Prenatal Nicotine Exposure Alters Genetic Profiles of Neurons in the Sub-regions of the VTA During Early Postnatal Development

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ABSTRACT

Brain growth occurs during the first two weeks of postnatal development in rats. This developmental period is equivalent to the third trimester of human gestation. Dendritic arborization, axonal growth, and gliogenesis are observed along with a significant maturation of neurotransmission during this critical development period. Furthermore, nicotine exposure during early development causes deficiencies in sensory and cognitive processing in adults. In this study, we further investigated the neuron populations and the influence of perinatal nicotine exposure on gene expressions of neurons within the sub-regions of the ventral tegmental area (VTA) in one week (P7), two week (P14) and three week (P21) old rat pups. We exposed pregnant rats to nicotine perinatally to investigate its effect in rat pups during early neuronal development (P7, P14, and P21). Real time PCR (RT-qPCR) was used to determine the relative expressions of GABA, dopamine (DA), and glutamate neuron markers within the sub-regions of the VTA including the paranigral nucleus (PN), parainterfascicular (PIF), and parabrachial pigmented nucleus (PBP). Our results indicated that during early maturation, the dopamine marker TH was not significantly expressed within the sub-regions of the VTA in the nicotine exposed P7 group. However, TH was significantly expressed within the PN sub-region compared to the PBP sub-region of the VTA in both the P14 and P21 groups and within the PN sub-region compared to the PIF sub-region in the P21 group. These results suggest that following perinatal nicotine exposure, VTA DA neurons, especially within the PN sub-region, are significantly excited after two weeks of maturation.

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CHAPTER 1: INTRODUCTION

Maternal smoking and nicotine usage are associated with higher rates of stillbirth, Sudden Infant Death Syndrome (SIDS), attention-deficit/hyperactivity disorder (ADHD), adverse effects on cognitive and behavioral development in children, and an increased risk of developing a dependency on nicotine later on in the life of the offspring[1]. On average, 15-25% of women smoke during pregnancy, and millions of pregnant women engage in smokeless tobacco use [1]. Furthermore, 25% of children are exposed to nicotine during gestation in industrialized countries [2]. Because nicotine can be passed to the offspring through the placenta, nicotine concentration levels exceeding maternal concentrations by 15% in fetal circulation and a concentration 88% higher than maternal plasma in amniotic fluid was detected in prenatal humans [1]. Additionally, nicotine accumulates in breast milk and has been shown to accumulate in the fetal brains of rats [3]. These findings suggest that prenatal nicotine exposure affects brain development since nicotine receptors are present in the first trimester [4]. The motor, sensory, and cognitive deficits observed in infants associated with in-utero exposure to tobacco also suggests that nicotine affects early neurodevelopment [1], [2].

The general reward circuit in the brain, illustrated in Figure 1.1, is also known as the dopamine pathway. Dopamine is produced in the ventral tegmental area (VTA) and travels to the reward center nucleus accumbens (NAc) and prefrontal cortex (PFC), where impulses and behavior motivation are derived [5]. The ventral tegmental area (VTA) of the midbrain is associated with reward and drug addiction which plays a crucial role in the release of dopamine and GABA, and its circuitry can be altered

when exposed to addictive drugs or behavior [5-6]. Nicotine use specifically targets the VTA where dopamine (DA) is released to the NAc and ɣ-aminobutyric acid (GABA) neurons provide inhibitory control along the same pathway [1], [8], [9]. In addition, the VTA DA neurons are controlled by glutamatergic synaptic inputs form the prefrontal cortex [10], [11]. Reward and motivation are associated with connections between the VTA and prefrontal regions in humans, which are active during rational thought [12]. Previously, in our lab, we have shown that exposure to nicotine increases DA neuron activity in the VTA, which increases neuronal firing complexity between the VTA and the prefrontal cortex (PFC) [13]. Interactions with addictive drugs have shown behaviors of aggression, compulsion, and lack of social play as a result of the mesocorticolimbic DA circuitry with the NAc and PFC [14]–[16]. Furthermore, doses of nicotine in mice have shown to activate the VTA to NAc dopamine pathway and induce a rewarding effect while simultaneously inhibiting the VTA to amygdala dopamine pathway [17]. These distinct signals are complex but traceable using optogenetics and immunofluorescent techniques [17], [18].

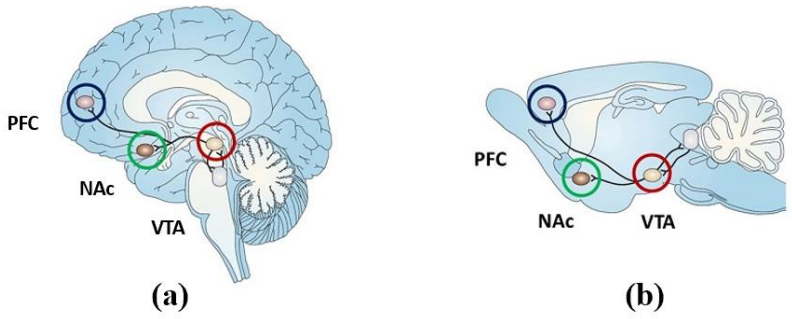


Figure 1.1. Illustration of the reward circuit in the (a) human brain and (b) rat brain. The reward circuitry in the brain consists of the ventral tegmental area (VTA), the nucleus accumbens (NAc), and the prefrontal cortex (PFC).

The neuronal makeup of the VTA is highly heterogeneous [8], [17], [18]. Neurons of the VTA consist of 60-65% dopaminergic (DA), 35% ɣ-aminobutyric acid (GABA)ergic, and up to 5% glutamatergic neurons [19]. Studies have isolated sub-regions of the VTA by analyzing the anterior versus posterior heterogeneity of the VTA or by tracing the origin of dopamine pathways to the NAc or amygdala [17], [20]. In our lab, we have isolated three horizontal sub-regions of the VTA that will be used in this study: the parabrachial pigmented nucleus (PBP), parainterfascicular nucleus (PIF), and paranigral nucleus (PN) shown in Figure 1.2 [13], [21], [22].

