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Sex differences in calbindin-D28K expressing cells in the brains of progesterone receptor knock out mice

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**Introduction:**

Sex differences in adult behaviors exist in almost all mammalian species. For example, males are typically more aggressive compared to females, whereas females often display more parental care than males. In humans, there are significant sex biases in the diagnosis of several psychiatric and neurological disorders. Males are more likely to be diagnosed early in life with developmental cognitive disorders such as Attention Deficit Hyperactivity Disorder (ADHD), autism, and dyslexia in comparison to females (Loke, 2015). Understanding the neural mechanisms underlying these disorders is crucial for our comprehension of how sex differences arise in the developing brain and the etiology of these disorders and why they affect males and females differently.

Differential exposure to gonadal hormones, such as androgens and estrogens, during development can alter the fundamental processes of neural development resulting in sex differences in adult behavior (Lonstein, 2001). Sex steroid hormones activate specific nuclear receptors, which then act as transcription factors to alter the developing brain. When an outside stimulus initiates an endocrine gland to produce a steroid hormone, the hormone is secreted into the bloodstream where it is met by a transport protein and carried throughout the circulatory system. Being lipophilic, steroid hormones can pass through membranes and eventually reach target cells located most often times inside nucleuses. Each steroid hormone will activate its own specific nuclear receptor, which will then create a receptor-hormone complex, which will then bind to DNA and affects gene transcription. Therefore steroid hormones can affect what genes are turned on, or expressed (Tetel, 2009).

Sex differences displayed due to the exposure of steroid hormones is evident in as early as in utero. During gestation, specifically embryonic days (E16-20), male testes produce
testosterone (Ward, 2003). In the brain, testosterone is aromatized to estradiol via the enzyme aromatase. Estradiol through the activation of estrogen receptor α, then induces progesterone receptor (PR) expression in the developing brain (Wagner, 2001). There is a dramatic sex difference displayed between the amounts of PR that is expressed during perinatal life. Males have higher levels of nuclear progesterone receptor expression in the medial preoptic nucleus (MPN) from E18 through at least postnatal day 10 (P10), representing one of the earliest and largest sex differences in the developing brain (Quadros, 2002) (Figure 1). It is not until P14 that the female ovary starts to secrete estradiol during the second week of life and the female MPN starts expressing PR (Quadros & Wagner, 2008). The expression of PR in males, but not females creates a developmental window during which the male brain is more sensitive to progesterone than the female brain, which could result in sex differences in the development of this region.

Based on the previous findings in our lab, we hypothesize that PR expression may play an integral role in the sexual maturation and differentiation of the MPN within the developing brain. In order to examine the importance of PR in development, progesterone receptor knockout (PRKO) mice, along with their wildtype (WT) counterparts, can be used as models to assess PR’s influence throughout development. Due to the insertional null mutation in the PR gene promoter, PRKO mice are unable to express the PR gene, and cannot transcribe either isoform of PRA or PRB throughout life (Lydon, 1995). As PRKO mice lack consistent PR expression from the moment of conception throughout adulthood, they are a superb model to assess how PR might exert its actions throughout to development to influence sexual differentiation.

The sexually dimorphic nucleus of the preoptic area (SDN-POA) is located inside the MPN, and is an area often studied to understand sexual differentiation of the brain. Since mice do not have a SDN-POA, the calcium-binding protein calbindin D-28K can be used as a...
biomarker for sexual differentiation in this area. In normal development with gonadal steroid modulation, a sexually dimorphic group of calbindin-immunoreactive (CALB-ir) cells within the SDN-POA develop within mice and can be called the calbindin-immunoreactive sexually dimorphic nucleus (CALB-SDN) (Sickel, 2000). Calbindin D-28K can be used as a marker to assess sexual differentiation within this area, because CALB-ir cell counts between P4 and P8 of male mice are seen to have a larger volume (Sickel, 2000). In addition, males exhibit more overall CALB-ir cells within the MPN compared to females (Gilmore, 2012). Finally, the distribution and quantity of cells expressing calbindin-D28K is sexually dimorphic in the adult mouse MPN (Wittmann, 2013). Therefore by counting the amount of CALB-ir cells and understanding their distribution in the MPN of mice, we can mark sexual differentiation within the developing brain.

