TIMING IS EVERYTHING: HORMONAL CONTROL OF THE DEVELOPING SPINAL NUCLEUS OF THE BULBOCAVERNOSUS (SNB) IN MICE

By

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PUBLIC ABSTRACT

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Hormones are responsible for much of our development and normal day to day functioning. From muscle growth, to aging, and our sleep wake cycle, hormone act throughout our body to maintain homeostasis. Testosterone is a hormone found in much higher concentrations in males than females, and thus acts predominately to promote “male-typical” development. Two conditions that arise when this signaling doesn’t function properly are androgen insensitivity syndrome (AIS) and congenital adrenal hyperplasia (CAH). In the case of AIS, a person produces normal amounts of testosterone, however, tissues, such as muscle or neurons, do not respond appropriately. As a result, a person may be genetically male, but show physical traits of a female. Conversely, CAH results in the body producing either too much or too little of a desired hormone. This can lead to abnormal development and infertility. In both cases, these developmental challenges can lead to difficulties throughout life. As a result, it is important to study how testosterone acts to promote development in order to understand how we can treat cases when something goes wrong. Here, I looked at the importance of when testosterone acts to promote normal masculinization. I show that cell number and cell size are influenced by testosterone at different times immediately after birth, suggesting they are controlled in different ways. This is useful going forward, as it gives us a better idea of when hormones are influencing development.

ABSTRACT

TIMING IS EVERYTHING: HORMONAL CONTROL OF THE DEVELOPING SPINAL NUCLEUS OF THE BULBOCAVERNOSUS (SNB) IN MICE

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In rats there is a sensitive period for the masculinization of soma size in motoneurons of the spinal nucleus of the bulbocavernosus (SNB), which extends beyond the sensitive period for masculinization of SNB cell number. The current study aimed to assess such sensitive periods in the SNB of mice. Female mice were exposed to testosterone propionate (TP) or vehicle during one of three potential sensitive periods of development; late embryonic (E 16-18), early postnatal (PN 1, 3, 5), or late postnatal (PN 7, 9, 11). Testosterone (T) was then provided to the females in adulthood via Silastic capsules to half of the animals in each group to serve as “activational” background from which organizational effects of the perinatal hormones could be assessed. Early neonatal TP treatment significantly increased the adult number of SNB motoneurons in females, and also increased the size of both the somata and nuclei of the motoneurons in adulthood. As expected, adult T treatment also significantly increased the size of SNB somata and nuclei in all groups, but not the number of SNB cells. There was no significant interaction of perinatal and adult hormone treatment on cell number, soma size, or nuclear size. These results begin to define the sensitive periods in which androgens masculinize the SNB system in mice, where genetic tools are available to perturb mechanisms of androgen action on SNB development.

This thesis is dedicated to everyone who has supported me on my journey to this point. Thank you to Anna for all your support and encouragement. Thank you to my parents, all of my family, my friends, and everyone else who has helped get me to where I am today.

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KEY TO ABBREVIATIONS

AIS Androgen Insensitivity Syndrome

CAH Congenital Adrenal Hyperplasia

SNB Spinal Nucleus of the Bulbocavernosus

TP Testosterone Propionate

T Testosterone

BC Bulbocavernosus

LA Levator Ani

AR Androgen Receptor

Tfm Testicular Feminization Mutant

CHAPTER 1

INTRODUCTION

The spinal nucleus of the bulbocavernosus (SNB) is a group of sexually dimorphic motoneurons in the lower lumbar spinal cord of both rats (S M Breedlove & Arnold, 1980) and mice (Zuloaga, Morris, Monks, Breedlove, & Jordan, 2007). These motoneurons innervate the bulbocavernosus (BC) and levator ani (LA) striated perineal muscles, which act to control penile reflexes important for male copulatory behaviors (Sachs, 1982). Initially shown to accumulate radiolabeled testosterone or its metabolites, SNB cells are more numerous in males than females, and this sex difference can be manipulated by altering neonatal, but not adult, androgen treatment (S. Marc Breedlove, Jacobson, Gorski, & Arnold, 1982; S M Breedlove & Arnold, 1981). Further, in genetically male rats with an inactivating mutation of the androgen receptor, the number of SNB cells is similar to that seen in females. This finding led to the conclusion that "the sexually dimorphic nature of the SNB depends on neither the adult hormone state nor the presence of a Y chromosome, but on the interaction of androgens with their receptors early in development" (S M Breedlove & Arnold, 1981).

In order to affect the developing animal, hormones such as androgens must not only be present, but they must be present when the animal is sensitive to any changes in hormone levels. This organizational effect of hormones usually occurs during a short critical period around the time of birth and acts to permanently alter the behavior or

neuroendocrine functioning of the animal (PHOENIX, GOY, GERALL, & YOUNG, 1959). Studies examining how hormones affect development can only be useful if we first understand when during development the hormones are effective, thus guiding experimental design. In the case of the SNB system, the hormone of interest is testosterone (T), acting via the androgen receptor (AR). Masculinization of the rodent brain is traditionally believed to be mediated by aromatized metabolites of T (T is converted to 17-β estradiol via aromatase, which then acts on estrogen receptors (Naftolin, Ryan, Davies, Petro, & Kuhn, 1975)). However, work with testicular feminization mutant (Tfm) rodents and the AR antagonist, flutamide, show that T can also act directly on ARs to promote masculinization of certain brain regions (Zuloaga, Puts, Jordan, & Breedlove, 2008). Similarly, sexual differentiation of the SNB is regulated by androgens acting via the AR (Swift-Gallant & Monks, 2017). Therefore, the SNB system is ideal to study the timing of androgens' effects as androgens, and androgens alone, are required for system survival. Given this circumstance, studying SNB system survival in females treated with testosterone at varying potential critical periods can tell us when testosterone is required for maintenance of the motoneurons, as the lack of T in females early in life normally leads to the cell death that is responsible for establishing a sex difference in SNB morphology (Nordeen, Nordeen, Sengelaub, & Arnold, 1985; Sengelaub & Forger, 2008).