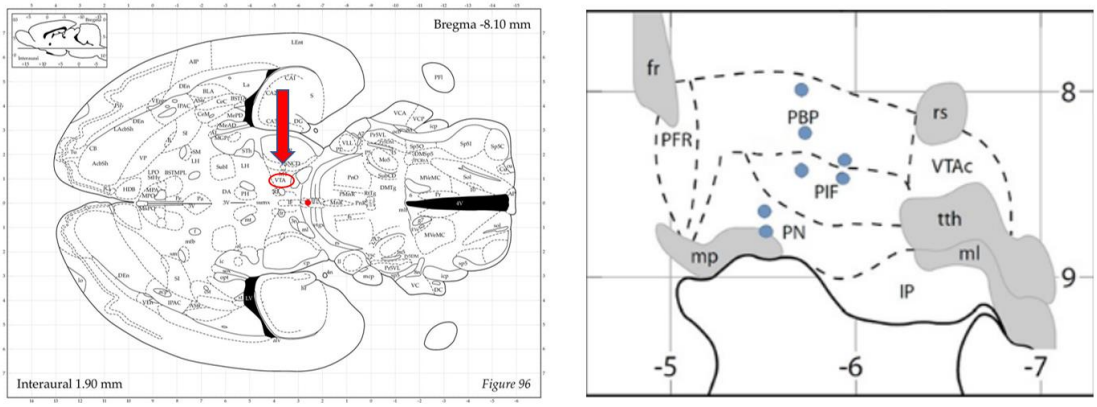


Figure 1.2. Location of VTA and sub-regions in a rat brain. (a) Horizontal cross section. The red arrow indicates the location of the VTA. (b) Lateral view of the PN (ventral), PIF, and PBP (dorsal) sub-regions of the VTA in the adult rat.

To gain insight concerning the dopaminergic composition of neurons in the VTA, studies have used immunohistochemistry to isolate the expression of tyrosine hydroxylase, as shown in Figure 1.3 [17], [18], [23]–[25]. Tyrosine hydroxylase (TH) is a rate-limiting enzyme in dopamine biosynthesis. The amino acid tyrosine is first converted into dihydroxyphenylalanine (DOPA) by TH and is then converted into dopamine by DOPA decarboxylase [26]. VTA dopaminergic neurons control

impulsivity, are integral in reward circuitry implicated by the dependance on addictive substances, and result in higher levels of TH in rats that were exposed to alcohol [7]. Immunolabelling of midbrain tissue, particularly the VTA, has included localizing neurons that contain mRNA for TH using immunofluorescence when comparing nicotine and saline exposed rats and mice since patterns of dopamine and TH immunoreactive neurons are similar [8], [26], [27].

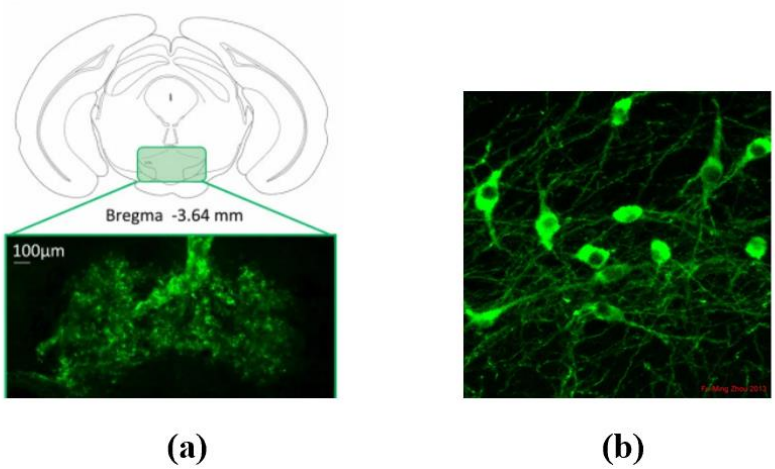


Figure 1.3. Examples of immunofluorescent images of neurons in rodent brains. (a) Coronal cross section of cells detected with eGFP gene antibody in a mouse brain. (b) Dopamine neurons detected with tyrosine hydroxylase antibody.

Our lab published a study that observed the three sub-regions of the VTA in rats following gestational exposure to nicotine at 28 days after birth [23]. Samples were collected from 28-day old rat pups following exposure to nicotine prenatally as well as through breast milk after birth [23]. Gene expression from these three sub-regions sought to show the expression of GABA (GAD1, GAD2), DA (Slc6a3, TH), and glutamate (Slc17a6) neuron markers when nicotine exposed pups were compared with saline exposed pups [23]. It was most notable that DA neurons were more

activated in the PN sub-region of the VTA. Our current study investigates neuron populations in the sub-regions of the VTA and gene expressions to gain insight of the dopaminergic development of the VTA in rat pups following perinatal exposure to nicotine at one week (P7), two weeks (P14), and three weeks (P21) after birth since most neuronal cell groups and early synaptogenesis occurs during this developmental stage [28]–[30]. To examine the population of neurons within the sub-regions of the VTA, we tested the specific gene expressions in rat neurons exposed perinatally with nicotine compared to rat neurons exposed perinatally with saline. Real time PCR (RT-qPCR) was used to find the relative expression of GABA, DA, and glutamate neuron markers. In addition, we examined the TH positive cell counts in the sub-regions of the VTA at all age groups to illustrate the development of DA neurons at early stages of neurodevelopment following perinatal nicotine exposure as compared to a saline exposed group.

CHAPTER 2: METHODOLOGY

2.1 Animal Preparation

All protocols and surgical procedure have been approved by the Institutional Animal Care and Use Committee (IACUC) and the University of Houston Animal Care Operations (ACO) and were performed in accordance with the accepted guidelines and regulations (protocol code 16-017 and date of approval: July 10, 2019). Pregnant, female Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were ordered to arrive on gestational day 4 and delivered to the University of Houston Animal Care Operations facility. On gestational day 7, 72 hours after arrival, an osmotic pump (Alzet, Cupertino, CA, USA) was implanted subcutaneously. Nicotine concentrations were calculated based on the weight of the adult rat and 6mg/kg/day of nicotine was released into the mother's system to be passed on to the pups. Nicotine was released via osmotic pump and passed on the pups from gestational day 6 to postnatal day 14 during pregnancy and through breastfeeding after birth. Saline filled osmotic pumps were used as control. The animals were observed and weighed daily while kept the ACO facility with a 12-hour light/12-hour dark schedule at a temperature of 22 ± 2ºC and 65% humidity. Access to standard food and water was provided.

2.2 Slice Preparation

On the appropriate postnatal day 7 (nicotine: n=7; saline: n=7), day 14 (nicotine: n=20; saline: n=14), or 21 (nicotine: n=18, saline: n=14), pups were anesthetized with isoflurane before decapitation. Brains were removed rapidly and sectioned using a VT1200 semiautomatic vibrating blade microtome (Leica, Nussloch,

Eisfeld, Germany) at a speed of .3 mm/s. Horizontal brain slices of the VTA were obtained by cutting from the ventral side of the brain (1500 µm deep) for ages P7, P14, and P21 before the VTA sub-region samples were taken. For the P7 group, a 400 µm thick slice was taken for the lower VTA sub-region and a 300 µm thick slice was taken for the upper VTA sub-region. For the P14 and P21 groups, a 400 µm thick slice was taken for the PN sub-region of the VTA and 300 µm thick slices were taken for the PIF and PBP sub-regions of the VTA. Two 1mm biopsy punches (Integra Miltex, VWR, Radnor, PA, USA) were used to collect the samples from the VTA on each brain hemisphere of each slice. Tissue samples were kept fresh in RNAlater (Invitrogen, Thermo Fisher Scientific, USA).

