

Age Impacts Perineuronal Net Density in the Somatosensory Genital Cortex Independently of
Gonadal Steroid Hormones

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ABSTRACT

A hallmark of pubertal development is the rise in gonadal hormone secretions and the subsequent onset of sexual behavior. The size of the somatosensory genital cortex increases in response to both this rise in gonadal steroid hormones and physical stimulation. However, whether specific markers of brain plasticity are similarly impacted by gonadal hormones during puberty is not known. Perineuronal nets (PNNs) are specialized extracellular matrix structures that ensheath neurons as they mature, and their maturation coincides with the closure of sensitive periods across several sensory systems. PNNs in somatosensory cortex, as an example, gate experience-dependent plasticity. Although experience-dependent changes occur in the somatosensory cortex, whether gonadal steroid hormones influence PNN development in the somatosensory cortex during puberty is not known. The current study tested the hypothesis that gonadal steroid hormones drive adolescent PNN development in the somatosensory genital cortex. At 22 days of age, before the pubertal onset of gonadal steroid hormone release, male and female mice were either gonadectomized or sham gonadectomized. Then at preadolescence (30d), adolescence (47d), or adulthood (70d), animals were transcardially perfused and had their brains removed. Brains were sectioned at 40 μ m and immunofluorescence labeling of Wisteria floribunda (WFA) was used to visualize and quantify PNN density. We found that the presence of gonads had no effect on PNN development in the genital cortex, but there were sex and layer-specific increases in PNNs with age. These findings suggest that despite gonadal steroid hormone release occurs during PNN maturation, separate processes contribute to changes in PNN density.

TABLE OF CONTENTS

INTRODUCTION.....1

CHAPTER 1 METHODS.....3

CHAPTER 2 RESULTS.....6

DISCUSSION.....7

REFERENCES.....10

APPENDICES.....13

VITA.....18

LIST OF FIGURES

Figure 1 Experimental design.....	13
Figure 2 Example of the genital cortex outlined with the use of the created template of the genital cortex.	14
Figure 3 Gonadal status and age effect on PNN density in the genital cortex.....	15
Figure 4 Gonadal status and age effect on PNN density in the genital cortex by layer.....	16
Figure 5 Age effect on PNN density in the genital cortex by layer.....	17

INTRODUCTION

Understanding neural plasticity, the brain's ability to make structural changes in response to experience, is important to understanding the neural changes observed during development (reviewed in Knudsen 2004). Heightened periods of neural plasticity are referred to as sensitive periods (reviewed in Knudsen 2004). During sensitive periods, sensory experience fine-tunes neural pathways to optimize behavioral function in adulthood, a process known as experience-dependent maturation (reviewed in Knudsen 2004). For example, light exposure during perinatal development drives the pattern of connectivity between the thalamus and visual cortex required for depth perception and visual acuity in the right and left eyes (reviewed in Hensch 2005; Pizzorusso et al. 2002). The opening of the sensitive period can be extended if animals are reared in the dark following birth (Mower 1991), and the closure of the sensitive period coincides with the maturation of perineuronal nets (PNNs). PNNs are structures found in the extracellular matrix that surround the soma and dendrites of neurons and inhibit neuronal signaling by acting as a physical barrier between neurons, locking neuronal connections in place (reviewed in Hensch 2005). Experience-dependent plasticity decreases across the sensitive period until PNNs reach full maturation, thereby closing the sensitive period (reviewed in Hensch 2005). Enzymatic degradation of PNNs reopens the window of experience-dependent plasticity in the visual system (Pizzorusso et al. 2002). Whether PNN development regulates sensitive period timing in brain regions outside the visual system is not yet clear.

The sexual differentiation of behavioral neural circuits is governed by gonadal hormone secretions during perinatal and adolescent periods of development (reviewed in Schulz and Forrester-Fronstin 2018). In males, behavioral masculinization (expression of mounts, intromissions, and ejaculations) and defeminization (suppression of lordosis) requires testosterone exposure during a protracted postnatal sensitive period that opens perinatally and closes following adolescence (Schulz et al., 2009). In females, maximal expression of lordosis behavior requires estradiol exposure during a more restricted periadolescent sensitive period (Bakker et al., 2002; Brock et al., 2011). The neural mechanisms underlying these sex-specific windows of sensitivity are unknown. The current study seeks to determine whether pubertal secretions of gonadal steroid hormones influence PNN development across the adolescent period.

