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Sex Differences and Organizational Effects of Androgen in Spinal Cord Motor Nuclei

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This article describes a laboratory module taught at UCLA and offers digitized microscope images that will allow instructors to recreate this module at their home institutions with only a computer required. This module allows for 1) an exploration of the effects of hormones on neural development, 2) the demonstration of sex differences in the nervous system, 3) the production of robust and statistically significant data by novice undergraduates, 4) the discussion of sophisticated statistical analyses (ANOVAs with significant main effects and an interaction), and 5) the understanding of at least

some of the neuroanatomy of the spinal cord. Specifically, this module both replicates and extends a previously published experiment on sexually dimorphic neurons in the spinal cord of rats (Grisham et al., 1992), which examined the effect of antiandrogen exposure (Flutamide) *in utero* on sexually dimorphic spinal motoneurons in male and female rats.

Key words: CNS development, sex differences, hormones, spinal nucleus bulbocavernosus, spinal cord, organizational effects

Undergraduate neuroscience laboratories rarely tackle developmental questions in mammals that require time for the animals to mature. Many undergraduate neuroscience laboratories do not have necessary resources to establish breeding colonies, perform histological preparations, and do analyses at a microscopic level. Also, finding laboratory experiences that naïve students can perform and obtain meaningful, statistically significant data are challenging. In order to address these needs, this article describes a module taught at UCLA that invariably produces statistically significant, meaningful data. This module involves blocking androgen receptors in development and examines the consequences on spinal motor neurons. The need for a breeding colony, histological preparation, and microscopes has been removed because this article and its supplement offer a set of digitized microscope images that will enable instructors to recreate the module at their home institution with only a computer (see Appendix A).

Steroid hormones, particularly testosterone and its metabolites (i.e. estradiol and dihydrotestosterone), play two different roles in development of the nervous system: organizational roles and activational roles (cf. Phoenix et al., 1959; Arnold & Gorski, 1984; Nelson, 1999). The organizing actions of steroid hormones happen early in life, during a critical/sensitive developmental period, and are relatively permanent. Activational actions happen later in life, are less closely tied to a given developmental period, and the effects are relatively transient, following the levels of steroids present (c.f. Nelson, 1999, chapter 3, for a review). This laboratory is designed to illustrate the organizational effects of gonadal steroids and the consequent sex differences that they can produce in the nervous system. Specifically, this module replicates the findings of a sex difference in neurons of the spinal nucleus bulbocavernosus (SNB) and the demasculinizing effect of Flutamide, an antiandrogen (Grisham et al., 1992). This module extends previous findings by looking for sex differences and the effects of antiandrogen exposure on

a second motoneuron pool, the retrodorsal lateral nucleus (RDLN).

SNB neurons are large, densely-staining neurons found in the lumbar spinal cord within about 200 microns ventral to the central canal (Figure 1). These motor neurons innervate the muscles of the penis, the bulbocavernosus (BC), and the levator ani (LA) (cf. Breedlove, 1984, for a review), which are sexually dimorphic: males have them but they are absent or vestigial in adult females. SNB neurons, not surprisingly, are larger and more numerous in males than they are in females (Breedlove & Arnold, 1980; Grisham et al., 1992). While this may seem like a trivial finding, the long-held belief was that there were no anatomical sex differences in the nervous system, only physiological sex differences (cf. Phoenix et al., 1959).

SNB neurons are under hormonal control in the course of development. Administration of testosterone in development masculinizes the female SNB (Breedlove, et al., 1982). Although this result suggests that androgens during development are responsible for the sex difference observed in adulthood, it does not necessarily prove that differential androgen circulation during development is necessary for the sex difference to occur. Documented sex differences in androgen levels occur prenatally and may be the source of sex differences in the developing central nervous system (Weisz, & Ward, 1980). The ideal experiment would eliminate the source of androgens by castrating the males *in utero* and examine the result. Since this surgery is a difficult if not impossible procedure, in place of castration, the actions of androgens can be antagonized by Flutamide, a drug that blocks androgen receptors by preventing the binding of androgens.

This module contrasts SNB neurons that innervate a highly sexually dimorphic BC/LA muscle with RDLN neurons, motor neurons that innervate the flexor digitorum brevis (FDB) muscle (Leslie et al., 1991). Although the FDB muscle is slightly different between the sexes (Leslie

probably will not) find sex differences in the number and size of RDLN neurons (cf. Jordan et al., 1982).

MATERIALS AND METHODS

Subjects

The subjects used are 18 males and 18 females, a sub-sample of the Sprague-Dawley rats used in Grisham et al. (1992). Half of the males and half of the females were treated *in utero* by injecting their mothers with a daily dose of 5 mg of Flutamide dissolved in a volume of 0.15 cc propylene glycol across days 11-21 of gestation. The mothers of the other half of the males and females were given injections of 0.15 cc propylene glycol alone.

