to fostering aversive conditioning (Root et al., 2014). Moreover, activation of Kappa-Opioid receptors (KORs) in the VTA may be required for aversive behaviors to be present (Ehrich et al., 2015). These receptors are necessary for regulating many neurochemical releases, specifically DA, within the central nervous system (Clark & Abi-Dargham, 2019); these results indicate the potential importance of both general VTA activation as well as VTA DAergic activation in order for aversive behaviors to be present.
Specifically, the VTA-NAc pathway is highly implicated in aversive behavior (Klawonn & Malenka, 2019), as the DA interacting in this pathway are associated with prediction error signaling (Yuan, Dou & Sun, 2019), a key factor in aversive behavior. Moreover, lower levels of DA are associated with more aversive behavior (Bromberg-Martin, Matsumoto & Hikosaka, 2010), indicating the role of DA in behavioral changes.

Tyrosine Hydroxylase (TH), a rate-limiting enzyme, is a dopamine precursor enzyme (Daubner, Le & Wang, 2011), and is crucial when considering negative-feedback regulation (Wasinski et al., 2020); lack of TH is associated with motor deficits (Minkley et al., 2020), in addition to being correlated to possible biosynthesis dysfunction (Kawahata & Fukunaga, 2020).

Though TH expression is found in a range of central nervous system (CNS) neurons (Weihe et al., 2006), it is the initial biosynthesis step for dopaminergic transmission (Salvatore, Calipari & Jones, 2016) making it a proximate indicator of the location of possible DA neurons (Daubner, Le & Wang, 2011). The biological reaction of phosphorylation of TH prompted the creation of DA (Struntz & Siegal, 2019). In other words, the concentration of DA in the brain is correlated with levels of TH found, ensuring the validity of utilizing TH as a method of DA level approximation (Kolacheva et al., 2022).

Though Gomes and Barr (2020) simulated the NICU experience on neonatal Long Evans rats in order to examine long term behavior, they did not examine the brains of the animals in an effort to observe neurochemical changes. Subject groups exposed to both pain and infection displayed the highest levels of aversive behavior relative to controls, while groups exposed to one or no trauma displayed no significant changes in aversive behavior relative to controls. These results are supported by previous research, as the VTA is part of the mesolimbic system, and this specific system may mediate the neuronal circuitry implicated in aversive behavior (Holly & Maczek, 2016); moreover, recent studies have demonstrated that aversive behavior may be generated through optogenetic stimulation and activation of Lateral Habenula DAergic neurons in the VTA (Danjo et al., 2014).

The goal of this study is to examine the possible impact early life exposure to pain and infection can have on neurochemical makeup, with an emphasis on measuring possible DA changes via TH levels. We evaluated the amount of TH in the VTA and SN of the brains of Long Evans rats; according to previous research, there seems to be a negative correlation between VTA DAergic activity and levels of conditioned aversion (Danjo et al., 2014), we hypothesized that

subjects with the highest level of aversion (pain + infection group) will display the lowest levels of TH in stained slices relative to controls. However, when considering TH in the SN, past studies indicated no change in DAergic activity and aversive behaviors (Brown et al., 2009); therefore, we hypothesized that subjects with the highest level of aversion (pain + infection group) will not have a significant change in TH levels in stained slices relative to controls.

Methods

Animals

The tissues examined within this project were collected from animals bred, treated, conditioned, and sacrificed by Gomes and Barr (2020); within the previous study, there were 25 Long Evans rat litters tested, with 7-9 subjects per litter. 8 subjects' tissue samples were examined for this current project.

Infection and Pain Exposure Protocol

On PN2, rats were exposed to either E Coli infection or saline via subcutaneous injection. On PN3, rats were given localized injections on their hind paw of either carrageenan or saline in order to simulate painful procedures and injections experienced by preterm babies in the NICU. Behavior tests were conducted at one of three different points in the rats lives (PN8, PN15, or PN65). PN8 simulates a full term infant, PN15 simulates mid-infancy, and PN65 simulates adulthood. In order to establish baseline pain thresholds, researchers completed a plantar thermal withdrawal test (done at PN8, PN15, and PN65); time until withdrawal of each paw from a localized heat source on plexiglass floor was measured, indicating the rats' nociceptive response to pain.