In addition to their presence within the motoneurons in the central nervous system and skeletal muscle in the periphery, androgen receptors have been found in other tissue types, suggesting additional potential sites of action. Using immunocyctochemistry and

reverse transcriptase-PCR, AR protein and mRNA has been shown in the sciatic nerve of adult rats (Jordan, Price, & Handa, 2002). More specifically, endoneurial and perineurial fibroblasts showed AR staining, whereas epineurial fibroblasts showed no signs of AR staining. Finally, at least within the sciatic nerve, Schwann cells do not seem to be a potential site of action, as they showed no specific AR stain, further suggesting they are not the source of AR mRNA (Magnaghi et al., 1999). Androgen receptor has also been observed in the prostate of male rats (Prins, Birch, & Greene, 1991). Using immunohistochemistry, luminal epithelial cells demonstrated strong AR positive staining, whereas basal epithelial cells were AR negative. Taken together, these studies remind us to consider the potential effects of direct T action on tissue types beyond SNB motoneurons and BC-LA skeletal muscle. This is an important caveat to keep in mind as research moves toward knockout lines to assess the importance T-AR interactions in different tissue types.

The historical understanding of testosterone's mechanism of action involves T entering the cell, being converted to dihydrotestosterone via 5α-reductase, and binding with AR in the cytoplasm. Following phosphorylation and dissociation from heat-shock proteins, the AR then translocates to the nucleus where it dimerizes, binds to DNA, and can alter gene transcription and translation (Gottlieb, Lombroso, Beitel, & Trifiro, 2005). This was sufficient when we believed T only acted on AR around the cell soma. As cell types in the periphery have been shown to be AR positive, there may be an additional mechanism of action for how T exerts its affects without translocation to the nucleus. One way this may occur is by activating MAPK signaling cascades that modulate

intracellular calcium levels (Heinlein & Chang, 2002). In addition to occurring in tissue-types lacking functional AR, these cascades lead to changes that occur too rapidly to involve gene transcription, further indicating a nongenomic effect. Further, physiological levels of T added to Sertoli cells can induce mitogen-activated protein kinase signaling pathways that lead to spermatogenesis within Sertoli cells (Fix, Jordan, Cano, & Walker, 2004), again indicating T can act on the AR in non-genomic ways.

In rats, adult hormone manipulation has varying effects on SNB cell number and cell size. Whereas adult hormone manipulation has no effect on the number of SNB cells, the size of these same neurons is sensitive to changes in hormone levels in adulthood, thus suggesting these two measures of SNB masculinity are regulated by different mechanisms (S. Breedlove & Arnold, 1983). These reports show that the critical period for the organizational masculinization of SNB cell size extends beyond the critical period for the masculinization of SNB cell number, indicating that, indeed, critical periods of development exist and also that these hormone-dependent changes do not all occur within the same window.

While these critical periods are established in rats, most genetic models today are more readily available in mice. Thus, it is worthwhile to investigate whether similar critical periods of development exist in mice, and if so, when those periods are. For example, Cre/loxP technology can be used to render the *AR* gene dysfunctional in a desired tissue type and/or at a particular point in development. Studying the resulting changes by disrupting the AR gene in different tissues could allow researchers to draw

conclusions about the importance of androgens and the AR in that type of tissue. However, if the critical period of development occurs before the disruption of AR in those tissues, such studies could be misleading.

MATERIALS AND METHODS

Adult wild-type C57BL6J male and female mice from Jackson Laboratories were housed in a temperature-controlled vivarium with a 12/12 light/dark cycle with food and water available *ad libitum*. All procedures were conducted in compliance with NIH guidelines and approved by the Michigan State University IACUC.

For breeding, a male was placed into a cage housing two females for no longer than 24 hours. During the next 14 days, the females were gently handled daily. Embryonic day 0 (E0) was defined as the day the parental animals were paired. After two weeks, pregnant dams were readily identified and randomly assigned to one of four treatment groups. Pups were exposed to testosterone propionate (TP) during one of three developmental periods: prenatal exposure (E16-18), early neonatal exposure (postnatal (PN) days 1, 3, and 5), or late neonatal exposure (PN 7, 9, and 11), or control animals given only sesame oil vehicle. Prenatal injection days (E16-18) were selected to provide maximum time to develop before treatment, while minimizing stress on the dam before birth. Early and late postnatal injection periods were selected based on known critical periods in rat androgen sensitivity. Prenatal hormone treatment was delivered by subcutaneous injections of the pregnant dam with 0.5 mg of TP in 50 ul sesame oil vehicle, daily. Postnatally, 0.1 mg of TP in 25 ul vehicle was injected subcutaneously to all pups in a litter on alternate days. These dosages were reduced from the 2mg TP (prenatal) and 1mg TP (postnatal) given to rats in order to account for the size difference between the species. 25 ul vehicle was determined to be the maximum

volume injectable into a newborn pup to avoid leakage. To control for the stress of handling/injections, all animals received an oil injection each day, with TP present only as defined by the treatment groups. Prenatal TP treatment hindered pup survival, so those pups were cross-fostered on PN day 1, the first light period after birth. Any cross-fostered female siblings were placed into different adult treatment groups in order to minimize the potential confounding effect of cross-fostering. All pups were weaned on PN day 21 and group-housed with same sex siblings (2-4/cage). On PN day 68, the female mice were anesthetized via isoflurane inhalation and subcutaneously implanted with a Silastic capsule (1.57mm inner diameter, 3.18mm outer diameter, effective release length of 6 mm, total tube length of 16mm) containing nothing (blank) or free T, to provide an "activational" background from which the organizational effects of perinatal hormones could be detected. Following capsule implant, all mice were treated with 0.1 mL ketoprofen (1 mg/mL) analgesic and monitored daily for 10 days. The Silastic capsule served to control the testosterone release rate, ensuring stable delivery throughout the duration of treatment.