Apparatus

Given that an instructor obtains the set of images offered (Appendix A), all that is necessary to run this exercise is a computer running some version of National Institute of Health (NIH) Image. Downloads of this program are available at <http://rsb.info.nih.gov/nih-image/> for Mac computers, and a JAVA version (ImageJ) is available for other platforms. A Beta version of Scion Image, another version of NIH Image that runs on Windows machines, is available at the Scion website, <http://www.scioncorp.com/>.

Procedure

Details of the tissue preparation are given in Grisham et al. (1992). Details of the dissection are given in Appendix B. Rats were sacrificed in adulthood at 120 days and perfused with 0.9% saline followed by 10% formalin in saline. The caudal spinal cords, including the lumbosacral enlargement, were dissected and stored in 10% formalin. Twenty-four hours before frozen-sectioning at 50 μ m, they were transferred to a solution of 20% sucrose in formalin, which cryoprotects the tissue by keeping ice crystals small and thereby minimizing tissue damage due to freezing.

Images of the SNB and RDLN neurons for each subject are available as supplemental material along with a spreadsheet with the sex and prenatal treatment for each subject (Appendix A). A calibration file is also provided. The large demarcations on the calibration file are 0.1 mm (100 μ m), the small ones are 0.01 mm (10 μ m). Only every fourth section was saved as a digital image in the interests of keeping supplementary material to a reasonable size. SNB neurons were digitized starting at their caudal-most extent and then sampled at rostral intervals of 200 microns until they were no longer present. RDLN neurons were sampled starting at their caudal-most extent then sampling a section every 200 μ m for five such intervals; after this they become indistinguishable from DLN neurons. Digital images of RDLN neurons were only made on the left side of the spinal cord. In instances where we could not frame all of the SNB or RDLN neurons on a given section in one picture, we provided several images of a given section, say section 6, and in such instances labeled them 6A, 6B, 6C.

Images provided in the supplementary materials (Appendix A) are from sections stained with thionin, which stains the rough endoplasmic reticulum (Nissl substance)

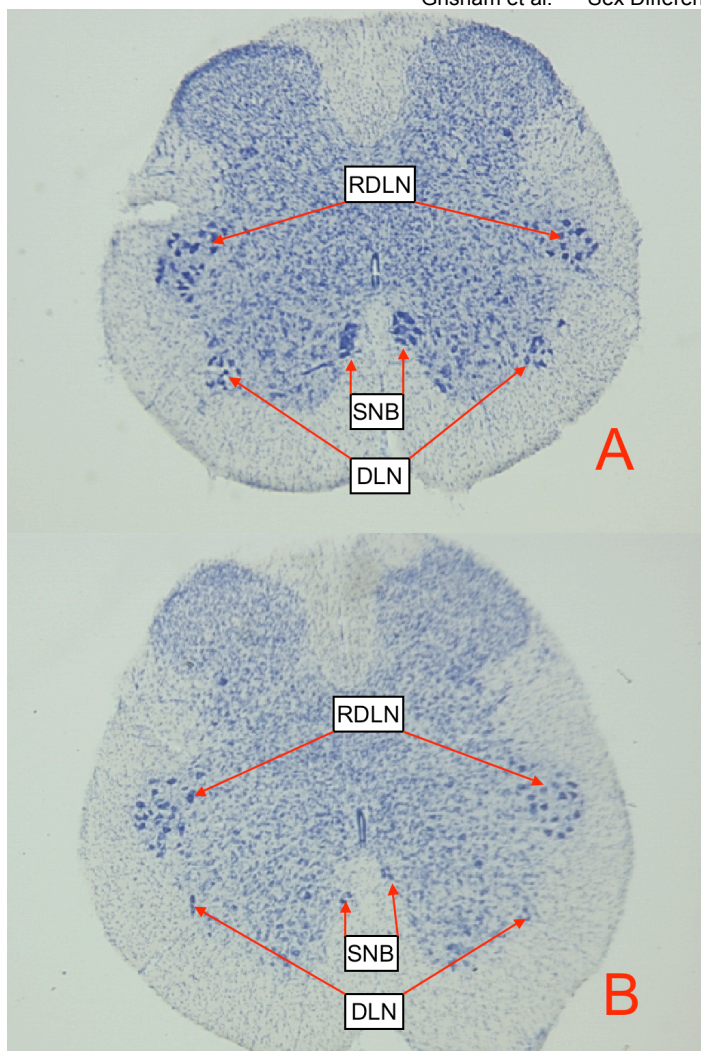


Figure 1. Photomicrograph of spinal cord cross sections. Male is shown in A and female in B. Larger, more darkly staining spots are cell bodies of SNB, DLN, and RDLN neurons as indicated by arrows. Males have larger SNB neurons than females do, and SNB and DLN neurons are more numerous in males.

et al., 1991), it lacks the dramatic sexual dimorphism found in the BC/LA muscles. RDLN neurons are also found in the lumbar region but are directly lateral to the central canal (Figure 1).