2.3 RNA Extraction and cDNA Preparation

Total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) following pestling of the tissue sample in a RLT buffer and Beta mercaptan ethanol solution (Sigma Life Science, Darmstadt, Germany) and sieving the sample through a 20-gauge syringe (Air-Tite Products Co., Inc., Virginia Beach, VA) ten times. Then, cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA) according to instructions and performed on a T100 thermal cycler (Bio-Rad, Hercules, CA, USA).

2.4 RT-qPCR

Taqman Gene Expression Assay primers, as shown in Table 2.1, GAD1 (Assay ID: Rn00690300_m1) and GAD2 (Assay ID: Rn00561244_m1) for GABA, Slc6a3 (Assay ID: Rn00562224_m1) and Th (Assay ID: Rn00562500_m1) for dopamine, and Slc17a6 (Assay ID: Rn00584780_m1) for dopamine and glutamate were performed in

triplicate with each sub-region sample. GAPDH (Assay ID: Rn01775763_g1) serves as the house keeping gene and was performed in triplicate for each sample as well. TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Carlsbad, CA) was used with associated assays and cycles of 2 minutes at 50 ºC, 2 minutes at 95 ºC, 40 cycles at 1 second at 95ºC and 20 seconds at 60ºC on the StepOnePlus Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific,
Carlsbad, CA).

Table 2.1. List of primer sequences for GABA, DA, and glutamate neural markers.

Target Gene	Primer Sequence	Category	Assay ID	Lot Number
Glutamate Decarboxylase 1 (GAD1)	CAACCTGTTTGCTC AAGATCTGCTT	GABA	Rn00690300_m1	1865386
Glutamate Decarboxylase 2 (GAD2)	CGATTAAAACAGGG CATCCCCGATA	GABA	Rn00561244_m1	1804450
Solute carrier family 6, dopamine transporter (Slc6a3)	GTGGCCACAGATGG ACCTGGGCTCA	DA	Rn00562224_m1	1881650
Tyrosine Hydroxylase (TH)	CAAGGACAAGCTCA GGAACTATGCC	DA	Rn00562500_m1	1865386
Solute carrier family 17, glutamate transporter (Slc17a6)	GGACAGATCTACAG GGTGCTGGAGA	Glutamate	Rn00584780_m1	1764173
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGGAGTCCCCATCC CAACTCAGCCC	Housekeeping Gene	Rn01775763_g1	1894565

2.5 Data Analysis

Comparative Ct method (ΔΔCt) was calculated using the StepOnePlus Real-Time PCR System, comparing the GAPDH and saline samples. These values are

shown in Figure 3.1 and Table 3.1, displaying the relative gene expression for each neural marker. Preparation of the data includes the calculation of standard deviation and t-test of $p<0.05$ and $p<0.01$ significance between nicotine and saline treatment groups and between each sub-region for all age groups.

2.6 Immunohistochemistry

Immunohistochemistry protocol by Karadottir et al. was followed [31]. Pups from each age group (n=2) and treatment group (n=1) were anesthetized with isoflurane before decapitation, and whole brains were harvested and placed in 4% PFA (Alfa Aesar, Haverhil, MA, USA) at 4°C overnight. The next day, VTA samples sliced at a thickness of 100 µm were placed in 24-well plates, and washed 3 x 15 minutes in 0.1M PBS gently on a shaker. The slices were then placed in 1 mL of blocking buffer (Immunofluorescence Blocking Buffer #12411, Cell Signaling Technology, Danvers, MA, USA) in 24-well plates, covered, and incubated for 4 hours on a shaker at room temperature. Primary anti-Tyrosine Hydroxylase (ab209921, Abcam, Cambridge, MA, USA) was diluted to a 1:1000 ratio in a solution of 0.1M PBS and 0.05% NaN_3. Aside from blank controls, the slices were each placed in 500 µL of primary antibody in 24-well plates and incubated in the dark 12-15 hours or overnight on a shaker at 60-80 rmp. The next day, slices were washed 4 x 20 minutes in 0.1M PBS gently on a shaker. Secondary antibody, Goat Anti-Rabbit IgG (ab150077, Abcam, Cambridge, MA, USA), was diluted to a 1:500 ratio in a solution of 0.1M PBS and 0.05% NaN_3. Slices were each placed in 500 µL of secondary antibody in 24-well plates and incubated in the dark for 30 minutes on a shaker at 60-80 rmp at room temperature. The slices were then kept in the dark overnight at 4°C.

The next day, slices were washed 4 x 20 minutes in 0.1M PBS and mounted on microscope slides for fluorescent imaging as shown in Figure 2.1a. Figure 2.1b shows TH positive cells in green obtained using a fluorescent microscope (Olympus, Tokyo, Japan) for images. Next, web-based image processing using Image-J analysis that maintained a constant threshold of 42 with a dark background and identified cells 15 pixels or greater was performed for each image individually and is shown in Figure 2.1c.

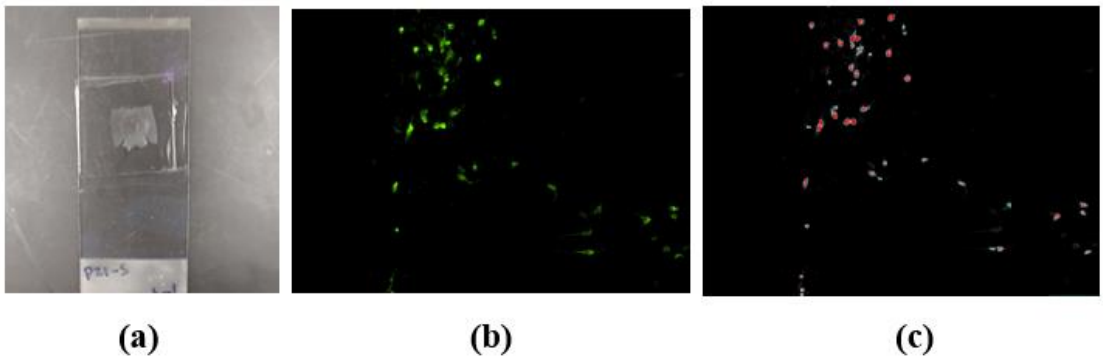


Figure 2.1. Process for TH positive cell counts. (a) Slide of 100 µm thick horizontal brain slice. (b) Image of TH positive cells (green) captured with fluorescent microscope. (c) Results from image processing using Image-J, cell count is 52.