The genital cortex, a component of the somatosensory cortex, plays an indirect role in reproductive behavior function by processing tactile information from the genitalia (Lenschow et al. 2018). This region also undergoes maturational changes in response to physical stimulation in the presence of adolescent gonadal steroid hormones (Sigl-Glockner et al. 2019; Lenschow et al. 2017). These maturational changes are defined as a nearly doubling in size of the genital cortex due to an increase in thalamic afferents to neighboring dysgranular cortex that respond to genital touch as well as an increase in cells receiving thalamic input in layer IV of the genital cortex (Lenschow et al. 2017; Sigl-Glockner et al. 2019). The maturation of the genital cortex during adolescence is a change not observed in the other regions of the somatosensory cortex which typically are fully mature soon after birth (Van Der Loos & Woolsey 1973; reviewed in Feldman & Brecht 2005). In female rats, prepubertal ovariectomy prevents increases in genital cortex size, indicating that ovarian release of estradiol plays a key role in genital cortex development (Lenschow et al. 2017). Although PNNs are abundant in the adult somatosensory cortex, whether PNNs play a role in steroid-dependent maturation of the genital cortex is unknown. The current study sought to characterize the development of genital cortex PNNs and test the hypothesis that

gonadal steroid hormones drive the development of PNNs during adolescence. This hypothesis predicts that only gonad-intact animals will display adolescent increases in PNN densities. Our strategy was to gonadectomize (GDX) or sham gonadectomize (referred to as intact) male and female mice prior to the pubertal onset of gonadal hormone secretions and quantify PNN densities before, during, and after adolescence. The results of this study will advance scientific understanding of gonadal steroid hormones' potential involvement in genital cortex PNN maturation.

CHAPTER 1

METHODS

Animals

72 male and female C57Bl/6 mice were bred from Envigo stock and weaned at 21 days of age. Littermates of the same sex and treatment group were housed 2 mice/cage. Exogenous sources of steroid hormone exposure were reduced by providing animals with phytoestrogen-free chow (Teklad), shredded aspen bedding (Teklad), and by using BPA-free, glass water bottles. Animals were provided food and water ad libitum and maintained on a 12:12 light/dark cycle. All procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville.

Experimental Design

All mice were weaned on postnatal day 21. On day 22, prior to the onset of pubertal secretions of gonadal hormones, mice were gonadectomized or received a sham surgery. Brain collection occurred at 3 time points to assess whether gonadal status impacts PNN development before (30d), during (47d), or after (70d) the normal timeframe of adolescence. (Fig 1.)

Surgical Procedures

Mice were provided presurgical buprenorphine (0.1mg/kg) and gonadectomized or sham gonadectomized under isoflurane anesthesia and aseptic conditions. Testes were removed via a small midline abdominal incision that was closed with a sterile suture and wound clips. Ovaries were removed via 2 small bilateral flank incisions that were subsequently closed using sterile suture and wound clips. Immediately following surgery, animals were administered subcutaneous ketoprofen (3mg/kg) and sterile saline (0.3ml). Animals were returned to their home cage following recovery in a heated cage and provided a second injection of ketoprofen 24 hours later.

Brain Collection

Blood was collected during cardiac perfusion for confirmation of hormone levels and then cleared with saline. After all the blood was cleared from the body, 4% paraformaldehyde solution was pumped in to fix the brain. Mice were then decapitated, and the brain was extracted and placed into 4% paraformaldehyde for 24 hours before being moved to a 30% sucrose solution. Brains were in the 30% sucrose solution for 24 hours before the solution was changed for another 24 hours of exposure. Brains were then frozen with dry ice, wrapped in aluminum foil, and placed in the -80 degrees Celsius freezer until sectioning. Brains were given a number coding that was recorded to blind researchers to treatment conditions.

Tissue Collection and Immunohistochemistry

Brains were sectioned on a Leica cryostat at 40-micron thickness and transferred to positively charged microscope slides. Histochemistry procedures were conducted on slides at room temperature. The lectin, Wisteria floribunda agglutinin (WFA), is a widely used marker for visualization of PNNs as it marks one of the primary extracellular proteins that constitute PNNs (Härtig & Brauer 1992). Tissue sections were incubated for 30 minutes in a blocking solution containing 10% normal goat serum (G9023, MilliporeSigma), 5% triton-x (from 10% triton-x working solution), and 85% PBS to reduce nonspecific staining. Extra blocking solution was made and then diluted to 50% by adding equal parts PBS for use in primary and secondary antibodies (referred to as 50% diluted blocking solution). After the 30-minute blocking period, solution was removed and WFA (1:500, L1516, MilliporeSigma) in 50% diluted blocking solution was applied to the tissue for a 24-hour incubation period. After the 24 hours, sections were rinsed with PBS for three 5-minute washes and then incubated for 4 hours in Streptavidin Alexa Fluor 488 (1:1000, S32354, Invitrogen) in 50% diluted blocking solution. After the 4 hours, sections were rinsed with two 5-minute PBS washes and then incubated for 5 minutes with DAPI in PBS (1:1000, D1306, Invitrogen). After the 5-minute incubation period, DAPI was removed from the slides and a final 5-minute PBS wash was applied. PBS was then removed, and the slides were left to dry in a dark, dry drawer overnight. The next day slides were cover slipped using Fluoromount-G (0100-01, SouthernBiotech) and the edges of the cover-slip were sealed with clear nail polish (Sally Hansen). Slides were stored in 4-degree Celsius refrigerator until taken to the microscope for imaging.