Thirty days after capsule implants, animals were sacrificed with sodium pentobarbital and perfused intracardially with saline followed by buffered formalin. The spinal cord from lumbar segment 4 through sacral segment 1 was removed and postfixed in buffered formalin for at least 30 days. Spinal cords were trimmed and frozen-sectioned in the transverse plane at 30 um thickness and alternate sections were mounted on gel-subbed microscope glass slides, then Nissl-stained with thionine for SNB motoneuron number and size measurements. Animal weight (g) was recorded just prior to sacrifice.

Anogenital distance (AGD) was measured as the distance (mm) between the anus and the penis/vaginal opening, where a longer AGD is considered more masculinized. Blood was collected at sacrifice for the plasma testosterone (nmol/L) assay.

Cell counts were conducted prior to size measurements in order to allow for the distribution of SNB cells to be visualized. Once counts were complete, 20 SNB cells distributed throughout the rostrocaudal extent of SNB cells were selected for soma and nuclear size analysis. In order to be selected, a cell had to exhibit a clear nuclear profile. Cell soma and nuclei were traced using MetaVue software, and data were exported to Microsoft Excel to calculate averages within an animal.

Sample sizes for each of the measured variables was as follows (Perinatal Treatment+Adult Treatment- N). *Body Weight*; Control+Blank- 10, Control+T- 10, Prenatal+Blank- 7, Prenatal+T 9, Early Postnatal+Blank- 10, Early Postnatal+T- 14, Late Postnatal+Blank- 15, Late Postnatal+T- 8. *Anogenital Distance*; Control+Blank- 10, Control+T- 10, Prenatal+Blank- 6, Prenatal+T 9, Early Postnatal+Blank- 10, Early Postnatal+T- 14, Late Postnatal+Blank- 15, Late Postnatal+T- 8. *Plasma Testosterone*; Control+Blank- 8, Control+T- 7, Prenatal+Blank- 5, Prenatal+T 7, Early Postnatal+Blank- 8, Early Postnatal+T- 8, Late Postnatal+Blank- 10, Late Postnatal+T- 5. *SNB Cell Number*; Control+Blank- 9, Control+T- 9, Prenatal+Blank- 7, Prenatal+T 8, Early Postnatal+Blank- 9, Early Postnatal+T- 13, Late Postnatal+Blank- 13, Late Postnatal+T- 7. *Cellular Size*; Control+Blank- 9, Control+T- 9, Prenatal+Blank- 7, Prenatal+T 8, Early Postnatal+Blank- 9, Early Postnatal+T- 13, Late Postnatal+Blank-

14, Late Postnatal+T- 7. *Nuclear Size*; Control+Blank- 9, Control+T- 9, Prenatal+Blank- 7, Prenatal+T 8, Early Postnatal+Blank- 9, Early Postnatal+T- 13, Late Postnatal+Blank- 14, Late Postnatal+T- 7. (Table 1).

Statistical analysis consisted of 2-way Analysis of Variance (ANOVA) with factors of perinatal treatment (prenatal, early postnatal, and late postnatal) and adult treatment (testosterone and blank). Significant main effects of perinatal treatment were further analyzed using Bonferroni's post hoc tests. All graphs and/or tables present the mean ± SEM based on N = the number of animals in the group. Significance was considered at $p<0.05$.

RESULTS

Body Weight. Adult testosterone treatment significantly increased body weight ($F(1,82)=15.66$, $p<0.0001$), (Blank, 22.21 g $\pm$ 0.24 (SEM), Testosterone, 23.54 $\pm$ 0.24). In addition, early postnatal TP-treated females weighed significantly more at sacrifice than all other perinatal treatment groups ($F(3,82)=14.96$, $p<0.0001$). There was no interaction between the perinatal and adult treatments on body weight ($F(3,82=1.25$, $p=0.296$). (Table 2).

Anogenital Distance. Adult testosterone treatment slightly lengthened anogenital distance ($F(1,81)=5.98$, $p=0.017$), (Blank, 4.23 mm $\pm$ 0.12, Testosterone, 4.67 $\pm$ 0.12). Prenatal TP treated females had a more masculine anogenital distance at sacrifice than all other perinatal treatment groups ($F(3,81)=15.15$, $p<0.0001$). There was no interaction between the two factors ($F(3,81)=1.185$, $p=0.321$). (Table 3).

Plasma Testosterone Levels. As expected, females given testosterone capsules in adulthood had significantly higher plasma levels of the hormone than females given blank capsules, independent of perinatal treatment ($F(1,57)=68.33$, $p<0.0001$), (Blank, 5.58 nmo/L $\pm$ 1.42, Testosterone, 22.63 $\pm$ 1.5). There was no effect of perinatal treatment on plasma T concentrations ($F(3,57)=0.50$, $p=0.683$), nor any interaction between the two factors ($F(3,57=1.91$, $p=0.14$). (Figure 1).