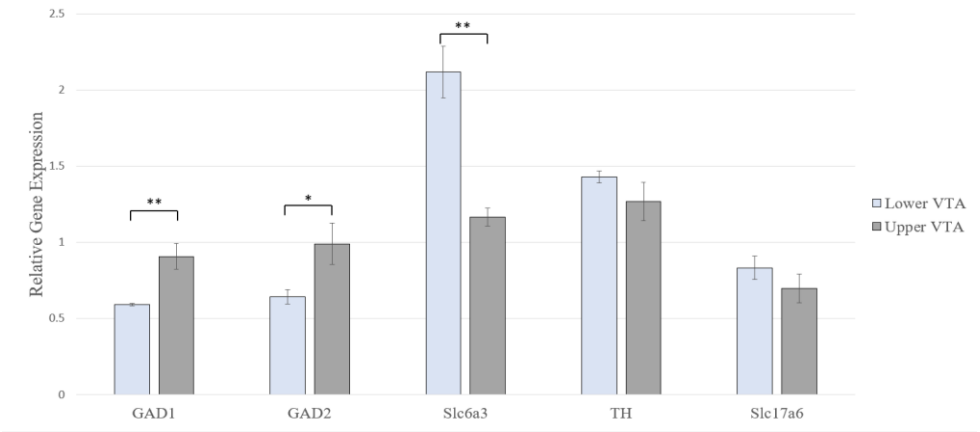
CHAPTER 3: RESULTS

3.1 Relative gene expression of the VTA by sub-region

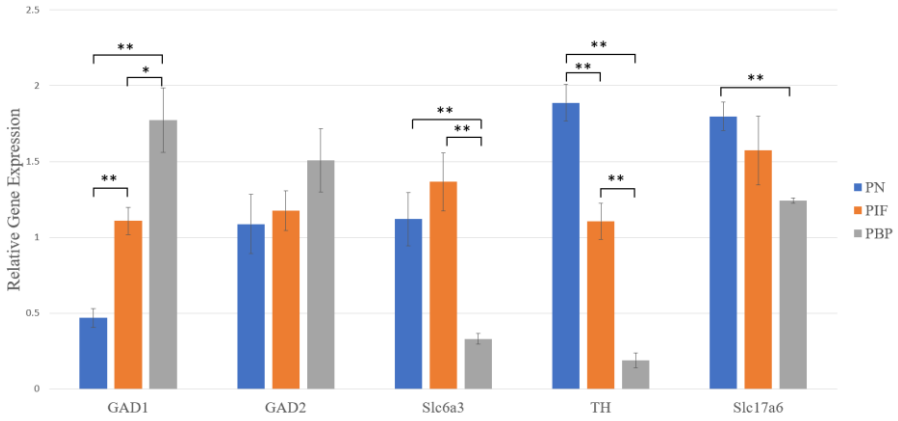
To understand the neuron distribution and development of DA, GABA, and glutamate neurons following prenatal nicotine exposure in each sub-region of the VTA, samples were taken 7 (P7), 14 (P14) and 21 (P21) days after birth and were compared with saline groups of the same age. Samples were obtained by individual brain slices from each sub-region, and their relative gene expression was analyzed using the primers for RT-qPCR listed in Table 2.1. The expression of each gene listed in Table 2.1 compared nicotine exposure with the saline group as well as between each sub-region for each age group. The results follow RT-qPCR analysis relative to housekeeping gene, GAPDH. Two-way analysis of variance was used to indicate the significance between gene expressions in each sub-region of the VTA as well as each gene for each age group. Results from the relative gene expression analysis using Comparative Ct Method (ΔΔCt) are shown in Figure 3.1 and their significance was determined using the t-test method and is listed in Table 3.1.

3.1.1 P7 Group

Sub-regions of the VTA in the P7 group were shown as the lower and upper VTA sub-regions, where the lower VTA contains the PN and PIF sub-regions of the VTA, approximately, and the upper VTA contains the PBP sub-region since the development of the sub-regions of the VTA have not been documented at this age. Expression of GAD1 and GAD2 was significant in the upper VTA sub-region compared to the lower VTA sub-region with *p*-values of 0.0034 and 0.0315, respectively. Slc6a3 expression was significant in the lower VTA sub-region



(a)



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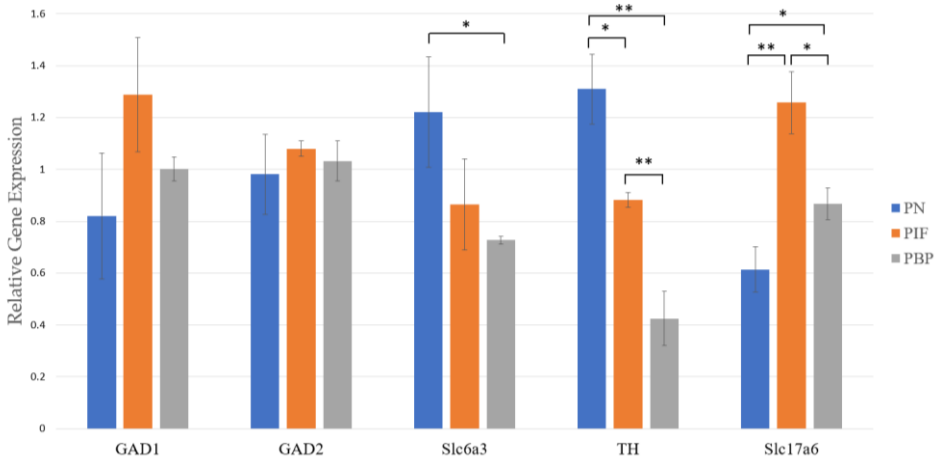

(c)

*Figure 3.1. Relative gene expression of GABA, DA, and glutamate markers by sub-region of the VTA following nicotine exposure as compared to the saline group. (**a**) age P7 (nicotine: n=7; saline: n=7) (**b**) age P14 (nicotine: n=20; saline: n=14), and (c) age P21 (nicotine: n=18; saline: n=14). * denotes p < 0.05, ** denotes p < 0.01, ± SEM*

compared to the upper VTA with a *p*-value of 6.9335 x 10^{-5}.

Additionally, in the P7 group, Slc6a3 expression was significant in the lower and upper VTA sub-regions in the nicotine exposed groups compared to the saline exposed groups with a *p*-values of 0.0348 and 0.0092, respectively. GAD2 expression was significant in the upper VTA of the nicotine exposed group compared to the saline exposed group with a *p*-value of 0.0199.

Table 3.1. Relative Gene Expression Summary and Statistics from RT-qPCR. NS is not significant.