The questions for this module are (1) Are the sex differences in SNB neurons replicable? (2) Does the sex difference in androgen levels *in utero* determine the sex difference in adulthood for SNB neurons? (3) Is there a sex difference in RDLN neurons that innervate a muscle that is not extremely different between the sexes? and (4) Do androgens *in utero* influence the development of RDLN neurons? Students compare the spinal cords of both sexes of rats treated during development *in utero* with an antiandrogen, Flutamide, relative to controls. Accordingly, they should find robust sex differences and effects of Flutamide on the number and size of SNB neurons, replicating previous findings (Breedlove & Arnold, 1980; 1983; Grisham et al., 1992), but they may not (and

so that the stain defines the cell bodies but not the axons and dendrites.

Measuring neurons

NIH Image (see above) is ideal for measuring neurons in this task. It is relatively easy to learn and comes with a downloadable manual. Essentially the student needs only to trace the perimeter of a given neuron and then ask the program to calculate the cross-sectional area, which it does by converting pixels according to the calibration selected.

One of the problems in teaching this module will be getting students grasp the distinction between SNB and non-SNB neurons, as well as between RDLN and non-RDLN neurons. In the supplemental materials, both relevant and irrelevant neurons will be found in the same image, as shown in Figures 2 and 3.

As can be seen in Figures 2 and 3, SNB and RDLN neurons are much more darkly stained and tend to be larger than non-SNB or non-RDLN neurons. SNB neurons are very distinct and obviously different from non-SNB neurons in males (Figure 2A); the distinction is more subtle in females (Figure 2B) and Flutamide-treated males because the SNB neurons are so much smaller than in control males, but the discrimination is still easily accomplished. We have endeavored to exclude as many irrelevant neurons as we reasonably can, but instructors should emphasize that students should select neurons that meet the criteria (more darkly staining and usually larger) and that not every neuron in a given image is an SNB or RDLN neuron (Refer to Figures 2 & 3, which are representative of the supplementary materials described in Appendix A). Students should also choose neurons to trace in which the outlines can be distinctly seen and in which they can see the nucleus of the neuron. The former restriction is to avoid confusing two adjacent neurons and interpreting them as one cell; the second restriction is to allow for a cross-sectional area to be taken more or less through the middle of the cell and to avoid measuring cell fragments. Notably, the neurons meeting the criteria that will allow them to be measured will not be a random sample of the neurons of a given nucleus. Though the criteria for tracing clearly precludes random selection, students will often insist that they randomly chose neurons, which provides a good starting point for discussing what random selection means and what basing a sample on a non-random selection can mean.

Despite detailed instructions, students will show wide variation in how they trace neurons. Figure 4 is provided as a guide. There is some subjective element in determining the boundaries, and instructors will find that some students will be wildly generous and some will be extremely stingy. Luckily, the effect sizes in this study are so large in the SNB neurons that even if the inter-student variation contributes a great deal to the variance in the data, some significant effects will likely be found. Nonetheless, this wide variability in tracing perimeters can

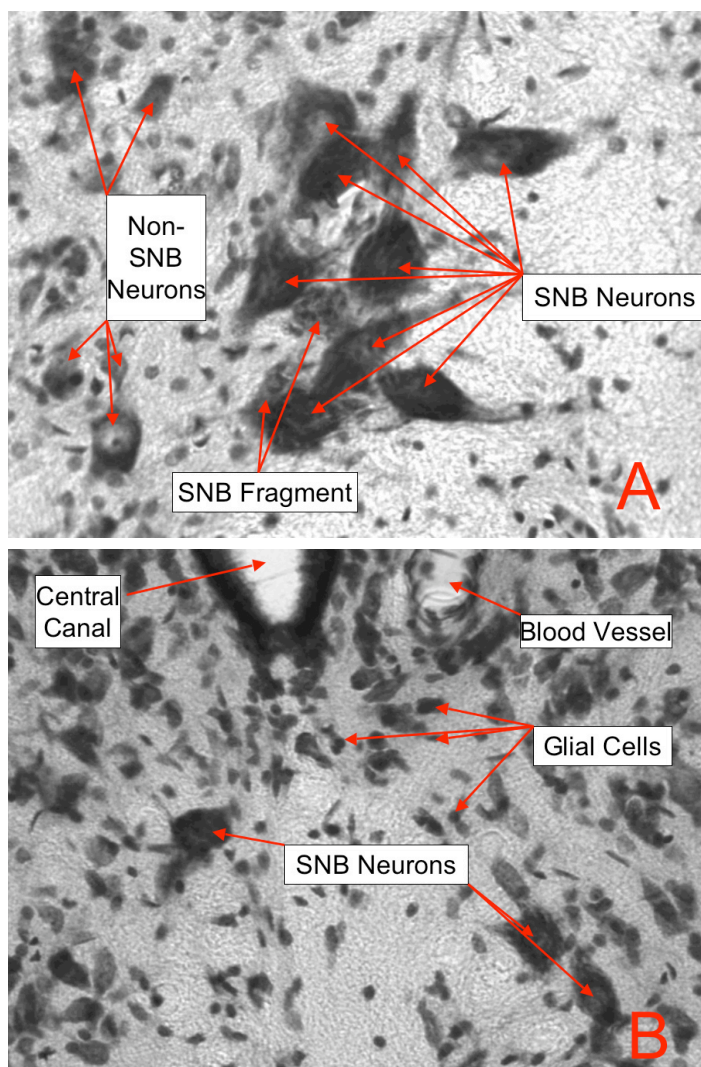


Figure 2. Photomicrograph of SNB Neurons. Control male (A) and control female (B) spinal cord indicating SNB and non-SNB neurons as well as other features that are encountered (glial cells, central canal, blood vessel). All SNB neurons are labeled, but only a few of the non-SNB neurons are labeled. SNB fragments should not be counted or measured.