Konigsmark-adjusted SNB Cell Number. As previously reported, SNB cell number was unaffected by adult hormone treatment ($F(1,74)=0.052$, $p=0.820$), (Blank, 42.61 ± 3.26, Testosterone, 43.67 ± 3.31). However, TP treatment during the early postnatal period masculinized SNB cell number, compared to control females ($F(3,74)=4.975$, $p=0.004$). There was no interaction between the two factors ($F(3,74)=1.455$, $p=0.235$). (Figure 2).

Cellular Size. As previously reported, adult testosterone treatment increased SNB cell size, independent of perinatal treatment ($F(1,75)=26.446$, $p<0.0001$), (Blank, 395.79 ± 13.04, Testosterone, 491.69 ± 13.33). In addition, TP treatment during the early postnatal period also increased cell size, as compared to control females ($F(3,75)=4.342$, $p=0.007$). There was no interaction between the two factors ($F(3,75)=1.588$, $p=0.200$). (Figure 3).

Nuclear Size. Adult testosterone treatment also increased the size of the nuclei of SNB cells, independent of perinatal treatment, compared to blank treated females ($F(1,75)=16.678$, $p<0.001$), (Blank, 124.49 ± 4.69, Testosterone, 151.86 ± 4.79). There was a marginally significant main effect of perinatal treatment ($F(3,73)=2.576$, $p=0.061$), but no interaction between the two factors ($F(3,75)=0.633$, $p=0.596$). (Figure 4).

DISCUSSION

Here I present evidence that there are unique critical periods for different aspects of masculinization of the SNB system in mice. The number of SNB cells can be masculinized by early postnatal TP treatment, however, this critical period is over around PN day 7, as late postnatal TP treatment of female mice yields no difference in cell number, compared to control. In addition, SNB number is not affected by adult testosterone treatment in mice, suggesting that this aspect of the SNB system is determined within the first week of life. SNB cell size is also masculinized by TP during the early postnatal period, however, this variable continues to remain sensitive to testosterone into adulthood, as adult females treated with testosterone for a month before sacrifice had larger SNB cells than animals given blank capsules. This sensitivity to testosterone in adulthood suggests that the mechanism of action for cell size is different than that for cell number. While the size of SNB motoneuronal nuclei can still be enlarged by adult testosterone treatment, this variable was unaffected by perinatal treatment, suggesting nuclear and cell size masculinization may be controlled in different ways during the first weeks of development. It should be noted, however, that the effect of perinatal treatment on nuclear size was just under the level of significance ($p=0.061$), and therefore it is possible that larger sample sizes would have revealed an effect.

Maternal behavior can have an effect on SNB system survival. In particular, changes in pup licking behavior can lead to changes in SNB cell number, size, and dendritic length,

with decreases in licking leading to decreases in variable (Lenz & Sengelaub, 2006; Moore, Dou, & Juraska, 1992). Further, cross-fostering has been shown to increase pup licking (Van Der Veen, Abrous, Ronald De Kloet, Piazza, & Koehl, 2008). If decreased pup licking leads to fewer and smaller SNB cells, it is reasonable to ask if increased licking can lead to more and larger SNB cells. If so, one could argue that pups prenatally treated with TP and then cross-fostered would show an increase in SNB cell number and size due to cross-fostering alone. There may even be evidence of this in our analysis, as prenatally TP-treated females that received blank capsules in adulthood had increased SNB cell numbers, similar to those of females treated with TP neonatally. While cross-fostering only 2 out of our 8 groups was not ideal, it was deemed necessary to achieve our desired group numbers. While it is possible this may have affected our findings, it is likely our handling/injection schedule would have masked any effect of cross-fostering simply due to the increased handling required to inject pups. That said, in order to eliminate this variable in any future studies, it would be advised to cross-foster all pups.

As previously shown in rats, the sensitive period for the masculinization of soma size in motoneurons extends beyond the sensitive period for masculinization of SNB cell number, suggesting these variables are controlled by separate mechanisms. The goal of this project was to examine if similar critical periods of development exist in mice, and if so, when those periods are. I have shown that androgen exposure masculinizes cell number and soma size when given during the same early postnatal window, however, only cell and nuclear sizes are sensitive to the activation dose of androgens given in

adulthood, thus suggesting different mechanisms regulate the development of these aspects of the SNB system, as seen in rats. In addition, I provide new evidence that soma size and nuclear size may be masculinized by different mechanisms, as perinatal androgen treatment was able to masculinize soma, but not nuclear, size.

In addition to furthering our understanding of the mechanisms involved in masculinization of the nervous system, this study also provides useful information on when the critical period windows are open. I found that masculinization of the SNB system extends after birth, and is complete by PN day 7. These data are important to remember when working with genetic tools that alter the function of the androgen receptor. For example, these results indicate that Cre/loxP-induced AR dysfunction must occur before birth if it is to effectively prevent androgens from masculinizing the SNB system.

APPENDICES

APPENDIX A:

Tables

	Body Weight	Anogenital Distance	Plasma T	Cell Number	Cell Size	Nuclear Size
Control + B	10	10	8	9	9	9
Control + T	10	10	7	9	9	9
Prenatal + B	7	6	5	7	7	7
Prenatal + T	9	9	7	8	8	8
Early Postnatal + B	10	10	8	9	9	9
Early Postnatal + T	14	14	8	13	13	13
Late Postnatal + B	15	15	10	13	14	14
Late Postnatal + T	8	8	5	7	7	7

Table 1: Samples sizes for each of the measured variables. Y-axis labeled as Perinatal Treatment + Adult Treatment.