Previously in our lab, we examined CALB-ir cells in the MPN of adult male and female PRKO mice and their WT counterparts in adulthood. Our lab observed a significant sex by genotype interaction in the distribution and cell numbers present in the MPN, although a lack of consistent anatomically matched sections led to three continuations of this study. In the first study, we compared the number and distribution of CALB-ir cells of adult male WT and PRKO mice in order to confirm that the genotypic interaction occurring previously was real. In order to understand if PR was actually influencing development, females were excluded from the study to limit the amount of variables present. Females express PR at different periods throughout development in comparison to males, due to the developing ovary secreting steroids later in life (Wagner, 1998). In our second study, we compared the CALB-ir cells of adult male WT and PRKO mice using sagittal sections as opposed to coronal sections. It is difficult to discern whether PR exerts its actions during development to influence sexual differentiation, so in a third
study we assessed development by comparing the CALB-ir cells of P10 WT and PRKO male and female mice.

**Hypothesis:**

Progesterone receptor expression during development is essential for the proper sexual differentiation of the medial preoptic nucleus.

**Methods:**

*Animals:*

All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee at the University at Albany. Mice in this study were of a mixed background (129SvEv X C57Bl6J), and were either homozygous for an insertional mutation in the PR gene (PRKO) or homozygous for the wildtype PR gene (WT). Heterozygous (HZ) males and females were used as breeders to generate animals of each genotype. PRKO mice were created by the insertion of a neomycin resistance gene and a lacZ reporter gene into Exon 1, preventing PR transcription of both PR isoforms (PRA / PRB) (Lydon et al., 1995). Animals were housed at the Life Sciences building at the University at Albany on a reverse 12-h light, 12-h dark cycle at a constant temperature of $25 \pm 2^\circ C$, with food and water available *ad libitum*.

*Genotyping*

Tail samples were collected at the time of euthanization and placed in labeled eppendorfs. Tissue was first lysed using 180 µl of ATL buffer and 20 µl of proteinase K and then placed in a hot water bath overnight at 55°C. Genomic DNA was purified and amplified using the Qiagen...
DNeasy Blood and Tissue kit, specifically rinses of AL buffer and ETOH, AW1, AW2, and finally the elution AE buffer before storage for amplification via polymerized chain reaction (PCR). To amplify the DNA, PCR was performed on purified DNA using three separate primers for PR and neomycin (PR: 5’- TAGACAGTCTTACTCGTTGTTG-3’; 5’-GATGGGCACATGGATGAAAC-3’; Neomycin: 5’-GCATGCTCCAGACTGCTTTGGAAA-3’).

Once PCR was complete, using gel electrophoresis, genotype was determined by the molecular weight of the extracted PCR product. On a pre-prepared 2.2% agarose gel the dyed samples were run in a presence of a molecular standard for ~5 minutes at 250V. The gel was then visualized on the Bio-Rad ChemiDoc MP imager under UV illumination and analyzed using the program Quantity One. The bands present on the gel for each sample were then analyzed, and an appropriate genotype was assigned to each animal (Figure 7).

**Tissue Collection:**

Male and female WT and KO tissue was collected for immunocytochemistry. Groups of male and female both PRKO and WT were used for these experiments (N 8-12 per group). For the adult confirmation studies, brain tissue was collected at P60-90. For the developmental study, brain tissue was collected at P10. Animals were anesthetized using Euthanasia III, brains collected, and immersed in 5% acrolein 0.1 M phosphate buffer for 6 hours to achieve fixation. The brains were then transferred into a 30% sucrose in 0.1 M phosphate buffer until sectioning. Brains were either coronally or sagitally sectioned on a freezing microtome at 50 µm, and stored at -20°C until immunocytochemical processing.