PNN Imaging and Quantification

Brain sections were imaged on a BZ-X700 Keyence microscope to capture images of the region of interest (genital cortex). These images were loaded into the Image-J software (NIH, Version 1.53q) where the genital cortex was identified and traced using a tracing template created using the bregma coordinates of the genital cortex provided by Lenschow et al. 2016 (Fig. 2). The template was aligned with the selected tissue using the curvature of the cortex and the hippocampus as landmarks. The genital cortex was then divided into layers 1-3, 4, and 5-6 because of functional differences in cortical layers (Lenschow et al. 2017). 4',6-diamidino-2-phenylindole (DAPI) was used to identify and trace individual layers in accordance with somatosensory layer divisions (Paxinos and Franklin, 2019). The cross-sectional area of each layer was calculated in ImageJ using the measure function. Slices selected for PNN counting were separated by 120 microns distance to ensure PNNs were not double counted. PNNs were counted manually if they met the criterion of surrounding at least 80% of the soma and one neurite extension. The volume of the traced region was calculated by taking the measured region and multiplying it by the thickness of the tissue (40 microns). PNN densities were calculated as PNN number divided by the volume collected (sum of cross-sectional areas x section thickness). PNN densities were calculated separately for each layer grouping.

Statistics

Genital cortex PNN density was examined by conducting a 2-factor univariate ANOVA separately for males and females. Specifically, age (prepubertal, adolescent, adult) and gonadal status (GDX and intact) were between-subjects factors. Alpha was set as 0.05 and error bars used standard error (SE). LSD posthoc tests were run on age and gonadal status. Gonadal status did not impact PNN density, nor did gonadal status interact with age. Therefore GDX and intact data were combined to examine layer-specific changes in PNN density across age and a one-way ANOVA was used to separately analyze PNN density in male and female mice.

CHAPTER 2

RESULTS

The presence of gonadal steroid hormones had no effect on PNN maturation

Contrary to predictions, gonadal status did not impact PNN densities in the genital cortex in female ($F_{2,15} = 0.030$, $p = 0.865$) or male ($F_{2,11} = 0.033$, $p = 0.859$) mice. In addition, age did not impact PNN densities in the genital cortex of both females ($F_{2,15} = 0.881$, $p = 0.435$) and males ($F_{2,11} = 1.241$, $p = 0.327$). No interaction between gonadal status and age was observed for either sex (Fig. 3).

PNN densities were then quantified in cortical layers of the genital cortex (Fig. 4). In females, there were no significant differences between GDX mice and intact mice for PNN density in genital cortex layers 1-3 ($F_{2,14} = 0.053$, $p = 0.821$), layer 4 ($F_{2,15} = 0.133$, $p = 0.720$), or layers 5-6 ($F_{2,15} = 0.038$, $p = 0.848$). Similarly, in males, there were no significant differences between GDX mice and intact mice for PNN density in layers 1-3 ($F_{2,10} = 0.322$, $p = 0.583$), layer 4 ($F_{2,11} = 0.081$, $p = 0.781$), or layers 5-6 ($F_{2,11} = 0.128$, $p = 0.727$).

Statistically significant age effects on PNN density were observed in females in cortical layers 1-3 ($F_{2,14} = 4.906$, $p = 0.024$), such that adults exhibited greater PNN densities than prepubertal ($p = 0.011$) and adolescent females ($p = 0.036$). An effect of age was not seen in females in layer 4 ($F_{2,15} = 0.360$, $p = 0.704$) or layers 5-6 ($F_{2,15} = 1.120$, $p = 0.352$). No significant age effect was seen in males in layers 1-3 ($F_{2,10} = 1.223$, $p = 0.335$), layer 4 ($F_{2,11} = 0.717$, $p = 0.510$), or layers 5-6 ($F_{2,11} = 1.481$, $p = 0.269$).

Varying patterns of PNN maturation across age within layers and sex

No significant differences were observed between GDX and intact mice. Therefore, data from the GDX and intact mice groups were combined to increase statistical power for investigating PNN maturational changes across age and within cortical layers. The results show that PNN density increases as animals age, but there are varying patterns of this between cortical layers and sex (Fig. 5).

In females, age significantly impacted PNN density in layers 1-3 ($F_{1,17} = 5.566$, $p = 0.014$), such that adults exhibited greater PNN densities than prepubertal females ($p = 0.006$). Age did not influence PNN density in layer 4 ($F_{1,18} = 0.349$, $p = 0.710$) or layers 5-6 ($F_{1,18} = 1.254$, $p = 0.309$).

In males, age marginally impacted PNN density in layers 1-3 ($F_{1,13} = 2.302$, $p = 0.139$) such that adult mice exhibited greater PNN densities than prepubertal males ($p = 0.051$). Age did not significantly influence PNN density in layer 4 ($F_{1,14} = 1.073$, $p = 0.369$). Age did not significantly alter PNN density in layers 5-6 ($F_{1,14} = 2.312$, $p = 0.136$) although adult mice exhibited significantly greater PNN densities than prepubertal males ($p = 0.050$).