	Blank	Testosterone
Control	21.68 ± 0.47	22.25 ± 0.47
Prenatal	20.79 ± 0.56	22.84 ± 0.5
Early Postnatal	23.59 ± 0.47	25.49 ± 0.4
Late Postnatal	22.77 ± 0.38	23.56 ± 0.53

Table 2: Body weights. Average body weight (g) at sacrifice presented as (mean ± SEM).

	Blank	Testosterone
Control	3.97 ± 0.23	4.02 ± 0.23
Prenatal	5.23 ± 0.3	5.82 ± 0.24
Early Postnatal	3.87 ± 0.23	4.67 ± 0.2
Late Postnatal	3.97 ± 0.19	4.17 ± 0.26

Table 3: Anogenital distances. Average anogenital distances (mm) at sacrifice presented as (mean ± SEM).

APPENDIX B:

Figures

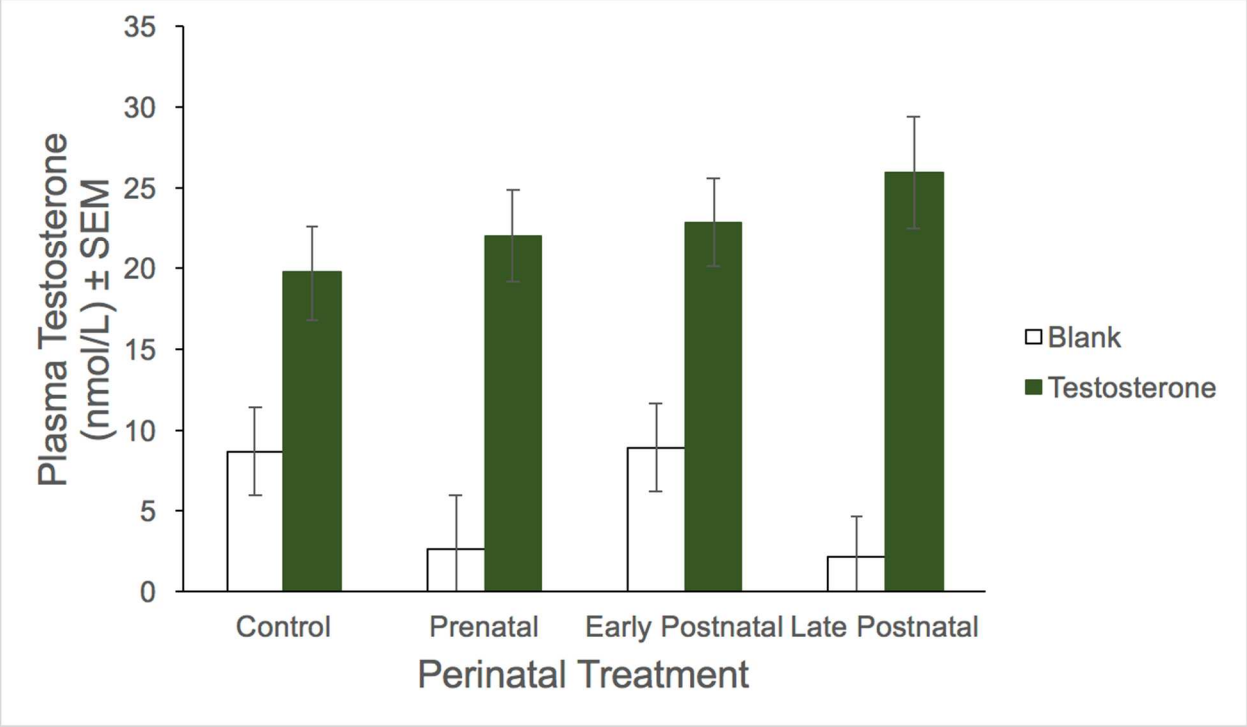

Figure 1: Plasma testosterone levels. Females treated with Testosterone in adulthood had significantly higher levels of plasma testosterone than those given Blank capsules, independent of perinatal treatment ($p<0.0001$). There was no significant effect of perinatal treatment on this measure ($p=0.366$) and no interaction between the two factors ($p=0.157$).