**Immunocytochemistry:**
Samples containing the MPN were placed in designated wells and rinsed 3 times with tris buffered saline (TBS) (5 mins each), incubated in 1% sodium borohydride in TBS for 10 mins, followed by 6 rinses in TBS to remove the sodium borohydride solution remaining within the wells. Sections were then blocked in 20% normal goat serum (NGS), 1% hydrogen peroxide (H₂O₂), TBS, and 1% bovine serum antibody (BSA) for 30 mins. This solution was added into each well to stop all non-specific binding from occurring when the primary antibody was added. Sections were then incubated in the primary antibody mouse monoclonal anti-calbindin-D28K antiserum at a 1:5000 dilution for a 24 hour period in tris triton X and goat (TTG) buffer, in order to induce a greater state of permeability. After primary incubation, the samples were incubated in a bioinylated goat anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) at a concentration of 250µg/ml, once again the presence of TTG. The samples were then incubated in avidin-biotin complex (ABC) solution for 90 minutes to allow it to bind to the secondary antibodies. The samples were then exposed to a nickle-enhanced diaminobenzede in TBS for a period of 3 minutes and 45 seconds to create visible chromogen staining (Figure 7). Sections were then rinsed 7 times (5 mins each) in TBS and mounted on gelatin covered slides.

Mounting and Coverslipping:

Once the ICCs were complete, the sections were mounted on the slides in anatomical order. The brains were then dehydrated and cleared in xylene. The first solution was 100% distilled water for 3 minutes, followed by 4 solutions of ethanol and distilled water at the different concentrations of 50%, 70%, 80%, and 95%. The slides soaked in the solutions for 5 minutes each, and then were incubated in 100% ethanol twice for 5 minutes. The slides were
then incubated in Hemo D twice for 5 minutes. Once removed, the slides were coverslipped using Permount (Fisher Scientific, Pittsburgh, PA, USA) and dried for at least 3 days.

**Data Analysis:**

The number of CALB-ir cells were quantified within a 3x3 grid (each grid box 100µm²) using a brightfield illumination microscope (Figure 7). The total number of immune-reactive cells were compared across groups using two-way ANOVA (sex x genotype) followed by Student Newman-Keuls post hoc analysis for multiple comparisons (p<0.05). During the first study conducted, we compared the total number of CALB-ir cells counted on the 3x3 grid. The middle box was placed over the highest accumulation of CALB-ir cells in the MPN in line with the third ventricle (Figure 5 A). The cell counts were then compared across groups using two-way ANOVA (genotype x grid #), where through Student Newman-Keuls post hoc analysis a significant genotype by grid number interaction was found when examining cells within the MPN. Grid cells 3, 4, 5, and 7 had a significant genotypic effect (Figure 5 B). An outlier was present in this study to make the results less significant, although a Wilcoxon Outlier test is underway to determine that this outlier is indeed a statistical outlier and can be removed from the study. Data analysis and method of analysis for the second study is still underway. For our third study the total number of CALB-ir cells in the MPN was compared across all P10 WT and KO females and males (Figure 6 A). In addition, a two-way ANOVA (sex x grid #5) was conducted. A significant sex interaction was found to take place in the center grid box (grid #5) with males having a higher CALB-ir cell count in comparison to females (Figure 6 B).
Results:

Through analysis of cell counts in experiment 1, WTs displayed increased CALB-ir clustering, and CALB-ir cell counts in comparison to PRKO males, illustrating how PR’s influence can be observed into adulthood (Figure 2). WT adult males exhibited CALB-ir cell clustering one anatomical section caudal in comparison to KO adult males (Figure 3). In addition, there was a significant genotypic effect (genotype x grid # interaction) illustrated in adult WT male MPN CALB-ir cell clustering and distribution with grid numbers 3, 4, 5, and 7 (Figure 5 B). Furthermore, WT adult males exhibited a higher number of CALB-ir cells per grid box.