Behavior Tasks

Researchers conducted Conditioned Place Aversion tasks during one of the three ages (PN8, PN15, PN65); rats were placed in a chamber divided into three zones. A lemon scent was placed in one zone, and no scent was placed on the opposite chamber. In the zone with the lemon scent, rats were injected with Formalin (in order to induce an inflammatory, localized pain response). 24hrs after injected with formaline, rats were placed in the same 3-chambered testing zone. Length of time spent in each chamber was recorded; less time spent in the lemon-scented formalin chamber was associated with an increase in aversive behavior.

Immunohistochemistry for TH Expression in SN and VTA

Once the rats were anesthetized and the brains were collected and preserved, the current study collected slices containing the substantia nigra and ventral tegmental area, placing relevant tissue samples on a slide warmer at 113 degrees Fahrenheit for 30 minutes. Slides were then placed under the fume hood and incubated for 30 minute in 4% PFA solution; they were then washed 3 times for 5 minutes each wash in the fume hood, and waste was dumped into the PFA waste container. Slides were then washed 5 times, each wash completed for 5 minutes, on a rocker in the wet lab. Calculations were completed for the primary antibody, Rabbit Anti-TH, at 1:2000. 1 mL of Phosphate-buffered saline (PBS) was added to each jar, in addition to 1 mL of H_2O_2. Slides were incubated for 15 minutes on rocker. Slides were then washed 5 times, each wash for 5 minutes, in PBS solution on rocker. Slides were then added to a black chamber, and the primary antibody was added to each slide; slides were cover-slipped, a hydrophobic pen was used to outline the samples, and the ink dried for 30 seconds before water was added to the chamber. The incubation period for the primary antibody was 48 hours.

After incubation in the primary antibody, slides were washed 5 times, each wash completed for 5 minutes. The secondary antibody, Goat Anti-Rabbit, was added to each slide at 1:1000. Slides were then placed on a tray and 250μL of the secondary antibody was added to each slide. Slides were cover-slipped. Water was then added to the chamber and slides were incubated for one hour. Slides were then washed 5 times, each wash completed for 5 minutes in PBS on rocker. The slides were then placed on a black tray and 260μ of ABC solution was added; slides were then incubated for an hour. Slides were washed 4 times, each wash completed for 5 minutes in PBS. Slides were then washed 2 more times, each wash for 5 minutes, in detergent (Triz). 60 mg of Diaminobenzidine (DAB) solution and 300 mL of Triz was created; 10 mL of each solution was added to each jar and 83 mL of H_2O_2 and slides were incubated for 10 minutes on the rocker. Slides were then washed 5 times for 5 minutes each wash in PBS. Waste was discarded. After drying overnight, slides were cleared and cover-slipped with mounting media before tissue images were captured.

Tissue Analysis and Cell Counts

Images of tissue samples were taken with a 1x and 4x magnification, overlaid with a template as seen in The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 2013). Brain slices were categorized into two subclassifications, slice 1 and slice 2, as each subject had 2 samples. Tissue samples were then further separated by spatial location of brain region: slice 1 left, slice 1 right, slice 2 left, and slice 2 right. Samples were analyzed with the Java program, ImageJ. Images were uploaded to the software, and results were collected utilizing the Optical Density Calibration protocol adapted from the NIH (*OD Calibration*). In order to calculate Optical Density (O.D.), the Epson Expression 1680 Professional scanner was uploaded to

ImageJ, with the range being set at .05 to 3.05. The mean gray value of the first 18 steps were scanned and then entered into the ImageJ program, the images of the samples were calibrated, and a calibration curve was created.

Statistical Analyses

A paired t-test was conducted in order to compare the right and left side of each tissue sample, determine if the location of the brain region captured showed significant differences in TH expression. Equal variance was examined via the Levene's Test in an effort to . A One-Way Anova was utilized to demonstrate if the optical density between subjects' tissue samples were significant.