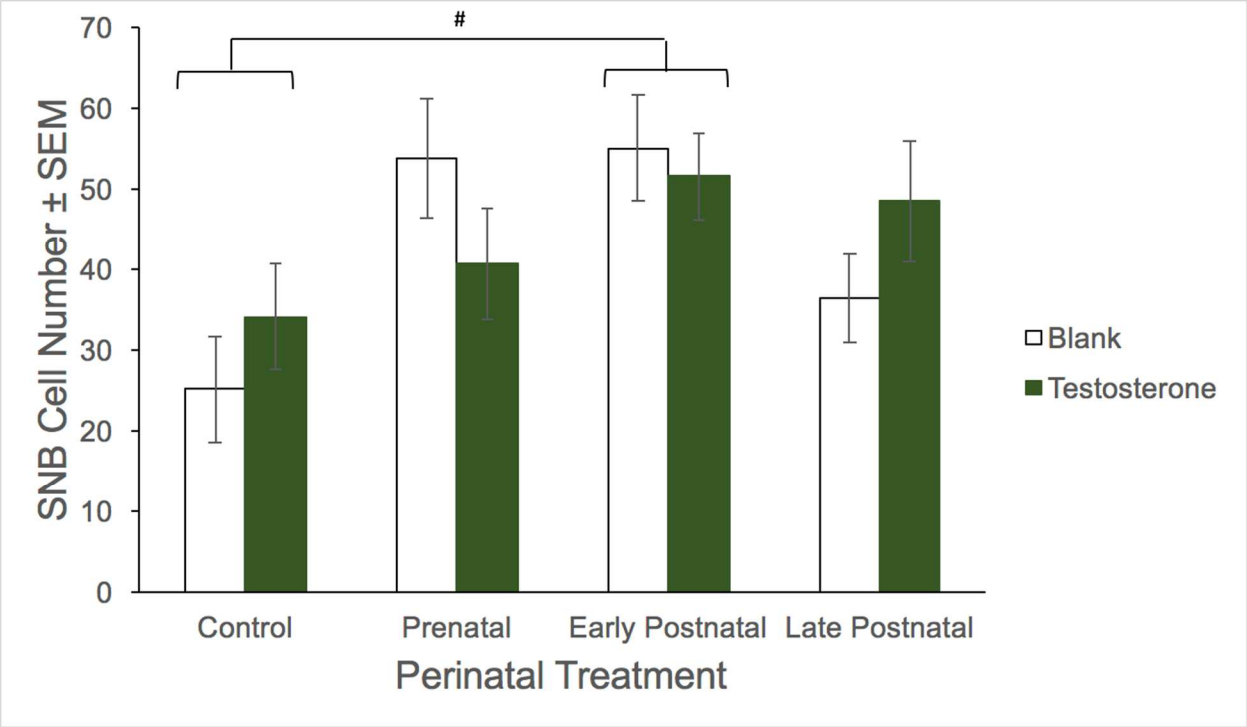

Figure 2: Average number of SNB cells. There was no significant main effect of adult hormone treatment on SNB cell number (p=0.820). Females given TP during the Early Postnatal period had significantly more SNB cells than females in the Control group (p=0.04). There was no interaction between the two factors (p=0.321).

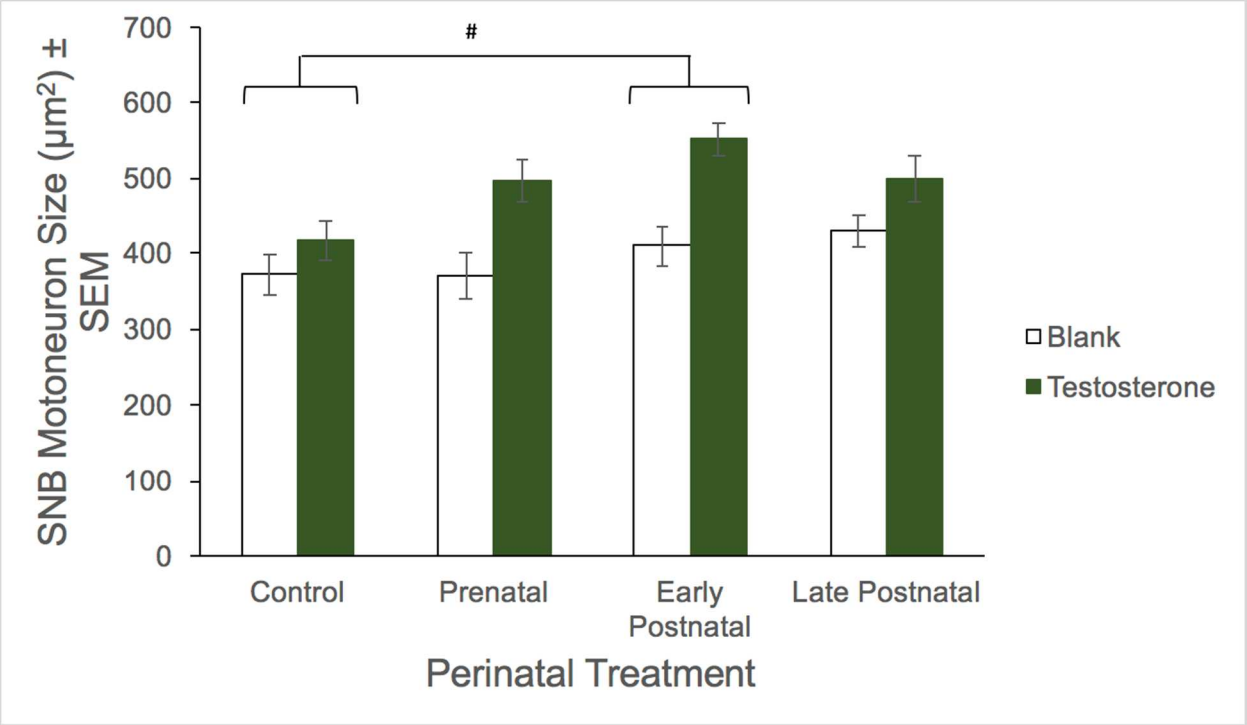

Figure 3: Average cell sizes. Females treated with Testosterone in adulthood had significantly larger SNB motoneuron cell bodies than those given Blank capsules, independent of perinatal treatment ($p<0.0001$). In addition, females given TP during the Early Postnatal period had significantly larger SNB cells than females in the Control group ($p=0.007$). There was no interaction between the two factors ($p=0.200$).