Age	Sub-region	GAD1	GAD2	Slc6a3	TH	Slc17a6
P7	Lower VTA	0.5916	0.6425	2.1191	1.4294	0.8324
	Upper VTA	0.9074	0.9899	1.1646	1.2670	0.6980
	p-values					
	Lower vs. Upper VTA	0.0034	0.0315	6.9335e-5	NS	NS
P14	PN	0.4678	1.8072	1.1195	1.8815	1.7980
	PIF	1.1086	1.1765	1.3671	1.1069	1.5723
	PBP	1.7730	1.5072	0.3298	0.1895	1.2440
	p-values					
	PN vs. PIF	0.0003	NS	NS	0.0036	NS
	PN vs. PBP	0.0013	NS	0.0067	0.0001	0.0026
	PIF vs. PBP	0.0174	NS	0.0034	0.0011	NS
P21	PN	0.8201	0.9810	1.2206	1.3098	0.6145
	PIF	1.2885	1.0780	0.8646	0.8817	1.2578
	PBP	1.0025	1.0323	0.7272	0.4250	0.8679
	p-values					
	PN vs. PIF	NS	NS	NS	0.0175	0.0048
	PN vs. PBP	NS	NS	0.0475	0.0023	0.0412
	PIF vs. PBP	NS	NS	NS	0.0056	0.0218

3.1.2 P14 Group

GAD1 expression was significant in the PBP sub-region of the VTA compared to the PIF and PN sub-regions with *p*-values of 0.0174 and 0.0013, respectively, and in the PIF sub-region compared to the PN sub-region with a *p*-value of 0.0003. GAD2 expression showed no significance between any sub-regions of the VTA. Expression of Slc6a3 was significant in the PN and PIF sub-regions of the VTA compared with the PBP sub-region with *p*-values of 0.0067 and 0.0034, respectively, and expression of TH was significant in the PN and PIF sub-regions compared to the PBP sub-region with *p*-values of 0.0001 and 0.0011, respectively, as well as in the PN sub-region of the VTA compared with the PIF sub-region with a *p*-value of 0.0036. Expression of Slc17a6 was significant in the PN sub-region of the VTA compared with the PBP sub-region with a *p*-value of 0.0026. Additionally, GAD1 expression was significant in the PN sub-region of the VTA in the nicotine exposed group compared to the saline exposed group with a *p*-value of 0.04866. Slc6a3 and TH expression was significant in the PBP sub-region of the VTA in the nicotine exposed group compared to the saline exposed group with *p*-values of 0.0052 and 0.0021, respectively.

3.1.3 P21 Group

Expressions of GAD1 and GAD2 showed no significance between sub-regions of the VTA. Expression of Slc6a3 was significant in the PN sub-region of the VTA compared to the PBP sub-region with a *p*-value of 0.0475. TH expression was significant in the PN and PIF sub-regions compared to the PBP subregions with *p*-values of 0.0023 and 0.0056, respectively, and TH expression was significant in the

PN sub-region of the VTA compared to the PIF sub-region with a *p*-value of 0.0175. Also, Slc17a6 expression was significant in the PIF sub-regions compared to the PN and PBP subregions with *p*-values of 0.0048 and 0.0218, respectively, and Slc17a6 expression was significant in the PN sub-regions of the VTA compared to the PBP sub-region with a *p*-value of 0.0412. Additionally, TH expression was significant in the PIF and PBP sub-regions of the VTA in the nicotine exposed groups compared to the saline exposed groups with *p*-values of 0.0002 and 0.0460, respectively. Slc17a6 expression in the PN sub-region of the VTA in the nicotine exposed group was significant compared to the saline exposed group with a *p*-value of 0.0343.

3.2 Relative gene expression of TH in the sub-regions of the VTA compared to saline treated whole midbrain VTA samples by age group

We further investigated the dopamine neuron marker TH in the VTA as it is the most well-known genetic marker for dopamine neurons. RT-qPCR was used to identify higher neuron populations by determining the relative TH expression within each of the sub-regions of the VTA in both the nicotine and saline exposed groups when compared with saline treated whole midbrain VTA samples.

3.2.1 P7 Group

Our results suggested that the nicotine exposed groups had higher expressions of TH and were statistically significant compared with saline exposed group when both groups were compared with whole midbrain samples in the lower VTA sub-region of the VTA with a *p*-value of 7.773×10^{-8} as shown in Figure 3.2a and in the upper VTA sub-region with a *p*-value of 0.0038 as shown in Figure 3.2b.

3.2.2 P14 Group

At age P14, our results suggested that there was an increase of TH expression in both the nicotine and saline exposed groups when compared to the saline whole midbrain samples for all sub-regions of the VTA. In the PN and PIF sub-regions of the VTA, shown in Figures 3.3a and 3.3b, the nicotine exposed group had a higher expression of TH than the saline exposed group and was statistically significant in the PN sub-region with a *p*-value of 1.6535×10^{-5}. In the PBP sub-region of the VTA, the saline exposed group had a higher expression of TH compared with the nicotine exposed group, as shown in Figure 3.3c, and was statistically significant with a *p*-value of 0.0002.

3.2.3 P21 Group

As shown in Figure 3.4a, our results suggested that nicotine exposed groups had significant expression of TH in the PN sub-region of the VTA compared with the saline exposed group with a *p*-value of 0.0466 in the P21 group when compared to the saline whole midbrain samples. Although the expression of TH in the nicotine exposed group was slightly increased compared to saline exposed samples in the PIF sub-region of the VTA, it can be seen in Figure 3.4b that there was not a statistically significant diference in the TH expression following perinatal nicotine exposure. However, TH expression in the PBP sub-region was slightly reduced in the nicotine exposed group compared to saline treated group as shown in Figure 3.4c.

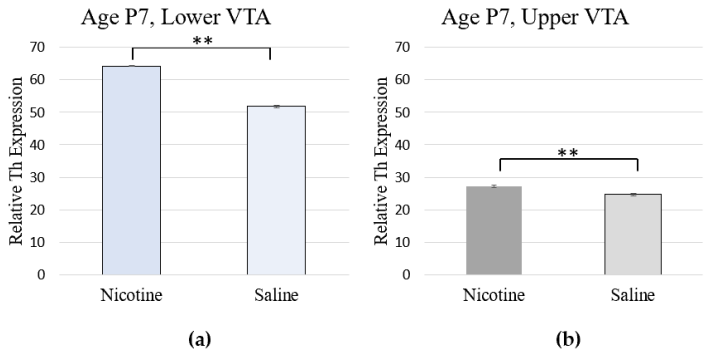

*Figure 3.2. Relative gene expression of TH in nicotine and saline exposed pups at age P7 (nicotine: n=7; saline: n=7) compared to saline treated whole VTA midbrain samples in (a) lower and (b) upper VTA. **denotes p < 0.01, ± SEM*

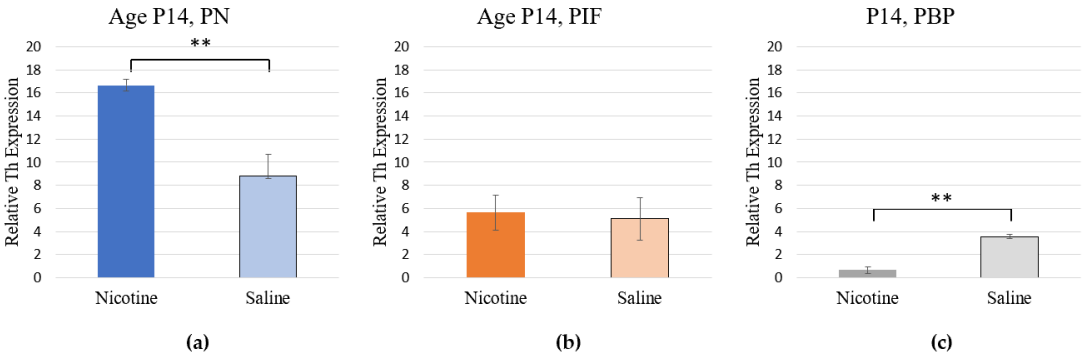

*Figure 3.3. Relative gene expression of TH in nicotine and saline exposed pups at age P14 (nicotine: n=20; saline: n=14) compared to saline treated whole VTA in the (a) PN, (b) PIF, and (c) PBP sub-regions. **denotes p < 0.01, ± SEM*