DISCUSSION

Despite gonadal steroid hormones being released at the same time PNN maturation occurs, the results suggest the presence of gonadal steroid hormones has no effect on the maturation of PNNs in the genital cortex during adolescence. This was illustrated by data revealing that GDX and intact mice were not significantly different in PNN density in the genital cortex at any point in development. So while gonadal steroid hormones do cause structural changes in the genital cortex during adolescence (Lenschow et al. 2017), it seems there is a separate process that underlies PNN maturation. Importantly, this finding does not rule out the possibility that PNNs regulate the timing of steroid-dependent maturation of genital cortex volume. While it is known that the absence of estradiol during adolescence results in an immature genital cortex in adulthood (Lenschow et al. 2017), it is not known if estradiol re-administered during adulthood would cause the steroid-dependent maturation of the genital cortex that we typically see in intact animals. There is a possibility that PNNs in adulthood may act as a physical barrier and block the increase in thalamic input to layer 4 and the subsequent expansion of the genital cortex. A future study that would support this idea is if gonadal steroid hormone-naïve mice are exposed to gonadal steroid hormones in adulthood, after the adolescent development of PNNs, and then genital cortex maturation is measured. Such a study would provide a better understanding of the role PNNs have in the developing genital cortex.

While we predicted that gonadal steroid hormones would be the experience that matures PNNs in the genital cortex (Lenschow et al. 2017), it may be genital grooming that is the driver of PNN maturation. This possibility is supported by the finding that in the somatosensory cortex, PNN maturation is driven by sensory experience (Ueno et al. 2017). Although gonadal steroid hormones increase grooming during adolescence (Moore 1986), it may be that gonadal steroid hormones do not directly promote the maturation of the genital cortex, but mediates genital grooming which provides the tactile experience needed for genital cortex expansion and PNN maturation. This idea may seem contradictory to our results, but it could indicate that the reduced grooming of the genitals that is seen in GDX mice (Moore 1986) may produce sufficient sensory experience to spur PNN formation, but not enough so that it promotes the maturation of the genital cortex. Alternatively, genital cortex maturation may require both gonadal steroid hormones and tactile touch, while PNN maturation only needs tactile touch. More research needs to be done to elaborate the relationship between gonadal steroid hormones, genital grooming, and the various maturational changes observed in the genital cortex throughout adolescence.

Not only were different patterns of PNN maturation between the layers across development observed, but differences in PNN density between males and females were also apparent.

For both females and males, layers 1-3 are responsible for coordination of the movement aspect of sexual behavior and are sexually monomorphic (Lenschow et al. 2018). For females the related movement is lordosis and for males it is thrusting, both of which are something that only occurs after puberty (reviewed in Sisk et al. 2005). It is possible the role of PNNs in layers 1-3 is solidifying the responsible neural circuits for these movements that mature with exposure to gonadal steroid hormone. In females, an absence of estrogen during adolescence results in increased lordosis behavior in hamsters (Schulz et al. 2004), but an absence of estrogen also leads to no expansion of the genital cortex (Lenschow et al. 2017). Perhaps it is the expansion of the genital cortex that allows for the development of the needed neural circuitry responsible for

normal lordosis and thrusting behavior. The data proposes the idea that PNNs function to solidify the maturational changes made by gonadal steroid hormones to layers 1-3 of the genital cortex, whether or not the hormones are present.

The present results indicate that PNN density remained stable across development in layer 4 for female and male mice. Layer 4 of the genital cortex receives afferent input from the thalamus which informs the genital cortex of physical stimulation of the genitals (Lenschow et al. 2018). In males, there are no changes that occur in penile nerve innervation of layer 4 across development (Purkart et al. 2020). While the role of layer 4 in genital cortex function is still in the early stages of understanding, this consistent innervation may hint at a consistent function of layer 4 of the genital cortex across development. This may explain why PNN density changes in layer 4 were not seen.

In cortical layers 5-6, male mice, but not female mice, see an increase in PNN density with age. Layers 5-6 are responsible for processing and relaying neural information from layer 4 to subcortical regions of the brain. Like other subregions of the somatosensory cortex, it projects to various regions of the thalamus, the striatum, and the pontine nuclei. However, layers 5-6 of the genital cortex are unique as the genital cortex is the only subregion of the somatosensory cortex that projects to the anterior-dorsal amygdala, the endopiriform nucleus, the nucleus accumbens, and the dorsal part of the submedial nucleus of the thalamus (Lenschow et al. 2018). The amygdala and the nucleus accumbens are both regions known to play a key role in sexual behavior (Harris and Sachs 1975; Liu et al. 1998). During adolescence, the neural circuits involved with sexual behavior, including the amygdala and the nucleus accumbens, undergo drastic changes in neural circuitry (reviewed in Sisk et al. 2005). How the genital cortex and these thalamic projections interact during sexual behavior, as well as how these thalamic projections change across development, is unknown. This study contributes to this gap in knowledge by suggesting PNNs may be developing in layers 5-6 of the genital cortex to lock in possible maturational changes that occur during adolescence in males. Why no changes in PNN density in cortical layers 5-6 in females were not observed is not clear and may involve a number of causes. In females, there are differences in sexual behavior across the estrus cycle (Morin 1977). In addition, PNNs have been shown to change dynamically with the estrus cycle to control plasticity in the dentate gyrus (Laham et al. 2022). There also may be a connection between neural plasticity and PNNs in this region to allow for the various responses to sexual stimuli seen in the different phases of the estrus cycle. Repeating the present experimental design and noting which phase of the estrous cycle the mouse is at during brain collection may be clarifying.