Results

Though there were approximately 215 rodents utilized in the original experiment conducted by Gomes and Barr (2020), our study only analyzed 8 animals, with each category (E Coli exposure, carrageenan exposure, both E Coli and carrageenan exposure, or no exposure) containing 2 subjects. The decision to exclude certain samples was based on the viability of the brain tissue, as technical issues precluded the analysis of a majority of the specimens collected. Tissue from both the left and right side of the brains per subject were examined; in order to ensure there were no overall differences between the two sides, a paired samples t-test was conducted (Table 1). Results suggest there to be no differences between the left and right side of each subject's brain; therefore, we were able to combine the results from the left and right side, calculating an overall average (Table 1). Additionally, a Homogeneity of Variances test was

conducted between groups*;* results were significant (all under $p < .001$), indicating equal variance cannot be assumed (Table 2) .

In order to analyze the four different conditions, a one-way ANOVA was completed comparing optical density between groups; the results were deemed insignificant between all groups. No statistical differences were found, as all p values were above .05 (see Table 2). The control group's optical density for the averages of slice #1 (M=.2, SD=.0746) did not display significant differences when compared to the pain group (M=.218, SD=.0175), the infection group (M=.21, SD=.0125), and the group exposed to both pain and infection (M=.185, SD=. 004). The control group's optical density for the averages of slice #2 (M=..171, SD=.056) did not display significant differences when compared to the pain group (M=.223, SD=.02575), the infection group (M=.2, SD=.037), and the group exposed to both pain and infection (M=.194, SD=.027) (Figure 1).

Discussion

Results indicate early life exposure to pain, infection, or the combination of pain and infection has no significant effect on the production of tyrosine hydroxylase in the substantia nigra in neonatal long evans rats when compared to rats without exposure. This result supports our hypothesis, as we predicted no change in TH expression in the substantia nigra when compared to the control group. Our results are limited to the regions within the substantia nigra due to tissue degradation and extraneous complications.

When considering the portion of our hypothesis addressing the VTA and TH expression, in which we predicted a decrease in TH expression in the VTA of rodents exposed to both pain

and infection, it is crucial to analyze the relationship between the SN and the VTA. The two dopaminergic pathways are independent of one another, as research has illustrated obvious differences in function between the dopaminergic neuronal subpopulations in the two regions (Boekhoudt et al., 2016). Specifically the dopamine neurons in the SN are associated with the mesostriatal system (Gantz et al., 2017), a system categorized by its involvement in motor regulation (Ziolkowska, 2021); in comparison, the dopamine neurons in the VTA are connected to the limbic and cortical regions (Gantz et al., 2017), commonly associated with pain modulation and aversive behavior (Baliki & Apkarian, 2015). Therefore, should there be no significant increase or decrease in TH expression in one of these two areas, as seen in the results of our current experiment, this is not an indicator of a possible increase or decrease of TH expression in the other brain region. Consequently, despite the results being insignificant in the SN, we cannot assume the VTA results would also be in agreement with our hypothesis.

Previous research has indicated the impact of different forms of early life stress (ELS) on TH expression in the midbrain; categories of ELS vary, ranging from maternal separation (Nishi, 2020) and childhood maltreatment (Catale et al., 2022), to sexual abuse (Romens et al., 2014) and social defeat (Jensen Peña et al., 2019). Prior experiments have illustrated a reduction in TH expression in the VTA in rodents experiencing forms of ELS, while TH levels in the SN remained unchanged (Catale et al., 2022). Additionally, research has indicated an increase of TH expression in the VTA in adult rodents exposed to bacteria during infancy via intraperitoneal injection, while no significant change in TH expression was seen in the SN (Kooi Ong et al., 2017). It is crucial to note previous experiments have examined the impact of infection via peripheral lipopolysaccharide injections (LPS) instead of E Coli; E Coli is a bacteria which

produces a relatively controlled, direct activation in specifics pathways, while LPS produces a more general, broad activation in the brain (Gabay, Lamacchia & Palmer, 2010).