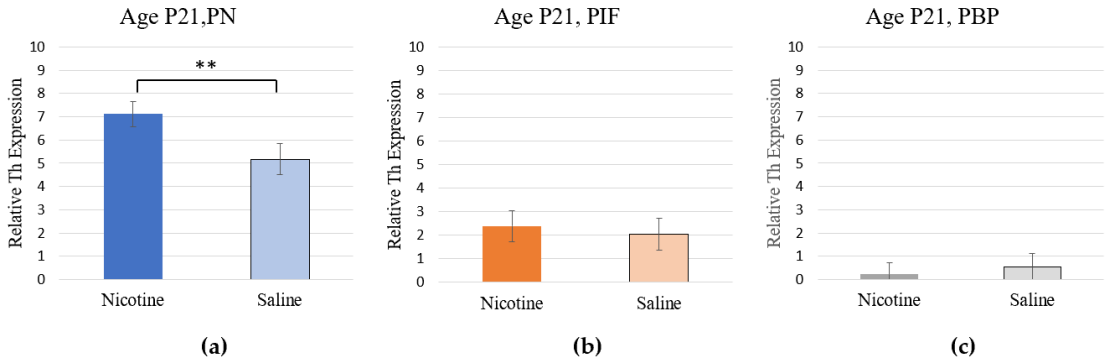

*Figure 3.4. Relative gene expression of TH in nicotine and saline exposed pups at age P21 (nicotine: n=18, saline: n=14) compared to saline treated whole VTA in the (a) PN, (b) PIF, and (c) PBP sub-regions. **denotes p < 0.01, ± SEM*

3.3 Immunohistochemistry staining of the nicotine and saline treated VTA sub-regions by age group

We continued to characterize the dopamine expression within the sub-regions of the VTA by obtaining fresh samples of each sub-region of the VTA at each age group and staining them using immunofluorescent (IF) markers. We labeled freshly obtained 100 µm slices of the VTA with TH antibody in both the nicotine and saline exposed groups and obtained images of the TH expression using a fluorescent microscope. Cell count was performed for each sub-region of the VTA at ages P7, P14, and P21 using Image-J, and the average cell counts of the 100 µm thick slice samples of each sub-region are shown in Figure 3.5.

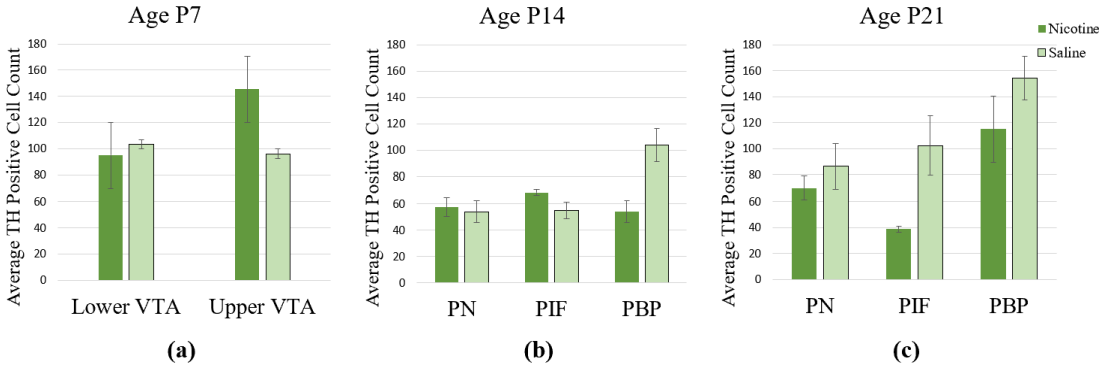

Figure 3.5. Average TH positive cell counts following immunofluorescent staining by VTA sub-region at ages (a) P7, (b) P14, and (c) P21.

3.3.1 P7 Group

The average cell counts per sub-region were 95.00 and 145.50 for the lower VTA and upper VTA of the nicotine exposed group, respectively, and 103.16 and 96.00 for the lower VTA and upper VTA of the saline exposed group, respectively. One 100 µm slice per sub-region of the VTA is shown in Figure 3.6. The imaged

samples show cell counts of 62 and 171 for the lower VTA and upper VTA of the nicotine exposed group, respectively, and 89 and 131 for the lower VTA and upper VTA of the saline exposed group, respectively. The TH positive cell count was greater in the saline treated group of the lower VTA when compared to the nicotine treated group, and the TH positive cell count was greater in the nicotine treated group of the upper VTA when compared to the saline treated group.

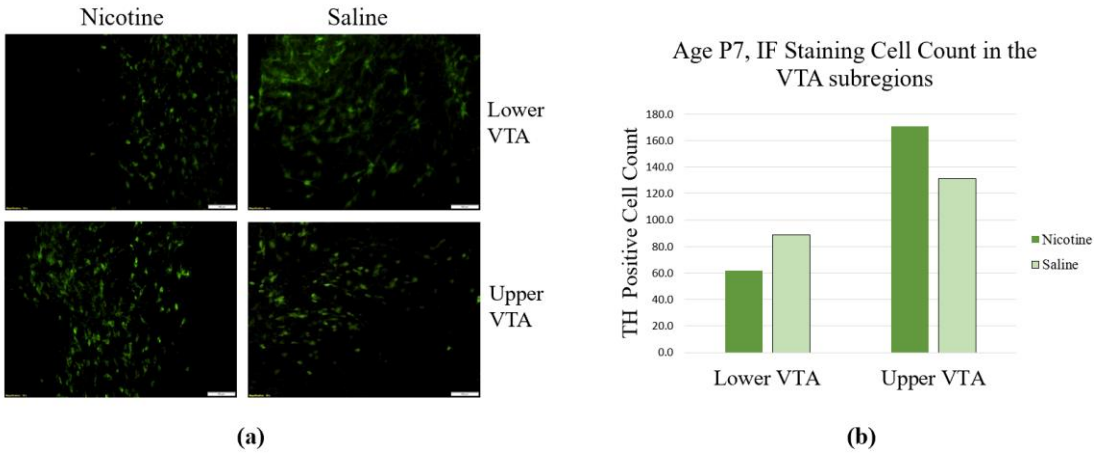

Figure 3.6. Immunofluorescence staining of TH positive cells in the lower and upper VTA sub-regions in the P7 group. (a) TH expressed neurons appear green. Scale bar represents 100 µm. (b) TH positive cell counts for images in (a).