Although the present study found no effect of gonadal steroid hormones on PNN maturation, the results encourage future studies to explore the effects of gonadal steroid hormones on the development of parvalbumin-positive (PV+) inhibitory interneurons in the genital cortex. PV+ neurons are the main cell type that PNNs surround (reviewed in Hensch 2005). In the hippocampus, the presence of gonadal steroid hormone alters the development of PV+ neurons in female mice (Wu et al. 2014). The role of PV+ neurons is currently unknown in the genital cortex, and future studies are needed to understand how gonadal steroid hormones modulate PV+ neuron development. While PNNs mostly surround PV cells in the somatosensory cortex (reviewed in Hensch 2005), other research shows excitatory neurons (Carstens et al. 2016) and other inhibitory interneurons, like somatostatin-positive interneurons, (McRae et al. 2007) are surrounded by PNNs as well in other regions of the brain. It is worth exploring the

relationship between PNNs and the various cell types PNNs surround in the genital cortex to fully understand the role of PNNs across development.

One problem this study encountered is that the shape of the genital cortex may have been altered under the various hormonal and age conditions this study employed. Although a representative area of the genital cortex was used, the present study could not measure possible rostral-caudal changes in PNN density that may occur in the genital cortex. This is of interest as the genital cortex expands rostrally through puberty when gonadal steroid hormones are present in addition to an increase in layer 4 thalamic afferents (Lenschow et al. 2016; Lenschow et al. 2017). So while no effects of age or treatment on PNN density in layer 4 were not observed, there may be rostral-caudal changes in layer 4 that this study cannot detect. A thorough mapping of the genital cortex under various hormonal conditions at each stage of development needs to be made before proper rostral-caudal changes in PNN density can be explored.

In conclusion, this study supports the interpretation that gonadal steroid hormones are unlikely to have a strong and direct role in PNN maturation in the genital cortex. What the results do show is layer-specific changes in PNN density across development between males and females. This study brings new ideas to how PNNs regulate plasticity in the genital cortex across development and its role in solidifying changes made to the genital cortex during adolescence. In addition, the results encourage future studies to clarify the relationship between the increase in genital grooming during adolescence as a potential mechanism for PNN maturation during adolescence.