Though our current study analyzed the impact of E Coli on neonatal rats rather than peripheral lipopolysaccharide injections, the impact of infection on the VTA, rather than the SN, may be similar. In studies examining the impact of opioid-exposure and pain modulation and regulation, changes in gene expression in the VTA have been observed while no significant alterations are noted in the SN, indicating the specificity of these regions (Doyle & Mazei-Robison, 2021). Our results support these findings, as the levels of TH expression in the SN regarding groups exposed to pain and infection, simulating NICU early life trauma, were not significantly different than those in the control group.

This study has potential limitations. The brain slices utilized were flash frozen improperly, resulting in water droplets crystallizing on the surface of the specimens; these water droplets then expanded, ultimately tearing the tissue, allowing for the contents of cells to diffuse throughout the slide. Many of the tissue samples were destroyed, resulting in 8 examinable brains. Due to this small sample size, results are not generalizable. Additionally, due to the tissue damage, the samples studied in this experiment did not contain the VTA; therefore, though the intention of this study was to compare TH expression in the VTA to expression in the SN, results were only collected from the SN. Future directions for this research may include the examination of TH expression in tissue containing both the VTA as well as the SN in an effort to determine if aversive behaviors displayed in adulthood are associated with a decrease in TH expression in the VTA.

Additionally, when considering future avenues of research, it may be beneficial to examine the connection between neonatal exposure to pain and infection and Parkinson's Disease (PD), a neurodegenerative disease commonly associated with dopaminergic neuronal degradation in the midbrain (Lew, 2012). Though a relatively small amount of PD cases have been linked to genetic dysfunctions (Warner & Schapira, 2003), the cause of the disease for the majority of patients remains relatively unknown (Tanner & Goldman, 1996). However, studies have begun to demonstrate a possible correlation between PD diagnoses and early life stress (ELS), as exposure to postnatal ELS has been seen to possible increase dopaminergic neuronal vulnerability, possibly resulting in later-life cell degradation (Esch et al., 2002). Though research has focused on the impact of ELS on PD diagnoses, there is little information examining the role of neonatal pain and infection on this disease. Future studies may benefit from analyzing possible changes in TH expression as a result of early life pain and infection exposure and accelerated symptoms in PD patients.

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Tables and Figures

Table 1

Paired Samples T-Test

O.D. Left	O.D. Right		statistic	df	p
Slice #1	Slice #1	Student's t	0.774	7	0.46
Slice #2	Slice #2	Student's t	1.344	7	0.22
Avg. Slice #1	Avg. Slice #2	Student's t	0.565	7	0.59

Paired samples t-test indicating there is no statistical significance between the optical density (O.D.) of the right and left brain slices of the subjects, as well as no significance between the averages of slice #1 and slice #2.

Table 2

Homogeneity of Variances Test (Levene's)

	F	df1	df2	p
Slice #1- Left	1.63E+30	3	4	< .001
Slice #1- Right	4.05E+30	3	4	< .001
Avg. Slice #1	3.51E+30	3	4	< .001
Slice #2- Left	3.28E+30	3	4	< .001
Slice #2- Right	6.99E+29	3	4	< .001
Av. Slice #2	1.24E+30	3	4	< .001
Final O.D.	1.40E+30	3	4	< .001

Levene’s test completed on the results of the optical density (O.D.) of right and left sides of subjects’ brain slices, indicating equal variance cannot be assumed.

Table 3

One-Way ANOVA (Welch's)

Variable	df1	df2	F	p
Slice #1- Left	3	2.07	0.5962	0.674
Slice #1- Right	3	1.84	0.042	0.985
Avg. Slice #1	3	2.03	0.1324	0.933
Slice #2- Left	3	2.07	0.1028	0.951
Slice #2- Right	3	2.05	1.3409	0.451
Av. Slice #2	3	2.16	0.2116	0.882
Final O.D.	3	2	0.17	0.908

One-Way ANOVA analyzing the optical density (O.D.) of the right and left side of subjects' brain slices, as well as the average optical density of the right and left, and final optical density of the two sides combined, indicating no significant difference in O.D. between right and left sides of slices, as well as slice # 1 when compared to slice #2.