3.3.2 P14 Group

The average cell counts for the nicotine exposed groups were 57.38, 68.50, and 53.83 for PN, PIF, and PBP sub-regions of the VTA, respectively, and the cell counts for the saline exposed group were 53.88, 54.83, and 103.83 for PN, PIF, and PBP sub-regions of the VTA, respectively. Illustrating this trend, one 100 µm slice per sub-region of the VTA is shown in Figure 3.7. The imaged samples show TH positive cell counts for the nicotine treated group of 62, 58, and 49 for PN, PIF, and PBP sub-regions of the VTA, respectively, and TH positive cell counts for the saline treated

group of 43, 50, and 90 for PN, PIF, and PBP sub-regions of the VTA, respectively. In the PN sub-region of the VTA, the nicotine treated group showed a greater TH positive cell count when compared to the saline treated group, whereas the PIF and PBP sub-regions of the VTA showed greater TH positive cell counts in the saline treated group when compared to the nicotine treated group.

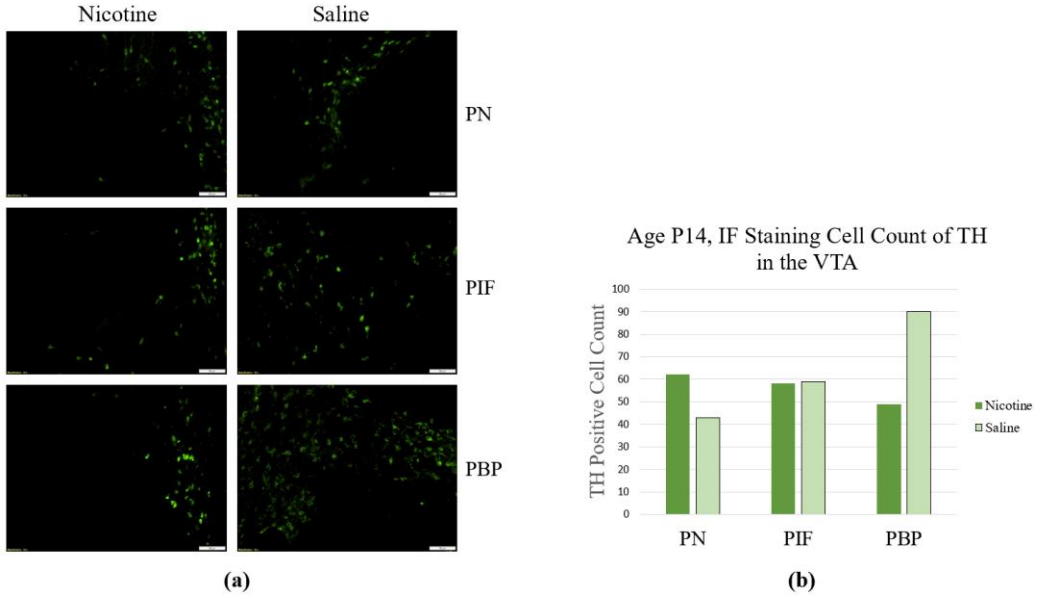

Figure 3.7. Immunofluorescence staining of TH positive cells in the PN, PIF, and PBP sub-regions of the VTA in the P14 group. (a) TH expressed neurons are green. Scale bar represents 100 µm. (b) TH positive cell counts for images in (a).

3.3.3 P21 Group

The average cell counts for the nicotine exposed groups were 70.00, 38.83, and 115.17 for PN, PIF, and PBP sub-regions of the VTA, respectively, and the cell counts for the saline exposed groups were 86.63, 102.50, and 154.17 for PN, PIF, and PBP sub-regions of the VTA, respectively. One 100 µm slice per sub-region of the VTA is shown in Figure 3.8. The imaged samples show TH positive cell counts for the nicotine treated group of 52, 66, and 89, for PN, PIF, and PBP sub-regions of the

VTA, respectively, and TH positive cell counts for the saline treated group of 69, 86, and 83 for PN, PIF, and PBP sub-regions of the VTA, respectively. In the PN and PIF sub-regions of the VTA, the nicotine treated group showed greater TH positive cell counts when compared to the saline treated group, whereas the PBP sub-regions of the VTA showed a greater TH positive cell count in the saline treated group when compared to the nicotine treated group.

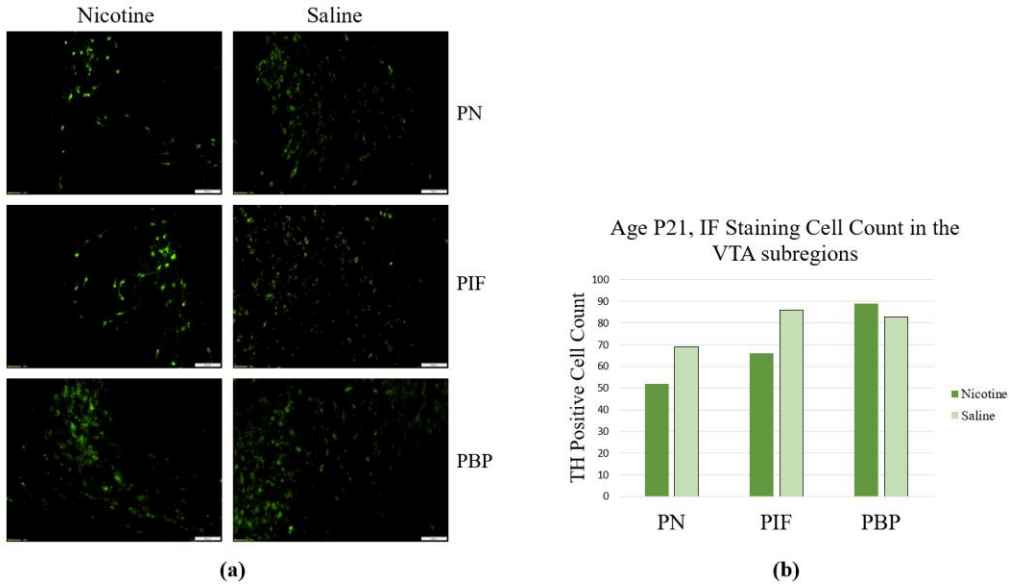

Figure 3.8. Immunofluorescence staining of TH positive cells in the PN, PIF, and PBP sub-regions of the VTA in the P21 group. (a) TH expressed neurons are green. Scale bar represents 100 µm. (b) TH positive cell counts for images in (a).