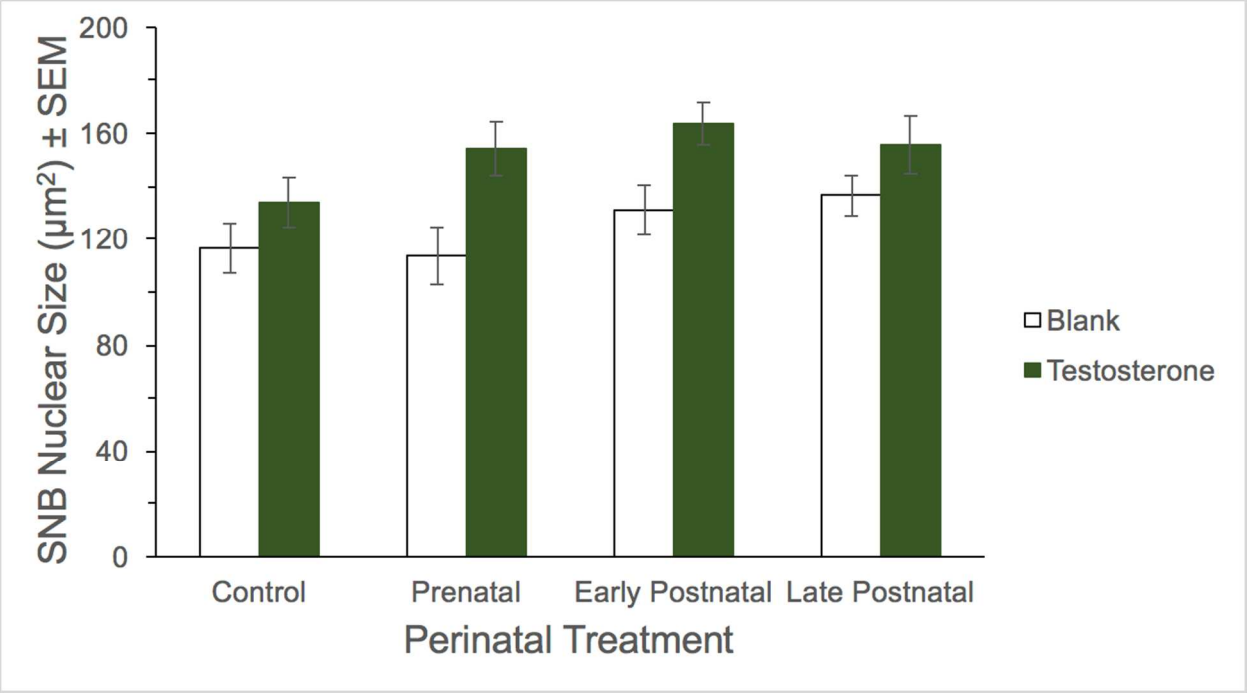

Figure 4: Average nuclear sizes. Females treated with Testosterone in adulthood had a significantly larger cell nuclei in SNB motoneurons than those given Blank capsules, independent of perinatal treatment ($p<0.001$). The effect of perinatal treatment was just under the level of significance ($p=0.061$). There was no interaction between the two factors ($p=0.596$).

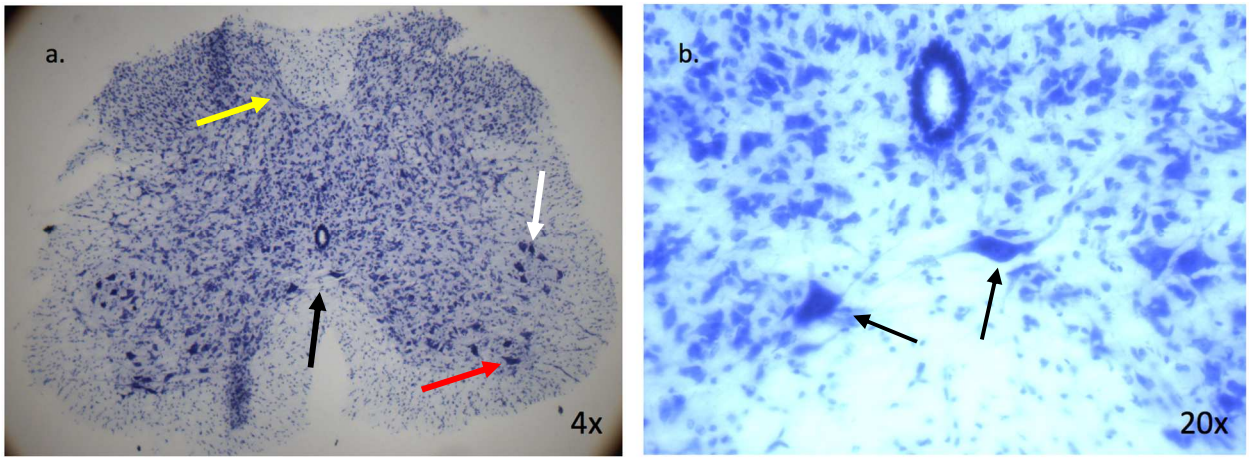

Figure 5: Thionine stained section and SNB cells. (a) Representative image of a thionine-stained lumbar spinal cord section showing the location of the SNB (black arrow), DLN (red arrow), and RDLN (white arrow), along the depth of the "U" the white matter (yellow arrow) makes on the dorsal side of the central canal in the region of the SNB. (b) Arrows indicate two SNB cells found just ventral to the central canal of the same slice shown in (a).