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APPENDICES

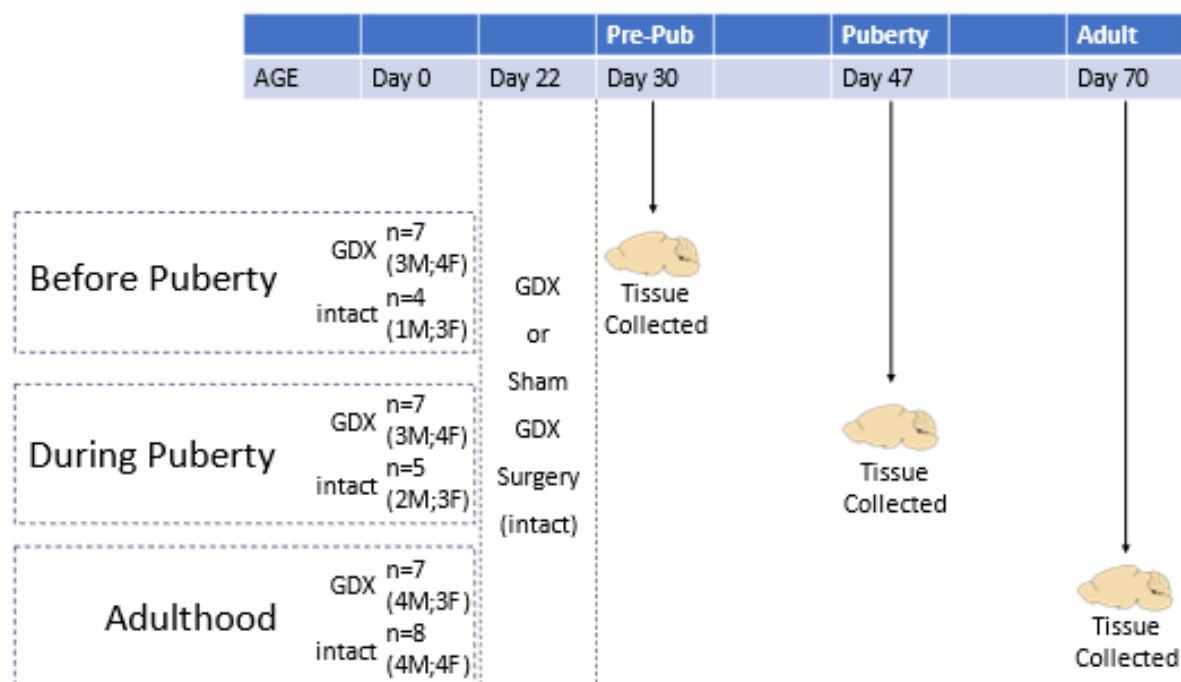


Figure 1. Experimental Design. All mice were weaned on postnatal day 21. On day 22, gonadectomized (GDX) mice had their gonads removed and intact animals received a sham surgery to control for surgical stress. On day 30, 47, or 70, representing before adolescence, during adolescence, or adulthood respectively, all mice were given a lethal dose of a ketamine and xylazine mixture and underwent cardiac perfusion. Brains were then collected and perineuronal nets were stained and counted in the genital cortex .

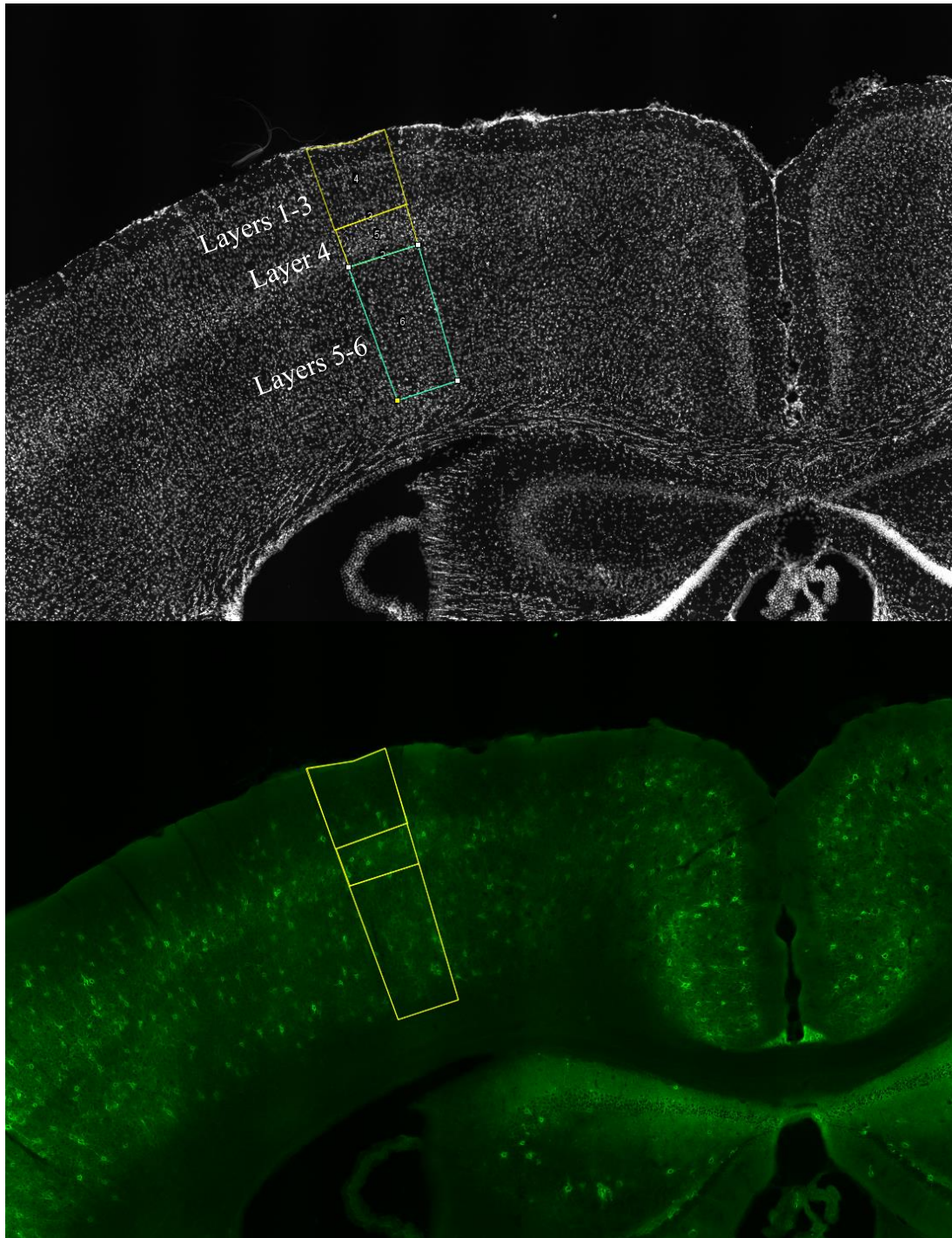


Figure 2. Example of the genital cortex outlined with a template created for the genital cortex. Top: DAPI channel in greyscale used to trace the region and to best visualize the layers. Bottom: fluorescence GFP channel to reveal WFA stained perineuronal nets.