Table 4

Group Descriptives

Slice 1	Condition	N	Mean	SD
Left	Both	2	0.189	0.0375
	Control	2	0.188	0.0594
	Infection	2	0.221	0.0226
	Pain	2	0.231	0.0163
Right	Both	2	0.181	0.0757
	Control	2	0.212	0.0898
	Infection	2	0.199	0.0127
	Pain	2	0.206	0.0332
Avg. O.D.	Both	2	0.185	0.0566
	Control	2	0.2	0.0746
	Infection	2	0.21	0.0177
	Pain	2	0.218	0.0247

Group descriptives comparing the optical density (O.D.) results of slices from the right and left sides of the first slice of subjects brains, as well as the average of the sides of the brain combined.

Table 5

Group Descriptives

	Condition	N	Mean	SD
Left	Both	2	0.213	0.0608
	Control	2	0.2	0.1336
	Infection	2	0.197	0.0559
	Pain	2	0.227	0.0332
Right	Both	2	0.175	0.0156
	Control	2	0.142	0.0247
	Infection	2	0.204	0.0488
	Pain	2	0.218	0.0396
Avg. O.D.	Both	2	0.194	0.0382
	Control	2	0.171	0.0792
	Infection	2	0.2	0.0523
	Pain	2	0.223	0.0364

Group descriptives comparing the optical density results of slices from the right and left sides of the second slice of subjects' brains, as well as the average of the sides of the brain combined.

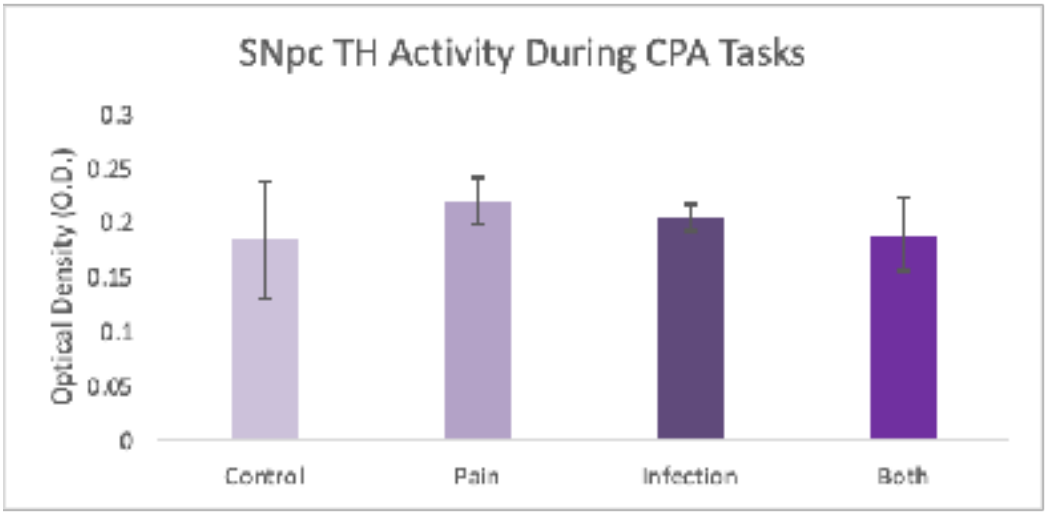
Table 6

Group Descriptives

	Condition	N	Mean	SD
Final O.D.	Both	2	0.19	0.0474
	Control	2	0.185	0.0769
	Infection	2	0.205	0.0173
	Pain	2	0.221	0.0306

Group descriptives indicating the final O.D. value of all conditions when combining both the first and second brain slices.

Figure 1



Optical density (O.D.) of brains does not vary significantly between subjects in control group, pain group, infection group, or pain & infection group.