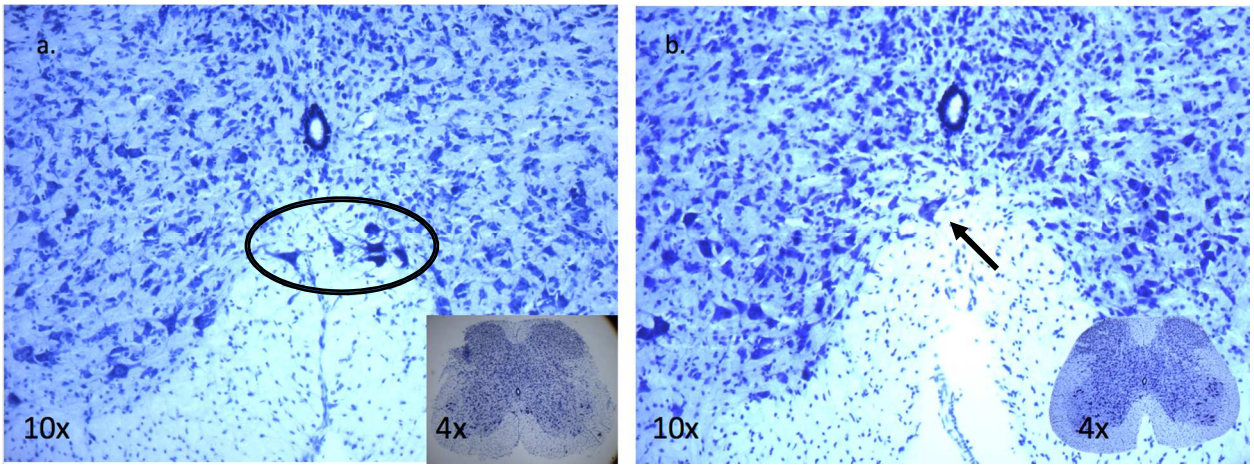

Figure 6: The difference between a section with many SNB cells and a section with 1 SNB cell. (a) Shows a section with a cluster of SNB cells (circled) located in the white matter just ventral of the central canal. (b) Shows a section with only 1 SNB cell. Inserts show that representative sections from similar regions of the spinal cord, given the similar depth of the white matter "U".

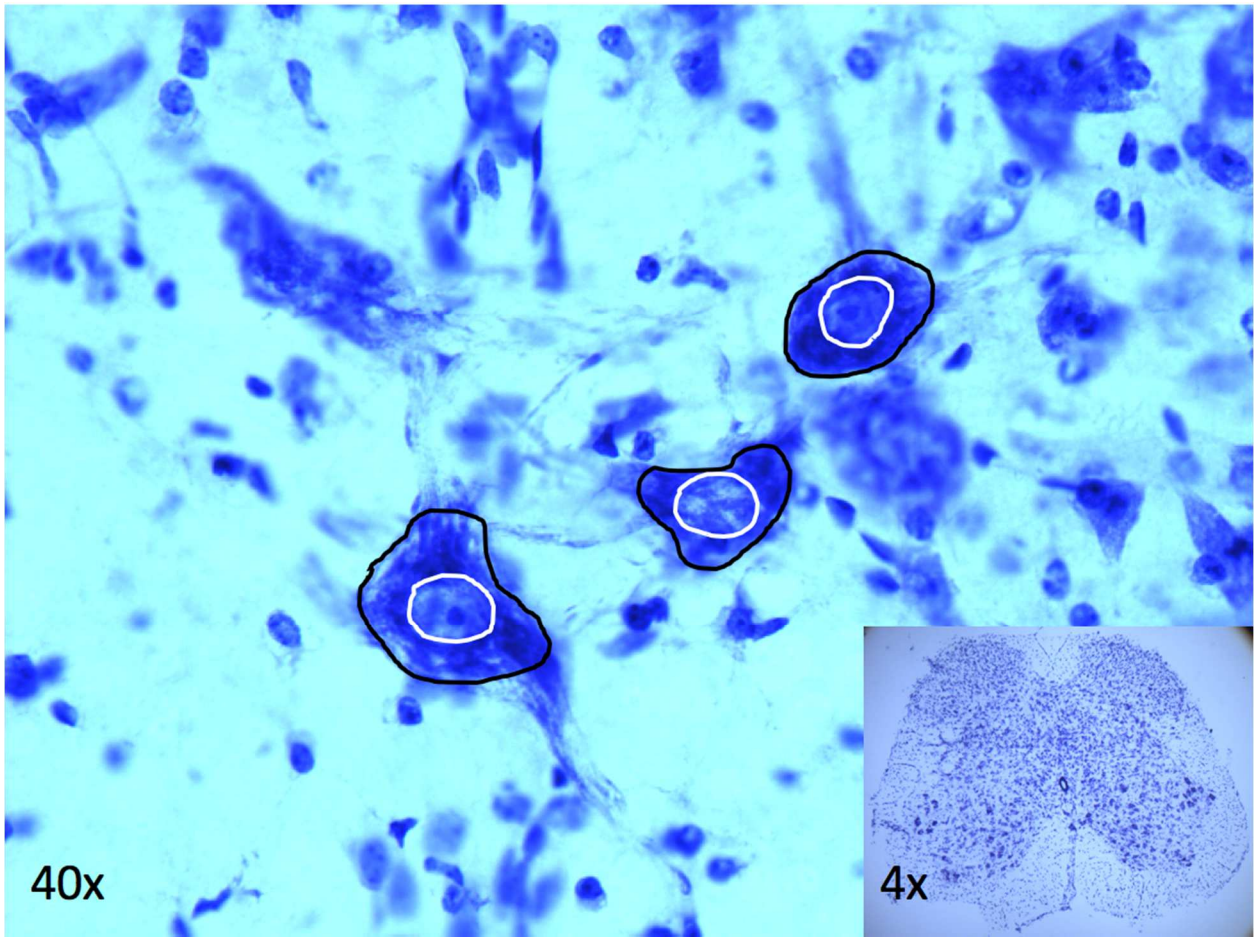

Figure 7: Examples of traced SNB cell bodies and nuclei. Cell body and nuclei sizes were measured by tracing the outline of the cell body (black trace) and nuclei (white trace) of 20 cells/animal, distributed throughout the length of the spinal cord section containing SNB cells.

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