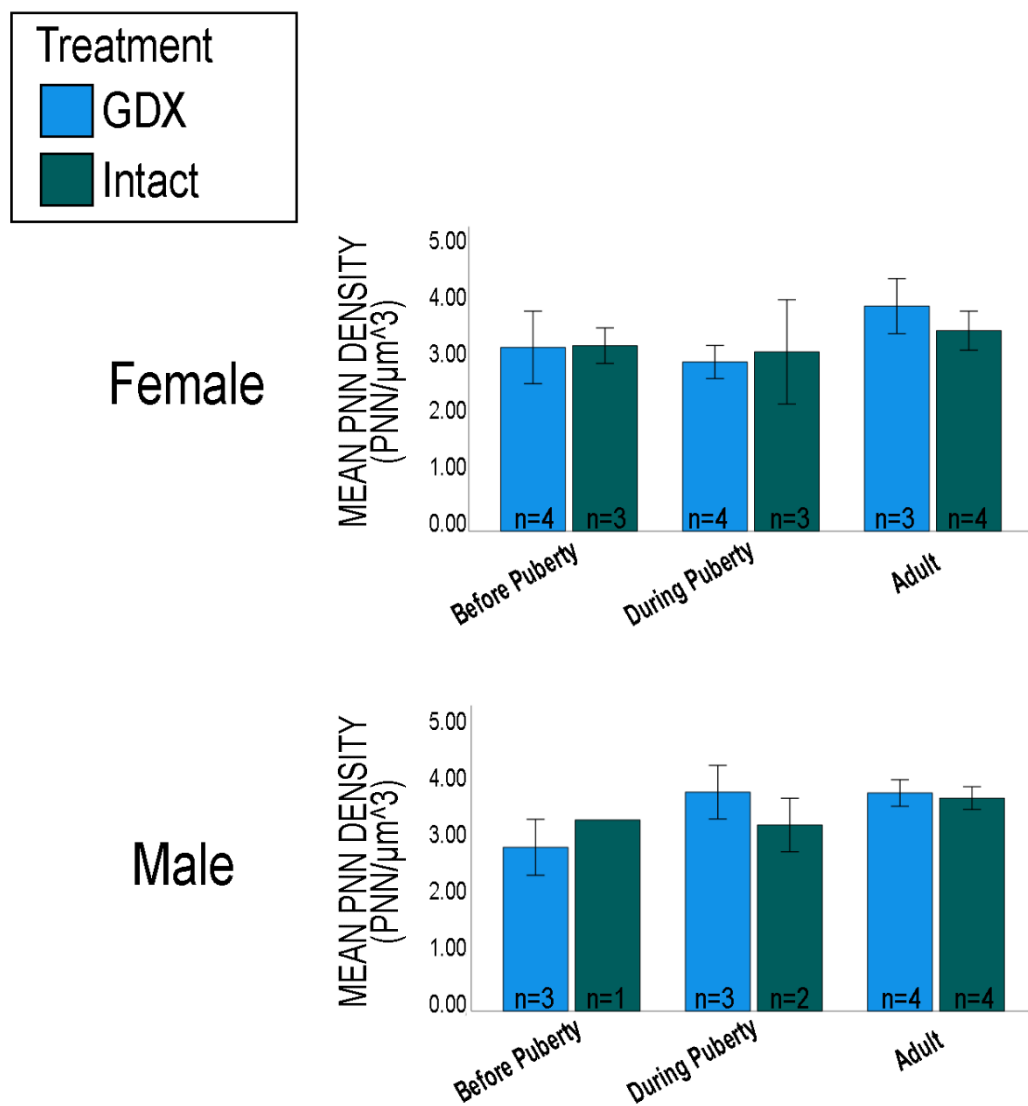


Figure 3. Both gonadal status and age have no effect on PNN density in the genital cortex.