Figure 2

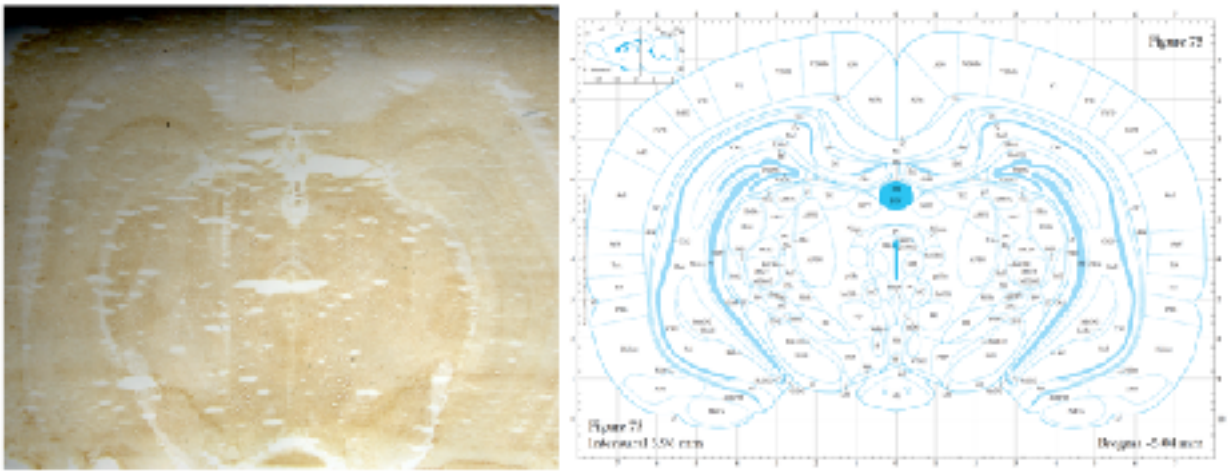
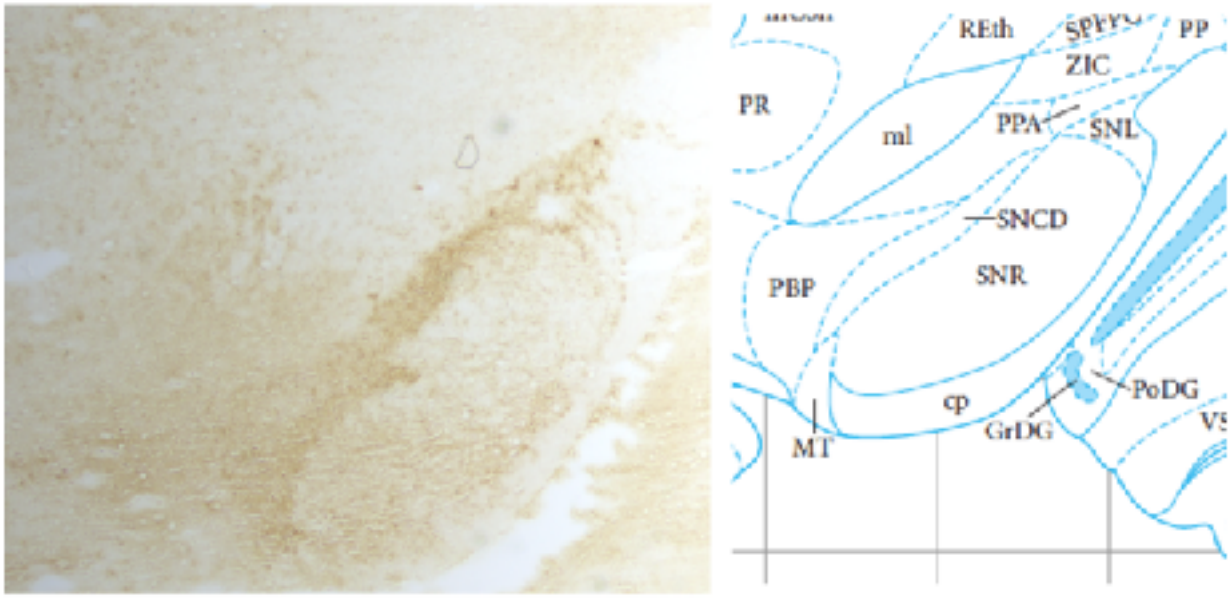


Image of a subject's brain taken on a microscope at 1x magnification, paired with slide 75 in the Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 2013), indicating the location of the brain region.

Figure 3



Magnified image of the substantia nigra from Figure 2 on a 4x microscope magnification of the right side of the region, paired with slide 75 in the Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 2013), indicating the precise location of the brain region.