CHAPTER 4: DISCUSSION

When rats are born, the stage of brain development is immature compared to humans. Around gestational day 13 (G13), dopamine neurons of the VTA originate and begin to innervate the neocortex by G15 [32]. Dopamine neurons in rats continue to develop and are formed approximately a week before birth, G15-G21 [33]. The first two weeks of a rat's life are comparable to the third trimester of human gestation, and most neuronal cell groups and early synaptogenesis occurs during this stage [28], [29]. Dendritic arborization, axonal growth, and gliogenesis also occur during this time [30]. In this study, we have focused on the development of neurons in the sub-regions of the VTA at P7, P14, and P21. It is known that prenatal exposure to nicotine can lead to permanent deficiencies in cognitive processing and that the reward seeking area of the VTA is rich in DA neurons. As we examined the early development of DA neurons in the brain and the effects of early nicotine exposure, we focused on sub-regions of the VTA: lower VTA and upper VTA, or PN, PIF, and PBP at three age groups: P7, P14, and P21. We hypothesized that the genetic expression of DA neurons will be higher in the nicotine exposed groups as compared to the saline exposed groups of rat pups and that these expressions will become more specific to certain sub-regions as the rats age [28]–[30].

At age P7, we noted that TH genes were not significantly expressed in any sub-region of the VTA but had a higher expression in both nicotine and saline exposed groups in all sub-regions of the VTA when compared to saline whole midbrain samples. Slc6a3 expression was significantly increased in lower VTA sub-region as

compared to the upper VTA. Additionally, GABA and glutamate neurons had a lower expression compared to the DA neurons in all sub-regions of the VTA.

At age P14, we continued to examine the three sub-regions of the VTA individually. It was observed that the PN sub-region significantly expressed an increase in DA expressing genes, TH and Slc6a3, when compared to the PBP sub-region of the VTA. We also observed that the gene expression of TH was significant in the PN and PIF sub-regions compared to the PBP sub-region, and the PBP sub-region is significantly expressed compared to the saline group. At this age of development, the expression of GABA neurons showed an increased expression of GAD1 and GAD2 in the PBP sub-region followed by the PIF sub-region. When comparing the expression of these genes between sub-regions, we noticed that the GABA neurons were more expressed in PBP sub-region of the VTA when compared with DA neurons, which were more highly expressed in the PN and PIF sub-regions. When comparing the TH expression of each sub-region with the whole saline midbrain, we found that the nicotine group was significantly expressed in the PN sub-region of the VTA, and the saline group was significantly expressed in the PBP sub-region at age P14. The average cell counts of TH positive cells following IF staining showed that the saline treated group had higher cell counts than the nicotine treated group in the PBP sub-regions of the VTA.

At age P21, we observed that the PN sub-region of the VTA was more significantly expressed for DA neurons when compared to the PBP sub-region of the VTA. For the genes Slc6a3 and TH, the PN sub-region increased expression compared to the PIF and PBP sub-regions, and TH expression was significant in the PIF and

PBP sub-regions compared to the saline treated groups. When comparing the expression of these genes between sub-regions, we noticed that the GABA and glutamate neurons were more expressed in PIF sub-region of the VTA followed by the PBP sub-region when compared with DA neurons which were expressed in PN sub-region, followed by the PIF sub-region. When comparing the TH expression of each sub-region with the whole saline midbrain, we found that the nicotine group was significantly expressed in the PN sub-regions of the VTA at age P21. In addition, average TH positive cell counts in the saline treated group were higher in the PN, PIF, and PBP sub-regions of the VTA when compared to the nicotine treated group.

This increase of expression in the DA neurons in the PN sub-region of the VTA in the P14 and P21 groups was consistent with our previous study of 28-day old rat pups, where there was a large increase of relative gene expression in DA neurons in the PN sub-region of the VTA compared to saline group [23]. In our current study, the glutamate gene Slc17a6 and GABA genes GAD1 and GAD2 showed an increased expression in the PIF sub-region compared to the PN sub-region. Also, the TH positive cell counts showed a similar trend to the previous study of 28-day old rat pups, where the cell counts for TH positive cells in the saline group were greater than that of the nicotine group.

In this study, we found that DA, GABA, and glutamate neurons were present in all three sub-regions of the VTA during brain development from postnatal day 7 to day 21 following prenatal and postnatal nicotine exposure. At age P7, we observed that DA expressions were present in both sub-regions of the VTA. Then, at age P14, the PN sub-region showed in increased expression of DA neurons while the PBP sub-

region increased expression of GABA neurons. Finally, at age P21, the PN sub-region was observed as being the richest in DA neurons, and the PIF sub-region had an increase in expression of GABA and glutamate neurons. Notably, TH expression was significantly increased in the PN sub-region of the VTA in all age groups when compared to the whole saline treated midbrain. While the findings of our study supported our hypothesis of increased DA expressing neurons in the VTA after two weeks of post-natal maturation in rat pups and we were able to observe the composition of the neuronal development of the sub-regions of the VTA, we also observed that GABA and glutamate neurons were also developing in the sub-regions of the VTA and dominated different regions of the VTA as complementary to the development of the DA neurons, which is consistent with our background research which describes the complex and heterogeneous nature of the VTA. We further examined the development and composition of dopamine neurons at each age group by comparing the cell count and gene expression of TH. It is important to consider the development of the whole network within the brain beyond what we can observe in individual neuron development.

As a summary, this experiment helps us understand the full circuitry of the VTA, PFC, and NAc in relation to reward and addiction during early maturation. By isolating the sub-regions of the VTA, where dopamine neurons are located during early brain development stages, we can create a better physiological map of how the brain is not only developing and responding to the influences of early exposure to addictive substances such as nicotine, but also how we could develop treatments or establish better timing on when interventions are necessary.

CHAPTER 5: CONLCUSIONS

We observed neuronal development in DA, GABA, and glutamate neurons in the sub-regions of the VTA in young rat pups (P7, P14 and P21) following prenatal and postnatal nicotine exposure. Samples were collected directly from each sub-region of the VTA at each age group, and relative gene expression of five significant genes were determined when comparing the nicotine exposed pups to saline exposed pups of the same age. Our results indicated that during early maturation, the dopamine marker TH was significantly expressed within all three sub-regions of the VTA in the P14 and P21 groups when compared with the saline group as well as in the PN sub-region when compared to the saline treated whole midbrain. Also, Slc6a3 expression was increased within the PN sub-region of the nicotine exposed group compared to the PBP sub-region in the P21 group. These results suggest that following perinatal nicotine exposure, VTA DA neurons within the PN sub-region are excited in the P14 and P21 groups.

Future studies for these experiments could include detection of the differences between samples of different sexes and observing the pathways leading from each sub-region of the VTA to the PFC [18]. Recent studies show that dopaminergic signaling pathways are increased in the NAc and decreased in the VTA of female mice rats following exposure to nicotine, whereas dopaminergic signaling pathways are decreased in the NAc and increased in the VTA of male mice following chronic nicotine exposure [34]. A study of this nature could also be expanded to include or compare other addictive substances such as alcohol or cocaine. Also, observing the experimental results from IF staining may help to further isolate the size of the VTA

during early development since the size and composition of the brain itself is still growing and forming from ages P7 to adult age of P28 [35], [36]. While the focus of this study was the expression of dopamine neurons during early development of the VTA, we included the gene expression of some GABA neurons which provide local inhibitory control of dopamine neuron activity to the NAc and could be used for future studies [8].

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