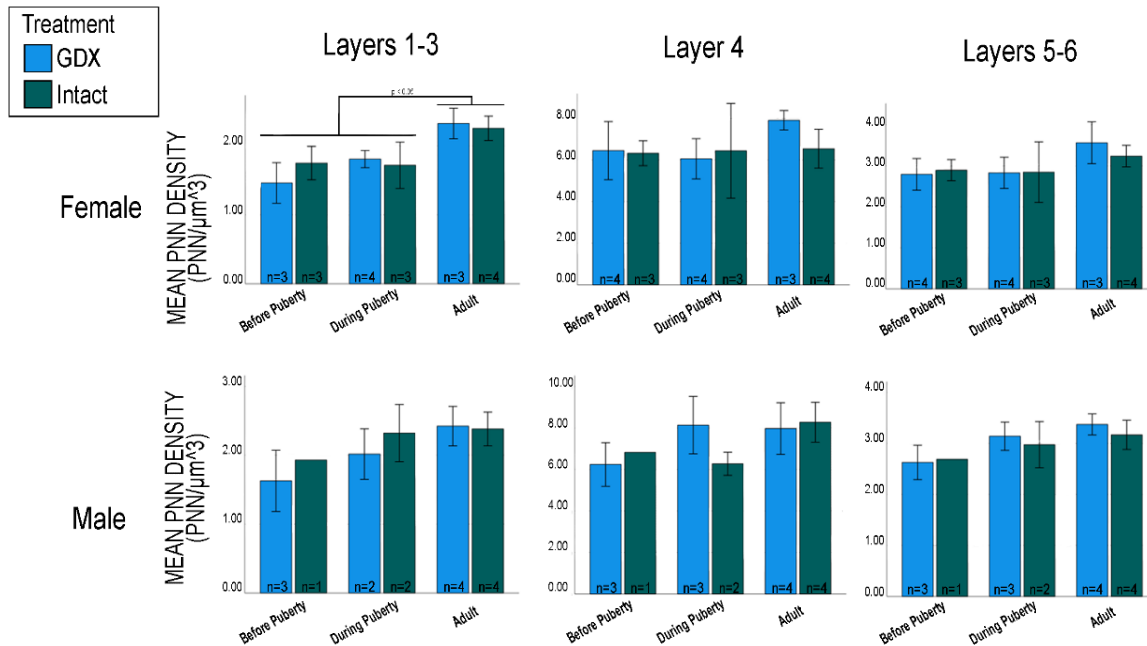


Figure 4. Gonadal status has no effect on perineuronal net maturation for males or females at any point in development in the genital cortex. An age effect was detected in females layers 1-3. This indicates that gonadal steroid hormones are unlikely to play a role in the adolescent formation of perineuronal nets.

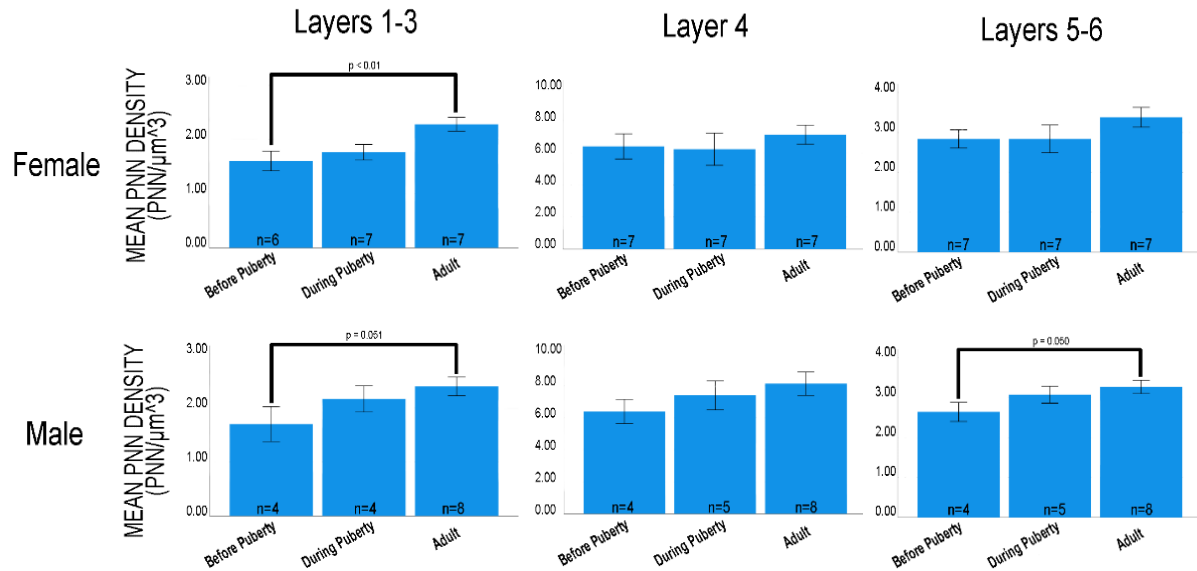


Figure 5. Perineuronal net density increases with age in specific layers differently for males and females in the genital cortex. For both males and females, layers 1-3 undergo an increase in perineuronal nets in adulthood. This increase in PNNs is not seen in layer 4 of the genital cortex for either sex. Layers 5-6 see sex-specific age increases as there is an increase in perineuronal net density in males, but not females.

VITA

Ian Warren started his research journey during his undergraduate years at the University of Tennessee at Knoxville in Dr. Kalynn Schulz's lab as an undergraduate research assistant. Originally a pre-med student that did it more for prestige and money than genuine interest, he found meaning in research and switched over to that as a career path. Ian would eventually become a master's student in Dr. Schulz's lab where he would develop his research interests and make connections with scientists around the world. He was accepted into the neuroscience PhD program at the University of California, Riverside and will start there in the Fall of 2023.