be a potential source of confounding: if a generously drawing student were to be assigned all of a given treatment group, say control males, then one could not discriminate between the observer characteristics and true differences among groups. From a didactic standpoint, this provides an excellent opportunity to discuss inter-observer variability as a source of error. To avoid confounding by this source of error, we usually assign a given student a rat from each of the four treatment groups, a procedure known as balancing.

Another possible problem can be caused by foreknowledge of the expected outcome of the experiment. Thus, students should remain blind to the treatment group from which their assigned rats came.

Given that the digitized images provide for only a sampling of the neurons for a given individual rat,

instructors may be relegated to using small sample sizes (five cells or even fewer) to determine the mean neuron area for a given individual. Using a greater number of cells will provide for a more accurate mean, decrease the variability among animals, and increase the probability of obtaining a significant result; thus the instructor has another opportunity to discuss a source of variability in the data and the possible consequences.

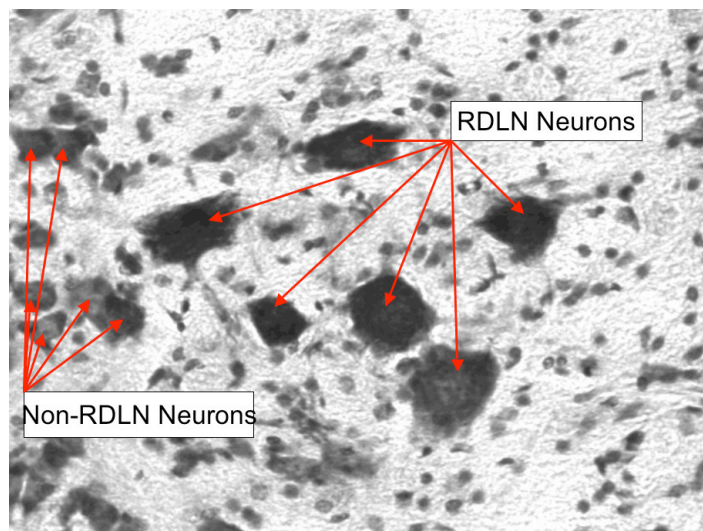


Figure 3. Photomicrograph of RDLN. Photograph from the more caudal region of the RDLN to show distinction between RDLN and non-RDLN neurons. All RDLN neurons indicated and some but not all non-RDLN neurons indicated.

Counting neurons

The biggest problem is operationally defining what will constitute a neuron that will increment the count. All operational definitions will result in an overestimate of the true number of neurons, but the overcounting error is made smaller by counting the smallest sub-cellular structure possible (c.f. Coggeshall, & Lekan, 1996, for a review). Overcounting errors get larger with larger structures, due to the greater likelihood that a larger structure would be split in sectioning. The resolution and section thickness in the digitized images provided require that nuclei be the unit of count, and students should probably increment the count if they see even a hint of a nucleus. (Nuclei have no endoplasmic reticulum and so appear as clear patches inside the cell body—see Figure 4.)

Although the raw counts will provide statistically significant data, those who wish to correct for overcounting may do so using the following formula (Konigsmark, 1970; Breedlove & Arnold, 1981):

$$N' = 4nt / \{t + \text{sqrt}(d^2 + k^2)\}$$

where N' is the corrected count, 4 is the multiplier because we sampled every fourth section, n is the raw count, t is the section thickness (here 50 μm), sqrt is square root, k is the smallest recorded nuclear diameter in the entire study, and

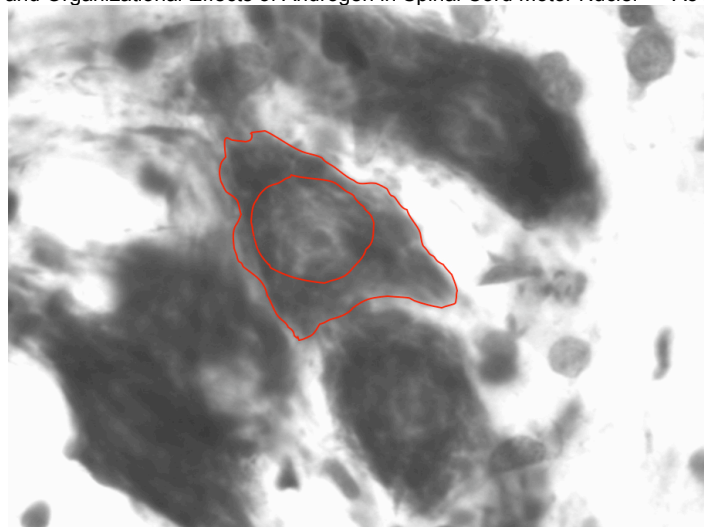


Figure 4. Photomicrograph of SNB Neurons. The cell body of a single SNB neuron and its nucleus (more lightly staining, clear area) are outlined in red. This figure is provided only as an example, the images available in Appendix A are the same magnification as Figures 2 and 3.

d is the mean diameter for a given animal which can be calculated from the mean area by using the formula:

$$d = 2 \text{sqrt}(\text{mean area}/\pi)$$

Correcting for overcounting requires finding the area of the nuclei of neurons (see Figure 4) to correct for the fact that they were split during sectioning. Areas of nuclei can be determined using NIH Image. Correcting for overcounting decreases the sex and treatment differences: because control males have larger nuclei than females and Flutamide-treated males, overcounting is much more likely to occur in control males and the correction factor will then cause their numbers to shrink disproportionately to females or Flutamide-treated males. Thus, it is possible to get significant differences with the raw numbers and to have these significant differences disappear after the correction factors have been applied. (In tests with the images provided in Appendix A, we did not find this happening.)

The dependent variables (SNB counts, RDLN counts, average SNB soma area, average SNB nuclear area, average RDLN area, and average RDLN nuclear area) should be analyzed in a 2 x 2 between subjects ANOVA using sex (male vs. female—a quasi-independent variable) and treatment (Flutamide vs. Control) as factors.

If students have had limited exposure to statistics and have not learned analysis of variance, instructors may consider using multiple t-tests, which can be performed by spreadsheet programs such as Microsoft Excel, to compare the means of the different groups on a given dependent measure. Despite the fact that multiple t-tests would lack the statistical power of an ANOVA and would result in a greater Type I error rate (false positive rate), the group differences are so large that students are likely to find the same pattern of results as when an ANOVA is used.

RESULTS

We have used the digitized images provided in four test runs with UCLA undergraduates, and the pattern of results has been absolutely consistent. Typical data reveal a sex difference in corrected SNB counts ($F(1,32) = 70.42, p < 0.0001$), a main effect for treatment ($F(1,32) = 43.86, p < 0.0001$), and a significant sex x treatment interaction ($F(1,32) = 40.84, p < 0.0001$) (refer to Figure 5A). Post-hoc orthogonal comparisons on the typical data revealed that control males had significantly more SNB neurons than did any other group (all comparisons $p < 0.0001$) but that there were no other significant comparisons among the four groups (all $p > 0.05$).

In our test runs, we consistently found a sex difference in the size of SNB neurons ($F(1,32) = 53.97, p < 0.0001$) but neither an effect of Flutamide ($F(1,32) = 1.88, p > 0.05$) nor a significant sex x treatment interaction ($F(1,32) = 0.17, p > 0.05$; refer to Figure 5B). The nuclear sizes of neurons showed a similar pattern with a significant sex difference ($F(1,32) = 8.01, p < 0.01$) but no treatment effect ($F(1,32) = 0.70, p > 0.05$) and no sex by treatment interaction ($F(1,32) = .50, p > 0.05$; refer to Table 1).

Corrected counts of the RDLN neurons from the digitized images typically revealed no significant differences due to sex ($F(1,32) = 0.47, p > 0.05$), treatment ($F(1,32) = 0.85, p > 0.05$), or sex x treatment interaction ($F(1,32) = 0.19, p > 0.05$; refer to Figure 6A). RDLN soma sizes similarly revealed no significant difference due to sex ($F(1,32) = 0.30, p > 0.05$), treatment ($F(1,32) = 0.44, p > 0.05$), or sex x treatment interaction ($F(1,32) = 0.87, p > 0.05$; refer to Figure 6B). RDLN nuclear sizes showed a similar pattern with no difference due to sex ($F(1,32) = .079, p > 0.05$), treatment ($F(1,32) = 0.78, p > 0.05$), or sex x treatment interaction ($F(1,32) = 0.90, p > 0.05$; refer to Table 1).

DISCUSSION

The SNB corrected counts obtained from the digitized images revealed three robust results, replicating previous findings (Breedlove & Arnold, 1980, 1983; Grisham et al., 1992). First, there was a significant main effect of sex: males had more SNB neurons. Second, there was a significant main effect of treatment: Flutamide-treated animals overall had fewer SNB neurons. Third and most importantly, there was an interaction due to the fact that Flutamide reduces the number of SNB neurons in males but has no significant impact on females.

We ask students to propose reasons for the dramatic decline in the number of SNB neurons in Flutamide-treated males and then to use their data and/or readings to determine the veracity of their conclusions. Typically, students suggest the following: 1) Flutamide could be a neurotoxin; 2) Flutamide could interfere with neural migration during development; 3) Flutamide could cause

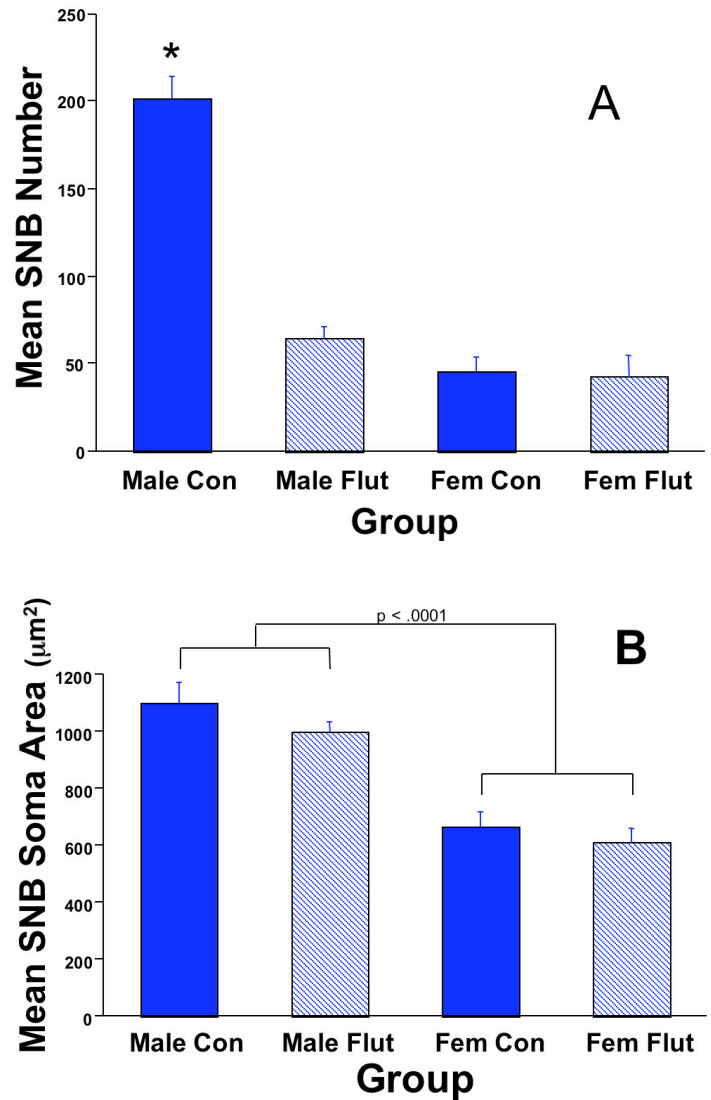


Figure 5. Mean number (A) and size (B) of SNB neurons as a function of group. Solid bars represent controls, hatched bars, Flutamide treated. Male Con = Male Control, Male Flut = Male Flutamide treated, Fem Con = Female Control, Fem Flut = Female Flutamide treated. Asterisk in A indicates different from all other groups, $p < 0.0001$. Brackets in B indicate males significantly different from females. Bars represent SEM.

neurons to differentiate into another, unrecognizable type; 4) Flutamide could prevent the birth of SNB neurons; and/or 5) Flutamide could block androgen action, which causes SNB neurons to die. These suggestions can be dealt with as follows:

1) *Flutamide could be a neurotoxin:* Their own data disallow this conclusion because there is no effect of Flutamide on female SNB neurons. They could then possibly argue that since females have so few SNB neurons, there is a floor effect and so cannot detect any toxicity. Nonetheless, RDLN neurons typically do not show any effect of Flutamide, so toxicity may be ruled out.

2) *Flutamide could interfere with migration during development:* SNB neurons migrate into place during

treated males. Alternatively, autoradiographic methods or *in situ* hybridization for androgen receptor might be used to see whether the same number of neurons is expressing androgen receptor in that region.

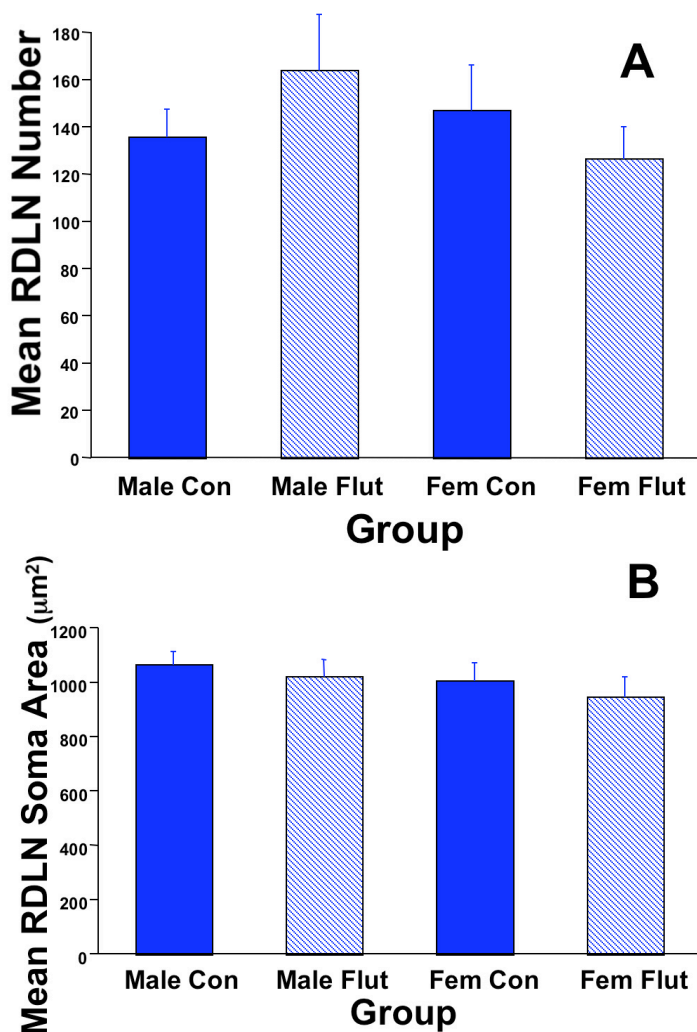


Figure 6. Mean number (A) and size (B) of RDLN neurons as a function of group. All abbreviations are as in Figure 5. Bars represent SEM.

development from the ventral-lateral locus of the dorsolateral nucleus (DLN—see Figure 1) (Sengelau & Arnold, 1986). If Flutamide merely disrupted migration, then we should find a greater number of DLN neurons in Flutamide-treated animals. Nonetheless, we see fewer DLN neurons in Flutamide-treated males (Grisham et al., 1992). This hypothesis could be also tested by injecting a retrograde tracer into the bulbocavernosus (BC) and the levator ani (LA) muscles and seeing whether the cell bodies were ectopically displaced.

3) *Flutamide could cause neurons to differentiate into another, unrecognizable type:* In other words, the SNB neurons are still there but they now look different and so do not stand out in a Nissl stain. Our data cannot directly address this possibility. Nonetheless, students should be urged to think about what experiments could be done to test this hypothesis: a retrograde tracer such as HRP could be applied to the BC/LA muscles to see if the same number of neurons is detectable as in non-Flutamide

	Mean	SEM
SNB Nuclear Area		
Male Control	224.93	34.42
Male Flutamide	199.00	21.77
Female Control	139.32	13.63
Female Flutamide	146.45	23.21
RDLN Nuclear Area		
Male Control	223.10	27.66
Male Flutamide	234.56	27.24
Female Control	218.98	24.30
Female Flutamide	223.65	32.40

Table 1. Mean and standard error of the mean (SEM) for SNB and RDLN nuclear areas. Males overall had larger nuclei in SNB neurons than females. Nuclei of RDLN neurons were not different.

4) *Flutamide could prevent the birth of SNB neurons:* The strongest argument against this possibility is the differential effect that Flutamide has on males and females: if Flutamide prevents the birth of neurons, why would it affect males more than females? Sex differences in steroids are not present until day 18-19 of gestation (Weisz & Ward, 1980), whereas spinal cord neurons are post-mitotic by day 14 of gestation (Breedlove et al., 1983). If Flutamide is acting as an antiandrogen, it seems unlikely that it had differential effects on the sexes before day 18-19.

5) *Flutamide could block androgen action, which causes SNB neurons to die in developing males:* This is the most likely explanation and is consistent with an organizational effect of androgens on this system. We know that SNB neurons die during early development in females and that exogenous androgen rescues these neurons from cell death (Breedlove et al., 1982; Nordeen et al., 1985). Thus, it is completely consistent that blocking androgen action in developing males with Flutamide would lead to the death of SNB neurons in these males. Incidentally, the site of action is probably the sexually dimorphic muscles that the neurons innervate: androgen spares these muscles from involution and the muscles in turn produce trophic factors that promote survival of the SNB neurons (cf Breedlove, 1992, for a review).

Although the main effects of sex, treatment, and the sex x treatment interaction replicate well in terms of SNB number, another more subtle effect did not. Grisham et al. (1992) found that Flutamide did not completely demasculinize SNB neurons in males: Flutamide-treated males still had significantly more SNB neurons than control females. Nonetheless, in every test with the digitized

images, students did not obtain this result. This failure to replicate mostly reflects the fact that when several observers are used, the operational definitions of what should be counted are less consistently applied and the variance in the data will be larger. Other contributing factors to the failure to replicate are the sample size of individuals and using a subset of the animals: Grisham et al. (1992) used sample sizes of up to $n = 16$ per group, whereas it is $n = 9$ per group in the set of digitized images provided. Additionally, Grisham et al. (1992) counted neurons in every section, whereas only every fourth section was digitized in the images provided. Given that the absolute difference between the mean number of SNB neurons in Flutamide-treated males and control females is smaller than the absolute difference between means in the main effects, these smaller differences may be obscured by greater variance or noise in the data.

Similarly, a main effect of sex proved robust in terms of the size of SNB neurons, but students consistently failed to find a main effect of treatment and sex \times treatment interaction in terms of the size of SNB neurons, thus failing to replicate Grisham et al. (1992). Given that there is some subjective nature to determining the boundaries of individual neurons, instructors may see rather large individual differences in how students approach this task. Thus, having several observers will greatly inflate the variance in the data. Nonetheless, instructors can use the inter-observer variability as an example of how increasing the variance in data can obscure effects. In our trials with the digitized images, students always measured five neurons per rat, whereas Grisham et al. (1992) measured ten. Measuring more neurons per animal will decrease the within group variance, and increase the probability of obtaining a statistically significant result. The fact that the sample of images was taken from fewer individuals and only every fourth section was sampled may also contribute to a failure to replicate.

Students found neither sex differences nor effects of Flutamide in the RDLN neurons when using the digitized images. This lack of sex differences in RDLN neurons is consistent with the fact that these neurons innervate a muscle that does not show a dramatic sex difference. We ask students to suggest reasons why we may not have found a significant sex difference or effect of Flutamide on RDLN neurons. Characteristically, the list is (1) the sample size was too small, (2) the dose of Flutamide was insufficient, (3) there was too much variance in the data, and (4) there really are no sex differences or effect of Flutamide in this system. Arguments 1-3 can be addressed by the fact that we obtained positive results in the SNB nucleus, where they were expected. Nonetheless, students could still argue that there are much more subtle differences in the RDLN and, if they had larger sample sizes, larger doses, or smaller variance in the data, they would have detected differences. The argument that there really are no sex differences or effect of Flutamide, while based on negative results, is reasonable. This lack

of a sex difference is consistent with some reports in the literature but not others. Females have been reported to have more RDLN neurons than males in some studies (Jordan et al., 1982, expt 1; Moore et al., 1996), but other studies have not found this sex difference (Jordan et al., 1982, expt 2; Leslie et al., 1991, Zup et al., 2003). Similarly, RDLN neurons have been reported to be larger in males (Leslie et al., 1991) or not different between the sexes (Moore et al., 1996; Zup et al., 2003). The difference between the SNB and RDLN probably lies in the fact that the muscles that the SNB neurons innervate are quite sensitive to androgens and produce trophic substances that sustain neurons in the course of development (cf. Breedlove, 1992), but the muscle innervated by the RDLN is not terribly sensitive to androgens (Leslie et al., 1991).

This article was inspired by discussions with other instructors at the PKAL/FUN conference held at Trinity College in 2001. In these conversations, we came to realize that in altogether too many cases the resources available to those teaching undergraduate neuroscience courses were quite limited, therefore narrowing the scope of topics that could be reasonably addressed in a student laboratory. This article attempts to remedy this situation by offering an interesting research topic: the dramatic impact of steroid hormones on aspects of the nervous system during development. To assist instructors in tackling this question, a set of carefully prepared digitized images has been made available. Since we have done the work to obtain and provide these images, this module can be run with minimal equipment, indeed just a computer. Furthermore, this module reliably generates highly significant, interpretable effects even in the hands of complete novices.

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APPENDIX A

Obtaining the resources

Resources for this module are available from the address below. These resources include images of the SNB and RDLN neurons as TIFF files, a TIFF file for calibrating NIH Image, a spreadsheet indicating sex and treatment group of each individual rat, a sample grading key, and PowerPoint files with figures from this article. The files can be found at www.funjournal.org in the supplementary materials section. If you would prefer a hard copy of the files, simply mail a self-addressed, stamped envelope that is large enough to hold a CD, along with a check for \$10 payable to UC Regents (to cover costs of burning the CD) to:

William Grisham, Ph.D.
Department of Psychology
1285 Franz Hall, Box 951563
Los Angeles, CA 90095-1563.

We will then send a CD with the TIFF file images, calibration file, spreadsheet, sample grading key, and PowerPoint images to you.

APPENDIX B

Tips on preparing spinal cords for those interested

The images provided are quite sufficient for a complete experiment and one would not have to supplement them by providing more tissue. Nonetheless, since sex differences in SNB are so readily obtained, those that have the resources might consider obtaining spinal cords from both sexes of rat and having students obtain data from the tissue that they prepared themselves. The lumbosacral spinal cord is amazingly easy to dissect: after perfusing a rat, simply cut coronally across the spinal column just anterior to the pelvic girdle and similarly across the spinal column just anterior to the second rib. We open the caudal spinal column slightly and insert a P1000 pipette tip that has been placed on a 1 cc syringe that in turn is connected to an air hose. When the air pressure is gently increased, to our continuous amazement the caudal spinal cord pops out of the rostral end almost always intact and often with the cauda equina attached. A sharpened small caliber cotton swab will also work well if one does not have a compressed air source available. Sectioning and mounting spinal cords is exceptionally easy and can be done well even without prior experience as long as thick sections (50 μ m) and a decent quality camel hairbrush are used.

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