In experiment 2, sagittal sections allowed for a more definite determination of CALB-ir cell clustering and rostral or caudal distribution in PRKO animals. The sagittal sections revealed how WT and PRKO adult males did not have CALB-ir cells in the same neuroanatomical location (Figure 4). Also, CALB-ir cells were seen to be more densely clustered in the WTs in comparison to the KOs. Analysis for this experiment is still underway.

In experiment 3, WT and KO males and females displayed no statistically significant main effect of sex or main effect of genotype. Furthermore, there was no significant interaction between sex and genotype (Figure 6 A). There was a significant main effect of sex on CALB-ir cell number in the center grid (grid #5) (Figure 6 B) (Figure 5 B).

Discussion:

Sexual differentiation of the brain is influenced by steroid hormones, and these hormones are what help establish fundamental processes for proper neural development. Steroid hormones influence the processes of neurogenesis, neuronal migration, connectivity, phenotypic
differentiation, and developmental cell death, and can ultimately alter what genes are expressed or turned on in any of these developmental processes (Chung, 2013). Through these mechanisms, steroid hormones can act on the brain to eventually alter adult behavior (Lonstein, 2001). My study confirms how the steroid hormone progesterone, and specifically its receptor, may play an integral role in the development of sexual differentiation within the brain throughout adulthood, and may be one mechanism by which the MPN is sexually differentiated. Figuring out how these steroid hormones fully affect the brain is imperative to our understanding of the mechanisms underlying many sex-based neurological disorders numerous people face today, and why they affect males and females differently.

My study proposes that PR may be a mechanism that sexually differentiates the brain later than P10 in development, such as during puberty. We originally used P10 as a day to attest CALB-ir in development for multiple reasons. At P2, CALB-ir cells are faintly present in male and female mice. At P4 there is no sexual dimorphism in the amount of CALB-ir cells present between both males and females. Apoptosis begins in MPN at P5, and is present more heavily in females. At P7 apoptosis is at its highest levels in females, where it eventually decreases by P12. In addition, from P8 to P26, males have a 2-4 fold larger volume of CALB-ir cells within the MPN (Sickel, 2000). Therefore studying CALB-ir cell counts and distribution at P10 when apoptosis is taking place in females, was a strong benchmark in determining if PR is influencing sexual differentiation throughout development and its effects on the developing brain. Finding no significant genotypic effect in experiment 3 promotes future studies involving ontogeny in determining where exactly a sex and or genotypic effect could take place with CALB-ir cells.

Finally, my experiments affirm calbindin-D28K as a successful biomarker for assessing sexual differentiation in the adult mouse brain due to the results found from experiment 1. This
could promote future experiments involving colocalization with calbindin-D28K and PR. Colocalization of both calbindin-D28K and PR cells could reveal if calbindin-D28K could be a marker of PR, and therefore provide more information on how the male and female brain sexually differentiates.
Figures:

1. Sex differences displayed in PR-ir cells of rats on the day of birth between males and females (Wagner, 1998).
2. Calbindin-ir cells in the MPN of adult male WT and PRKO mice (Right hemispheres)
3. Calbindin-ir cells in the MPN of adult male WT and PRKO mice (Coronal Sections)
4. Calbindin-ir cells in the MPN of adult male WT and PRKO mice (Sagittal Sections)
5. Figure
   a. Data Analysis: Calbindin-ir cell number and distribution box method
   b. Significant genotype x grid number interaction of calbindin-ir cells in the MPN of adult male WT mice
6. Figure
   a. Total Cell # of Calbindin-ir cells in the MPN of P10 male and female WT and PRKO mice
   b. Total Cell # of Calbindin-ir in P10 male and female WT and PRKO mice with the center grid box (*p=0.04)
7. Picture representing methods
Figure 1:

Male

Female

(Wagner, Nakayama, & DeVries, 1998)
Figure 2:

WT

KO

(Sarenski, unpub data)
Figure 3:

Rostral

Caudal

WT

KO
Figure 4:

WT       KO
Figure 5:
A.)
B.)
Figure 6:

A.)

B.)
Figure 7:
References:


