Published in final edited form as:

J Chem Neuroanat. 2015 ; 0: 19–27. doi:10.1016/j.jchemneu.2015.04.001.

Localization and Regulation of Reproductive Steroid Receptors in the Raphe Serotonin System of Male Macaques

Cynthia L Bethea, PhD^{1,2,3}, Kenny Phu, BS¹, Yelena Belikova, BS¹, and Sarah C Bethea¹

¹Divisions of Reproductive Sciences, Oregon National Primate Research Center Beaverton, OR 97006

²Neuroscience, Oregon National Primate Research Center Beaverton, OR 97006

³Department of Obstetrics and Gynecology, Oregon Health and Science University Portland, OR 97201

Abstract

We previously showed that tryptophan hydroxylase 2 (TPH2) and serotonin reuptake transporter (SERT) mRNAs are increased by the androgens, testosterone (T) and dihydrotestosterone (DHT) in serotonin neurons of male macaques. In addition, we observed that serotonin in axons of a terminal region were markedly decreased by aromatase inhibition and lack of estradiol (E) from metabolism of T. These observations implicated androgen receptors (AR) and estrogen receptors (ER) in the transduction of steroid hormone actions in serotonin neurons. Due to the longer treatment period employed, the expression of the cognate nuclear receptors was sought. We used single and double immunohistochemistry to quantitate and phenotypically localize AR, ER α and ER β in the dorsal raphe of male macaques. Male Japanese macaques (*Macaca fuscata*) were castrated for 5–7 months and then treated for 3 months with [1] **placebo**, [2] **T**, [3] **DHT** (non-aromatizable androgen) **plus** **ATD** (steroidal aromatase inhibitor), or [4] Flutamide (**FLUT**; androgen antagonist) **plus** **ATD** (n=5/group). After single labeling of each receptor, quantitative image analysis was applied and receptor positive neurons were counted. Double-label of raphe neurons for each receptor plus TPH2 determined whether the receptors were localized in serotonin neurons. There were significantly more AR-positive neurons in T- and DHT+ATD-treated groups (p= 0.0014) compared to placebo or FLUT+ATD-treated groups. There was no difference in the

Correspondence to: Cynthia L Bethea, PhD, Oregon National Primate Research Center, 5005 NW 185th Ave, Beaverton, OR 97006, betheac@ohsu.edu, 503-690-5327 tel, 503-690-5384 fax.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure

Cynthia L Bethea, PhD, has nothing to disclose.

Kenny Phu, BS, has nothing to disclose.

Yelena Belikova, BS, has nothing to disclose.

Sarah C Bethea (undergraduate summer intern) has nothing to disclose.

Ethical Statement

Cynthia L Bethea, PhD, has nothing to disclose.

Kenny Phu, BS, has nothing to disclose.

Yelena Belikova, BS, has nothing to disclose.

Sarah C Bethea (undergraduate summer intern) has nothing to disclose.

number of positive-neurons stained for ER α or ER β . Double-immunohistochemistry revealed that serotonin neurons did not contain AR. Rather, AR-positive nuclei were found in neighboring cells that are likely neurons. However, approximately 40% of dorsal raphe serotonin neurons contained ER α or ER β . In conclusion, the stimulatory effect of androgens on TPH2 and SERT mRNA expression is mediated indirectly by neighboring neurons containing AR. The stimulatory effect of E, derived from T metabolism, on axonal serotonin content transport is partially mediated directly via nuclear ERs.

Keywords

androgen receptors; estrogen receptors; serotonin; male; macaque

Introduction

The presence of a receptor for a particular ligand defines the cell as a direct target for the ligand. With this knowledge, the specific intracellular actions of a ligand can be unraveled. This laboratory has maintained a long-term interest in defining cognate reproductive steroid receptor target neurons in the brain with a focus on the nuclear receptors and their actions as gene transcription factors. Our steroid treatment paradigms in macaques extend a month or more, which is expected to engage nuclear receptors. In addition, each nuclear receptor has a membrane counterpart that mediates rapid actions of steroid hormones (Razandi *et al.*, 1999). Moreover, these rapid actions may initiate and support longer term actions on gene expression (Levin, 2015).

Previously, we demonstrated that serotonin neurons in female macaques express nuclear ER β (Gundlah *et al.*, 2000; Gundlah *et al.*, 2001). Estradiol (E), acting through ER β , *increased* expression of genes encoding progesterone (P) receptors (PR) (Bethea, 1994). E, with or without supplemental P, significantly altered gene expression in multiple pathways that increased serotonin neurotransmission (Pecins-Thompson *et al.*, 1998; Sanchez *et al.*, 2005), proliferation of dendritic spines (Bethea and Reddy, 2010; Rivera and Bethea, 2012), glutamate receptors (Bethea and Reddy, 2012b), synaptogenesis (Bethea and Reddy, 2012a), and neuronal resilience (Bethea and Reddy, 2015).

In serotonin neurons of male macaques, T and dihydrotestosterone (DHT) increased tryptophan hydroxylase 2 (TPH2) and serotonin reuptake transporter (SERT) mRNA expression in a manner that was not affected by aromatase inhibition (Bethea *et al.*, 2014). However, aromatase inhibition markedly decreased detection of serotonin in axons of a terminal region and markedly decreased serotonin/prolactin release in response to fenfluramine challenge (Bethea *et al.*, 2013). In addition, aromatase activity and metabolism of T to E suppressed MAO-A mRNA expression, which correlated with serotonin metabolites in cerebral spinal fluid (CSF; Phu *et al.*, 2014). Therefore, we surmised that the metabolites of T acted on different compartments of the serotonin system, and that both androgen receptors (AR) and estrogen receptors (ER) might be involved in the regulation of serotonin in males. However, definition of reproductive nuclear steroid receptors in

serotonin neurons of the male macaque raphe nucleus was lacking. This study examines the regulation and co-localization of AR, ER α and ER β in serotonin neurons of male macaques.

Materials and Methods

This experiment was approved by the IACUC of the Oregon National Primate Research Center and conducted in accordance with the 2011 Eight Edition of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Male Japanese macaques (*Macaca fuscata*) were utilized for study.

Troop

The Japanese macaques were born and raised in a 2-acre outdoor corral at ONPRC with approximately 300 individuals. The troop has been the subject of extensive behavioral studies since it arrived at ONPRC in 1965 (Eaton, 1974; Eaton *et al.*, 1990). The troop composition is relatively stable and the age structure is comparable to that of a natural troop (Maruhashi, 1982). Like other macaque species, the hierarchical organization of the troop is along matriarchal lineages. The matriarchal lines and dominance hierarchies within the troop are well documented, and have remained stable for the past 50 years. In the wild, males normally leave the natal troop and so their dominance is less a function of their mother's status and more a function of their age, size and social skills. Males cannot leave our troop on their own so in that respect, there are more males than a natural troop although they are removed for research and sales with attention to troop stability.

Study Animals and Treatments

The animals used in this study were the same animals used in previous studies as quoted below (Bethea *et al.*, 2014; Bethea *et al.*, 2013). Twenty adult male Japanese macaques were assigned to this project, castrated and housed as previously described (Bethea *et al.*, 2013). The experimental period was conducted for 3 months during the mating season when aggression is highest amongst males in the troop. Although the animals were housed indoors, their annual rhythms continue to be manifested (Rostal *et al.*, 1986). In year 1, half of the animals were treated with placebo or testosterone (**T**) (n=5/group) and then euthanized. In year 2, the remaining animals were treated with dihydrotestosterone (**DHT**) + aromatase inhibitor (**ATD**) or an androgen antagonist (**Flutamide; FLUT**) + **ATD** (n=5/group) and then euthanized. The **T** treatment achieved serum **T** concentrations previously reported in intact Japanese macaques (Eaton and Resko, 1974; Rostal *et al.*, 1986). Dosing and inhibition of brain aromatase with ATD administration were also previously reported in macaques (Ellinwood *et al.*, 1984). The dose of **DHT** significantly elevated DHT ~20-fold over the normal concentration observed in intact or T treated macaques (Bethea *et al.*, 2013).

The rationale for the different treatment groups was explained in detail previously (Bethea *et al.*, 2014). Briefly, **T** treatment exposed the brain to DHT via 5 α reductase metabolism and to E via aromatase metabolism, yielding high androgen receptor (AR) & high estrogen receptor (ER) activation. The **placebo** group would have little or no activity at AR, but independent *de novo* production of neural E from cholesterol could remain, yielding low AR & some ER activation (Mukai *et al.*, 2006; Tsutsui, 2012). The **DHT+ATD** group would

have significant androgen activity with ~90% inhibition of aromatase (Ellinwood *et al.*, 1984), yielding high AR and no ER activation. It was previously reported that ATD nonspecifically activated androgen receptors (AR) in castrated macaques (Resko *et al.*, 1993). Therefore, the **FLUT+ADT** group was expected to have no androgen activity and inhibition of aromatase, yielding no AR and no ER activation. Please see Bethea et al., 2014 for a diagrammatic summary of the treatments and their effects.

Blood samples were obtained for immunoassay of T, DHT, and E to verify treatment efficacy and the results were previously reported (Bethea *et al.*, 2014; Bethea *et al.*, 2013).

Weights, Ages, Ranks, Behavior

Each animal was weighted before treatment and at intervals during treatment. The ages were known from exact birth dates obtained from daily observations of the corral by ONPRC technicians. The dominance ranks were determined from win-loss recordings during focal observations after the animals were placed into group housing. The win-loss results have been published [supplement, (Bethea *et al.*, 2013)]. Focal observations using Observer Software (Noldus, Netherlands) were routinely conducted on all animals prior to a fenfluramine challenge and euthanasia. The results have been published (Bethea *et al.*, 2013).

Euthanasia

Monkeys were euthanized at the end of the treatment periods for collection of the brain and other tissues according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association and performed by an expert veterinary pathologist. They were transported to the necropsy suite under sedation and a CSF sample was obtained via puncture of the cisterna magna. They were then administered pentobarbital (30 mg/kg, i.v., Hospira, Lake Forest, IL) and exsanguinated with severance of the descending aorta.

Tissue Preparation

Perfusion, fixation and preparation of the brain has been previously described in detail (Bethea *et al.*, 2014). The midbrain blocks containing the dorsal raphe were cut on a sliding microtome at 25 μ m, and half of the sections were processed and stored at -80°C for *in situ* hybridization (ISH) assays. The rest of the sections were stored at -20°C in cryoprotectant for subsequent immunohistochemical (IHC) assays as utilized in this study.

Steroid Receptor Immunohistochemistry (IHC)

Midbrain sections containing the dorsal raphe were removed from -20°C storage in cryoprotectant buffer and washed in 0.2 M potassium phosphate buffered saline (KPBS) buffer 4 times for 15 min each (rinsed), immersed in 1% hydrogen peroxide in KPBS for 30 min, rinsed, and incubated for 60 min with normal serum diluted in KPBS based upon the secondary antibody used in each assay as follows: Vector normal horse serum (NHS; Vector Laboratories, Burlingame, CA) for AR, or Vector normal goat serum (NGS) for ER α , or Vector NHS for ER β . Normal serum was followed by Vector avidin for 20 min and then Vector biotin for 20 min. Sections were incubated for 24 h at 4°C in rabbit anti-human AR (SC-816, Santa Cruz, Dallas, Texas) or rabbit anti-human ER α (C1355, Cat #06-935,

Millipore, Billerica, Massachusetts) or mouse anti-human ER β (GTX70174, GeneTex, Irvine, California) diluted in 2% normal serum corresponding to the secondary antibody species, 0.02 M KPBS, and 0.4% Triton. The antibody to AR was used at a concentration of 1/400; the antibody to ER α was used at a concentration of 1/1000; and the antibody to ER β was used at a concentration of 1/400. The antibodies were characterized across a range of titers with positive and negative controls. Sections were then rinsed, and incubated for 60 min in Vector biotinylated anti-rabbit IgG (horse anti-rabbit serum), or anti- goat IgG (goat anti-rabbit serum) or anti-mouse IgG (horse anti-mouse serum) diluted in 0.02 M KPBS and 0.4% Triton at 1/500. The sections were rinsed, incubated with Vector ABC reagent, rinsed, incubated with 0.05% diaminobenzidine (Vector DAB kit) containing 3% hydrogen peroxide for 1–10 min and lastly rinsed. Sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific Inc., Waltham, MA) and dried overnight under vacuum. The sections were further dehydrated through a graded series of ethanols and xylene. The sections were finally mounted under glass with DPX.

Double immunostaining for TPH and steroid receptors

To determine the localization of the steroid receptors, double immunostaining was conducted. Tryptophan hydroxylase (TPH), the rate-limiting enzyme in serotonin synthesis, was used as the marker for serotonin neurons. The antibody against TPH cannot distinguish the 2 forms of TPH. However, in the brain, it is predominantly binding TPH2. Each antibody was processed as described above, with 1-hour formalin fixation between the steroid receptor reactions and the TPH reactions. All blocking and development processes were the same as in the single ICC protocol described above.

To double label AR + TPH, rabbit anti-human AR antibody (SC-816; Santa Cruz) was applied at a concentration of 1/400 followed by biotinylated horse anti-rabbit serum (Vector BA-1100; 1/500). The conjugate was developed with Vector ABC kit and Vector Nickel-DAB kit, yielding black nuclear staining of AR. Afterwards the sections were fixed in 4% formaldehyde for 1 hour and rinsed. Then, goat anti-TPH antibody (Sigma-Aldrich SAB2501377) was applied at a concentration of 1/1000 followed by biotinylated rabbit anti-goat (Vector BA-5000; 1/500). The conjugate was developed with Vector ABC reagents and Vector DAB kit, yielding brown cytoplasmic staining.

To double label ER α + TPH, rabbit anti-human ER α (C1355, Millipore #06-935) was applied at a concentration of 1/8000 followed by biotinylated horse anti-rabbit serum (Vector BA-1100; 1/500). The conjugate was developed with Vector ABC reagents and Vector Nickel-DAB kit, yielding black nuclear staining of ER α . TPH immunostaining proceeded as described above.

To double label ER β + TPH, mouse anti-human ER β (GTX70174, GeneTex, Irvine, California) was applied at a concentration of 1/200 followed by biotinylated horse anti-mouse serum (Vector BA-2000; 1/500). The conjugate was developed with Vector ABC kit and Vector Nickel-DAB kit, yielding black nuclei with significant cytoplasmic staining of ER β . TPH immunostaining proceeded as described above.

Positive controls

The antibody to ER α has been well characterized in mouse brain (McClellan *et al.*, 2010), and the antibody to ER β produced nuclear staining in the human lung (Taniuchi *et al.*, 2014). To engender more confidence for staining in monkey, further characterization of the specificity of the commercially available ER antibodies was obtained in macaques. The positive control tissue for ER α was the uterine endometrium from an E-treated rhesus macaque. The endometrium was in the proliferative phase and contained secretory glands, which are composed of cells known to contain ER α (Brenner and Slayden, 1994). In this demonstration, the endometrium was cut on a cryostat without fixation prior to processing for IHC.

The positive control tissue for ER β was the prostate gland. This tissue also contains glandular elements composed of cells known to contain ER β (Kuiper *et al.*, 1996). The prostate gland was immersion fixed in 4% formaldehyde prior to freezing and cut on a sliding microtome. The AR antibody has been well characterized by Santa Cruz (<http://www.scbt.com/datasheet-816-ar-n-20-antibody.html>) and in the literature (Satoh *et al.*, 2001; Saunders *et al.*, 2000; Vija *et al.*, 2014).

Densitometric Analysis of Steroid Receptors

Four sections at different morphological levels of the raphe were immunostained for steroid receptors and analyzed for each animal. The sections were morphologically matched between animals using anatomical reference points. The Marianas Stereological workstation with Slidebook 5.0 was used for image capture. A montage of the entire area of receptor immunostaining in the dorsal raphe was built by the workstation. The image was exported as a Tif file and ImageJ was used to perform the analysis. The raphe was defined, further cropped and kept constant across the levels and animals for each receptor. After contrast adjustment, the image was segmented into positive (highlighted with red) and negative pixels (not highlighted) with the same saturation parameters. The program provided the positive pixel area of the red highlighted nuclei. A filter was applied that counted objects (groups of pixels that are adjacent) within a range of sizes corresponding to nuclei. This provided the number of positive nuclei in the defined area. Therefore, for each section the following data were obtained: positive pixel area, positive nuclei (neuron) number, and total area examined (constant). The data were then averaged across the 4 sections from each individual animal.

Double-label images

Photomicrographs of the double-labelled sections were captured with a Zeiss Axioplan bright field microscope and a Zeiss AxioCam digital camera with Zen software at magnifications of 200 $\times$ and 400 $\times$.

Statistical Analysis

The steroid receptor measurements were averaged across the 4 sections from each animal, generating one overall value for the individual animal. Therefore, the variance around the mean of each group reflects the difference between animals. All data were compared with

analysis of variance (ANOVA) followed by Newman-Keuls post hoc pairwise comparison. The data were tested for unequal variance and if present, a non-parametric ANOVA was applied (Kruskal-Wallace).

Results

The ER α and ER β receptor staining in the positive control tissues are shown in Figure 1. The commercial antibody to ER α used in this study of the brain (C1355, Millipore, #06-935) detected nuclear staining in the endometrial glandular cells as previously reported (McClellan *et al.*, 1986; Slayden and Brenner, 2004) with other monoclonal ER α antibodies, such as H222 or D75 (Greene *et al.*, 1984) and 1D5 (al Saati *et al.*, 1993). This tissue had not been fixed and so the staining was somewhat diffuse compared to that observed in fixed tissue.

The commercial antibody to ER β stained the nuclei of the glandular cells in the macaque prostate (bottom panels) as reported in human prostate (Mak *et al.*, 2010). Similar results were reported with other antibodies such as CFK-E12 in epididymis (Choi *et al.*, 2001) or GC-17 in prostate (Leav *et al.*, 2001).

Figure 2 illustrates the immunostaining for AR, ER α β in the dorsal raphe of a T-treated animal. The area photographed was located in the rostral dorsal raphe. All 3 receptors were abundantly expressed in the male midbrain, both within and outside of the dorsal raphe. Cellular staining differed between the receptors. AR and ER α staining were robust with strictly nuclear staining. ER β staining was present, but it differed from the prostate in that it was not strictly concentrated in the nucleus. Positive neurons exhibited a range of staining patterns from largely nuclear to a mix of nuclear and cytoplasmic to largely cytoplasmic. In some neurons, the nuclear staining was intense and obvious. In other neurons, the nuclear staining was a darker area within the cytoplasmic staining, and even this was not always evident. When the dilution of the antibody increased, both types of staining disappeared, suggestive of specificity. Therefore, we think that the ER β staining is specific, and that it represents a difference between neurons and prostate glandular elements. There was no evidence of treatment effects on the staining in the nucleus versus cytoplasm. That is, we did not observe more neurons with intense nuclear staining or a shift in staining density between compartments with any treatment.

Figure 3 illustrates the effect of treatment on AR expression. There was a significant difference between the groups in the positive pixel area (F [3,14]=9.9; p=0.0009) and in the number of positive neurons (F [3,14]=9.1; p=0.0014). Post-hoc analysis indicated that the T and DHT+ATD groups were significantly higher than the placebo and FLUT+ATD groups in both positive pixel area and number of positive cells (p<0.05).

Figures 4 and 5 illustrate the effect of treatment on ER α and ER β . There was no difference between the groups in positive pixel area or number of positive neurons for either receptor type.

Double labeling for each steroid receptor and TPH is illustrated in Figure 6. AR did not co-localize in serotonin neurons. Rather, ARs were located in another cell type, probably

neuronal, in the vicinity of serotonin neurons. An occasional TPH2-positive neuron might have appeared to have AR staining in the nucleus, but changing the plane of focus revealed that the appearance was always due to overlapping cells (astericks).

Overall, there was robust ER α in the dorsal raphe of males, both within and outside of serotonin neurons. Nuclear ER α was easily observed in a subpopulation of serotonin neurons and the subpopulation was not limited to any one area of the dorsal raphe. ER α was also observed in neurons that did not contain TPH; and there were TPH expressing cells that did not contain ER α . Approximately 40% of the serotonin neurons contained ER α .

The bottom panels illustrate serotonin neurons that contain ER β . ER β was also observed in neurons that did not contain TPH; and there were TPH expressing cells that did not contain ER β . The TPH+ER β staining was hard to photograph due to the diffuse nature of the ER β staining. When the cytoplasm was stained for TPH (brown), it often covered up the gray ER β staining yielding an intensely stained dark brown neuron. If the neuron had ER β concentrated in the nucleus, then it was more clearly double labeled. Approximately 40% of the serotonin neurons contained ER β . The count included TPH-positive neurons with obviously ER-positive nuclei as well as the very dark brown neurons thought to represent TPH staining on top of cytoplasmic ER β staining.

Discussion

The actions of androgens on aggression were linked to low serotonin for many years (Bonson *et al.*, 1994; Brown *et al.*, 1982; Coccaro *et al.*, 1997; Higley *et al.*, 1996; McGinnis, 2004; Stanley *et al.*, 2000). Indeed, studies of different compartments of the serotonin system indicated that anabolic steroids decreased serotonin in terminal fields throughout the hamster brain (Grimes and Melloni, 2006); and elevated aggression correlated with low concentrations of the serotonin metabolite, 5HIAA, in male macaque cerebral spinal fluid (CSF) (Howell *et al.*, 2007). Our recent investigations have shed new light on these observations and provided a different interpretation of androgen action in the serotonin system. Using castrated male macaques; we devised a treatment paradigm that allowed activation of androgen receptors (AR) and estrogen receptors (ER) together or independently. T and its metabolite, DHT, plus aromatase inhibition (DHT+ATD), increased mRNA expression of the gene coding for TPH2 to the same extent (Bethea *et al.*, 2014). TPH2 is the rate-limiting enzyme in serotonin synthesis (Jequier *et al.*, 1969), and studies in females indicate that increased TPH2 mRNA is accompanied by increased TPH2 protein (Bethea *et al.*, 2000) and increased serotonin in forebrain areas (Sanchez *et al.*, 2013). T and DHT also increased the mRNA expression for the serotonin reuptake transporter (SERT) (Bethea *et al.*, 2014), which correlates with increased synaptic serotonin in human (Parsey *et al.*, 2006) and macaque studies (Sanchez *et al.*, 2013). The increase in TPH2 and SERT mRNAs occurred with aromatase inhibition, indicating that metabolism to estradiol (E) was not required. In marked contrast, detection of serotonin in axons was severely diminished by aromatase inhibition (Bethea *et al.*, 2014). That is, metabolism to E was required for serotonin to concentrate in axons. Moreover, serotonin axon density correlated with fenfluramine-induced serotonin release/prolactin secretion (Bethea *et al.*, 2014).

Altogether the data yield a different interpretation than postulated in earlier studies with rodents, primates and humans (Brown et al., 1982; Grimes et al., 2006; Howell et al., 2007). The use of DHT plus aromatase inhibition in our model enabled disassociation of TPH2 mRNA expression, and perhaps serotonin production, from axonal serotonin content. AR activation increased TPH2 mRNA while aromatase inhibition blocked axonal serotonin concentration. Thus, reports of nonaromatizable anabolic steroid-induced decreases in serotonin content or axon density may not represent serotonin function at all levels (Breuer *et al.*, 2001). It appears that the serotonin “system” is manifested in different compartments that may be regulated independently or in concert, but clearly, measurement of one compartment does not reflect the function of the entire system in male macaques.

Based upon these observations, it was of interest to examine AR and ERs, α β , in the serotonergic cell bodies of the male macaque dorsal raphe nucleus and to compare our results to rats and mice. Serotonin neurons did not contain nuclear AR. Nonetheless, AR-positive nuclei were prevalent in the dorsal raphe and the number of AR-positive cells increased with T and DHT treatment, in the presence or absence of aromatase inhibition. It may be questioned whether the AR-positive nuclei belonged to neurons or glia. We believe they are located in excitatory interneurons because of the stimulatory effect of androgens on TPH2 and SERT gene expression. Although the AR-positive nuclei appear small, this is only in comparison to the very large serotonin neurons. Moreover, the AR-positive nuclei do not resemble nuclei in astrocytes, oligodendrites or microglia. The phenotypic identity of the postulated excitatory neurons in the raphe is under investigation.

The stimulatory effect of androgens on AR expression has been documented in the hypothalamus of macaques (Roselli and Resko, 1989) and rodents (Roselli and Resko, 1984). The up-regulation of nuclear AR by androgens could increase responsiveness to androgens and further increase TPH2 and SERT mRNA expression in a feed forward manner.

Overall, nuclear AR expression in the dorsal raphe appears to occur in male primates, rats and mice (Sheng *et al.*, 2004), but it does not co-localize within serotonin neurons. Of note, we found very little AR in a crudely dissected block of the dorsal raphe in female macaques with RT-PCR (unpublished).

We found ER β -positive serotonin neurons in male (this study) and female macaques (Gundlah *et al.*, 2000). This is also true in mice and rats (Lu *et al.*, 2001; Sheng *et al.*, 2004; Yamaguchi and Yuri, 2011). ER β regulates numerous serotonin functions in primates; and ER β regulates TPH2 mRNA expression in serotonin neurons of rodents (Donner and Handa, 2009; Hiroi and Handa, 2013).

We also show ER α -positive serotonin neurons in male macaques. However, ER α has not been detected in the dorsal raphe of female macaques (Vanderhorst *et al.*, 2009). ER α was also undetectable by qRT-PCR in the raphe region of ovariectomized female marmosets treated with placebo or E for 6 months (Bethea *et al.*, 2014). ER α is found in the raphe in mice of both sexes, but detection of ER α in rats is sparse at best, and co-localization in

serotonin neurons has not been reported in rats of either sex (Alves *et al.*, 1998; Sheng *et al.*, 2004; Lu *et al.*, 2001).

Table 1 summarizes the studies quoted above. Macaques show a sex difference in the expression of AR (yes–males; no-females) and ER α (yes-males; no-females) in the dorsal raphe. Macaques, mice and rats are similar in the expression of AR in males and expression of ER β in both sexes in the dorsal raphe. Thus, male macaques have a cohort of reproductive steroid receptors (AR, ER α , ER β) similar to male mice. Female macaques differ from female mice in the expression of ER α in the raphe (yes-mice; no-monkeys). Data on ER α in rats are somewhat inconsistent.

The above information is important for understanding sex and species differences in steroid hormone action in the serotonin system and its downstream targets. Data to support this type of multi-analysis of membrane receptors on any particular neuronal phenotype is lacking. However, as studies emerge of diverse steroid binding proteins in the membrane that are present with, and without, the classical nuclear steroid receptors, it becomes important to examine these membrane proteins in serotonin neurons. This task would be daunting with macaques *in vivo*, but it may be feasible in our embryonic stem cell-derived serotonin neurons (Bethea *et al.*, 2009; Salli *et al.*, 2004; Tokuyama *et al.*, 2010).

In order to link steroid receptors with function in male macaques, it would appear that AR in neighboring neurons increased serotonin related gene expression, but intracellular ERs are needed to detect serotonin in terminal fields. This is more complex than in female macaques wherein ER β mediates increased TPH2 mRNA (Sanchez *et al.*, 2005), as well as gene expression related to transport (Bethea and Reddy, 2015). Our observations show the nuclear steroid receptors are present to transduce cognate ligand actions in serotonin neurons of male macaques; and they provide support for hormone therapy in men for treatment of serotonin related pathologies. Nonetheless, diet may impact the efficacy of T as it does in female macaques administered E (Bethea *et al.*, 2014) and a window of opportunity may exist in men as well as women.

ARs and ERs work together in males to elevate serotonin synthesis and axonal content. Since elevated androgens also increase aggression in male macaques during mating season, it follows that elevated serotonin and aggression coexist. The same conclusion has been reached in studies of MAO-A deletion (Brunner *et al.*, 1993; Cases *et al.*, 1998), MAO-A allelic transcription reduction (Caspi *et al.*, 2002; Huang *et al.*, 2004) and MAO-A promoter methylation (Checknita *et al.*, 2015). Androgens also reduce MAO-A expression in male macaques (submitted for publication). The androgen-induced reduction of MAO-A correlated with lower CSF 5HIAA (serotonin metabolite) and higher serotonin in axons. Thus, data are accumulating from several directions that suggest elevated serotonin accompanies aggression and not an androgen-induced reduction in serotonin.

The expression and co-localization of AR and ERs in the serotonergic dorsal raphe probably does not represent the entire midbrain. We observed AR in the PAG, ventral to the pons and lateral to the decussation of the cerebellar peduncles in male macaques. Kritzer reported that AR were also expressed in male rats in VTA, lateral terminal (LT) and pedunculular

nuclei (PPN), subregions of the substantia nigra, lateral margins of the retrorubral fields and others (Kritzer, 1997). We expect that similar observations will be made in male macaques.

We observed robust ER α in the raphe and lateral to the decussation of the cerebellar peduncles of male macaques. Therefore, we predict it will be prominent in the other midbrain areas of males, like VTA. In addition to the dorsal raphe, we previously noted ER β in the central linear raphe, substantia nigra, and pons in female macaques (Gundlah *et al.*, 2000), and we predict that male macaques will also express ER β in these other areas.

In conclusion, this study shows that AR are located in cells adjacent to serotonin neurons in the dorsal raphe, suggesting that the androgen-induced increase in TPH2 and SERT mRNAs may be mediated indirectly by neighboring excitatory neurons. However, nuclear ER α and ER β are expressed by a subpopulation of serotonin neurons, and E from the aromatization of T is necessary for maintaining serotonin in axons of terminal fields.

Acknowledgements

We are very grateful to Kevin Muller for training the animals, administering drugs and monitoring the health and wellbeing of the animals. We greatly appreciate Dr. Kris Coleman and Nicola Robertson for earlier behavioral observations and analysis. We thank the Primate Genetics Program at the Oregon National Primate Research Center for calculations of the relatedness of our animals. We are also grateful to Dr. Jay Welch and the technicians of the Division of Comparative Medicine (DCM), for the management and care of our animals. We thank the Surgery and Pathology Sections of DCM for their expertise and handling of our needed surgeries and necropsies. This work was funded by an NIH grant MH 86542 to CLB and P51 OD11092 for support of the Oregon National Primate Research Center.

Literature Cited

- al Saati T, Clamens S, Cohen-Knafo E, Faye JC, Prats H, Coindre JM, Wafflart J, Caveriviere P, Bayard F, Delsol G. Production of monoclonal antibodies to human estrogen-receptor protein (ER) using recombinant ER (RER). *Int J Cancer*. 1993; 55:651–654. [PubMed: 8406995]
- Alves SE, Weiland NG, Hayashi S, McEwen BS. Immunocytochemical localization of nuclear estrogen receptors and progesterin receptors within the rat dorsal raphe nucleus. *J Comp Neurol*. 1998; 391(3):322–334. [PubMed: 9492203]
- Bethea CL. Regulation of progesterin receptors in raphe neurons of steroid-treated monkeys. *Neuroendocrinology*. 1994; 60:50–61. [PubMed: 8090282]
- Bethea CL, Coleman K, Phu K, Reddy AP, Phu A. Relationships between androgens, serotonin gene expression and innervation in male macaques. *Neuroscience*. 2014; 274:341–356. [PubMed: 24909896]
- Bethea CL, Mirkes SJ, Shively CA, Adams MR. Steroid regulation of tryptophan hydroxylase protein in the dorsal raphe of macaques. *Biol Psychiatry*. 2000; 47:562–576. [PubMed: 10715363]
- Bethea CL, Reddy AP. Effect of ovarian hormones on genes promoting dendritic spines in laser-captured serotonin neurons from macaques. *Mol Psychiatry*. 2010; 15:1034–1044. [PubMed: 19687787]
- Bethea CL, Reddy AP. Effect of ovarian steroids on gene expression related to synapse assembly in serotonin neurons of macaques. *J Neurosci Res*. 2012a; 90:1324–1334. [PubMed: 22411564]
- Bethea CL, Reddy AP. Ovarian steroids increase glutamatergic related gene expression in serotonin neurons of macaques. *Mol Cell Neurosci*. 2012b; 49:251–262. [PubMed: 22154832]
- Bethea CL, Reddy AP. Ovarian steroids regulate gene expression related to DNA repair and neurodegenerative diseases in serotonin neurons of macaques. *Mol Psychiatry*. 2015 in press.
- Bethea CL, Reddy AP, Pedersen D, Tokuyama Y. Expression profile of differentiating serotonin neurons derived from rhesus embryonic stem cells and comparison to adult serotonin neurons. *Gene Expr Patterns*. 2009; 9:94–108. [PubMed: 18996226]

- Bethea CL, Reddy AP, Robertson N, Coleman K. Effects of aromatase inhibition and androgen activity on serotonin and behavior in male macaques. *Behav Neurosci*. 2013; 127:400–414. [PubMed: 23506438]
- Bonson KR, Johnson RG, Fiorella D, Rabin RA, Winter JC. Serotonergic control of androgen-induced dominance. *Pharmacol Biochem Behav*. 1994; 49:313–322. [PubMed: 7529925]
- Brenner, RM.; Slayden, OD. Cyclic changes in the primate oviduct and endometrium. In: Knobil, E.; Neill, JD., editors. *The Physiology of Reproduction*. Second Edition. New York: Raven Press, Ltd.; 1994. p. 541-569.2 ed.
- Breuer ME, McGinnis MY, Lumia AR, Possidente BP. Aggression in male rats receiving anabolic androgenic steroids: effects of social and environmental provocation. *Horm Behav*. 2001; 40:409–418. [PubMed: 11673914]
- Brown GL, Ebert MH, Goyer PF, Jimerson DC, Klein WJ, Bunney WE, Goodwin FK. Aggression, suicide, and serotonin: relationships to CSF amine metabolites. *Am J Psychiatry*. 1982; 139:741–746. [PubMed: 6177256]
- Brunner HG, Nelen M, Breakefield XO, Ropers HH, van Oost BA. Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. *Science*. 1993; 262:578–580. [PubMed: 8211186]
- Cases O, Lebrand C, Giros B, Vitalis T, De Maeyer E, Caron MG, Price DJ, Gaspar P, Seif I. Plasma membrane transporters of serotonin, dopamine, and norepinephrine mediate serotonin accumulation in atypical locations in the developing brain of monoamine oxidase A knock-outs. *J Neurosci*. 1998; 18:6914–6927. [PubMed: 9712661]
- Caspi A, McClay J, Moffitt RE, Martin J, Craig IW, Taylor A, Poulton R. Role of genotype in the cycle of violence in maltreated children. *Science*. 2002; 297:851–854. [PubMed: 12161658]
- Checknita D, Maussion G, Labonté B, Comai S, Tremblay RE, Vitaro F, Turecki N, Bertazzo A, Gobbi G, Côté G, Turecki G. Monoamine oxidase A gene promoter methylation and transcriptional downregulation in an offender population with antisocial personality disorder. *Br J Psychiatry*. 2015; 206:216–222. [PubMed: 25497297]
- Choi I, Ko C, Park-Sarge OK, Nie R, Hess RA, Graves C, Katzenellenbogen BS. Human estrogen receptor beta-specific monoclonal antibodies: characterization and use in studies of estrogen receptor beta protein expression in reproductive tissues. *Mol Cell Endocrinol*. 2001; 181:139–150. [PubMed: 11476948]
- Coccaro EF, Kavoussi RJ, Cooper TB, Hauger RL. Central serotonin activity and aggression: inverse relationship with prolactin response to d-fenfluramine, but not CSF 5-HIAA concentration, in human subjects. *Am J Psychiatry*. 1997; 154:1430–1435. [PubMed: 9326827]
- Donner N, Handa RJ. Estrogen receptor beta regulates the expression of tryptophanhydroxylase 2 mRNA within serotonergic neurons of the rat dorsal raphe nuclei. *Neuroscience*. 2009; 163:705–718. [PubMed: 19559077]
- Eaton GG. Male dominance and aggression in Japanese macaque reproduction. *Adv Behav Biol*. 1974; 11:287–297. [PubMed: 4475646]
- Eaton GG, Resko JA. Plasma testosterone and male dominance in a Japanese macaque (*Macaca fuscata*) troop compared with repeated measures of testosterone in laboratory males. *Horm Behav*. 1974; 5:251–259. [PubMed: 4474138]
- Eaton GG, Worlein JM, Glick BB. Sex differences in Japanese macaques (*Macaca fuscata*): effects of prenatal testosterone on juvenile social behavior. *Horm Behav*. 1990; 24:270–283. [PubMed: 2365304]
- Ellinwood WE, Hess DL, Roselli CE, Spies HG, Resko JA. Inhibition of aromatization stimulates luteinizing hormone and testosterone secretion in adult male rhesus monkeys. *J Clin Endocrinol Metab*. 1984; 59:1088–1096. [PubMed: 6541658]
- Greene GL, Sobel NB, King WJ, Jensen EV. Immunochemical studies of estrogen receptors. *J Steroid Biochem*. 1984; 20:51–56. [PubMed: 6200700]
- Grimes JM, Melloni RH Jr. Prolonged alterations in the serotonin neural system following the cessation of adolescent anabolic-androgenic steroid exposure in hamsters (*Mesocricetus auratus*). *Behav Neurosci*. 2006; 120:1242–1251. [PubMed: 17201468]

- Gundlah C, Kohama SG, Mirkes SJ, Garyfallou VT, Urbanski HF, Bethea CL. Distribution of estrogen receptor beta (ER β) mRNA in hypothalamus, midbrain and temporal lobe of spayed macaque: continued expression with hormone replacement. *Mol Brain Research*. 2000; 76:191–204. [PubMed: 10762694]
- Gundlah C, Lu NZ, Mirkes SJ, Bethea CL. Estrogen receptor beta (ER β) mRNA and protein in serotonin neurons of macaques. *Mol Brain Research*. 2001; 91:14–22. [PubMed: 11457488]
- Higley JD, Mehlman PT, Poland RE, Taub DM, Vickers J, Suomi SJ, Linnoila M. CSF testosterone and 5-HIAA correlate with different types of aggressive behaviors. *Biol Psychiatry*. 1996; 40:1067–1082. [PubMed: 8931909]
- Hiroi R, Handa RJ. Estrogen receptor-beta regulates human tryptophan hydroxylase-2 through an estrogen response element in the 5' untranslated region. *J Neurochem*. 2013; 127:487–495. [PubMed: 24033289]
- Howell S, Westergaard G, Hoos B, Chavanne TJ, Shoaf SE, Cleveland A, Snoy PJ, Suomi SJ, Dee Higley J. Serotonergic influences on life-history outcomes in free-ranging male rhesus macaques. *Am J Primatol*. 2007; 69:851–865. [PubMed: 17330868]
- Huang YY, Cate SP, Battistuzzi C, Oquendo MA, Brent D, Mann JJ. An association between a functional polymorphism in the monoamine oxidase A gene promoter, impulsive traits and early abuse experiences. *Neuropsychopharmacology*. 2004; 29:1498–1505. [PubMed: 15150530]
- Jequier E, Robinson DS, Lovenberg W, Sjoerdsma A. Further studies on tryptophan hydroxylase in rat brainstem and beef pineal. *Biochem Pharmacol*. 1969; 18:1071–1081. [PubMed: 5789774]
- Kritzer MF. Selective colocalization of immunoreactivity for intracellular gonadal hormone receptors and tyrosine hydroxylase in the ventral tegmental area, substantia nigra, and retrorubral fields in the rat. *J Comp Neurol*. 1997; 379:247–260. [PubMed: 9050788]
- Kuiper GGJM, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson J-A. Cloning of a novel estrogen receptor expressed in rat prostate. *Proc Nat Acad Sci*. 1996; 93:5925–5930. [PubMed: 8650195]
- Leav I, Lau KM, Adams JY, McNeal JE, Taplin ME, Wang J, Singh H, Ho SM. Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. *Am J Pathol*. 2001; 159:79–92. [PubMed: 11438457]
- Levin ER. Extranuclear steroid receptors are essential for steroid hormone actions. *Annu Rev Med*. 2015; 66:271–280. [PubMed: 25587652]
- Lu H, Ozawa H, Nishi M, Ito T, Kawata M. Serotonergic neurones in the dorsal raphe nucleus that project into the medial preoptic area contain oestrogen receptor beta. *J Neuroendocrinol*. 2001; 13:839–845. [PubMed: 11679052]
- Mak P, Leav I, Pursell B, Bae D, Yang X, Taglienti CA, Gouvin LM, Sharma VM, Mercurio AM. ERbeta impedes prostate cancer EMT by destabilizing HIF-1alpha and inhibiting VEGF-mediated snail nuclear localization: implications for Gleason grading. *Cancer Cell*. 2010; 17:319–332. [PubMed: 20385358]
- Maruhashi T. An ecological study of troop fissions of Japanese monkeys (*Macaca fuscata yaku*) on Yakushima Island, Japan. *Primates*. 1982; 23:317–337.
- McClellan KM, Stratton MS, Tobet SA. Roles for gamma-aminobutyric acid in the development of the paraventricular nucleus of the hypothalamus. *J Comp Neurol*. 2010; 518:2710–2728. [PubMed: 20506472]
- McClellan M, West NB, Brenner RM. Immunocytochemical localization of estrogen receptors in the macaque endometrium during the luteal-follicular transition. *Endocrinology*. 1986; 119:2467–2475. [PubMed: 3780534]
- McGinnis MY. Anabolic androgenic steroids and aggression: studies using animal models. *Ann N Y Acad Sci*. 2004; 1036:399–415. [PubMed: 15817752]
- Mukai H, Tsurugizawa T, Ogiue-Ikeda M, Murakami G, Hojo Y, Ishii H, Kimoto T, Kawato S. Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory. *Neuroendocrinology*. 2006; 84:255–263. [PubMed: 17142999]
- Parsey RV, Hastings RS, Oquendo MA, Huang YY, Simpson N, Arcement J, Huang Y, Ogden RT, Van Heertum RL, Arango V, Mann JJ. Lower serotonin transporter binding potential in the human brain during major depressive episodes. *Am J Psychiatry*. 2006; 163:52–58. [PubMed: 16390889]

- Pecins-Thompson M, Brown NA, Bethea CL. Regulation of serotonin re-uptake transporter mRNA expression by ovarian steroids in rhesus macaques. *Mol Brain Res*. 1998; 53:120–129. [PubMed: 9473622]
- Phu, K.; Reddy, AP.; Bethea, SC.; Bethea, CL. CSF 5HIAA Reflects MAO-A gene expression, which is suppressed by testosterone; and not TPH2 gene expression, which is increased by testosterone, in male macaques. Annual Meeting of the American College of Neuropsychopharmacology; Phoenix, AZ. 2014. p. abstract #M256.
- Razandi M, Pedram A, Greene GL, Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ER α and ER β expressed in chinese hamster ovary cells. *Mol Endocrinol*. 1999; 13:307–319. [PubMed: 9973260]
- Resko JA, Connolly PB, Roselli CE, Abdelgadir SE, Choate JV. Differential effects of aromatase inhibition on luteinizing hormone secretion in intact and castrated male cynomolgus macaques. *J Clin Endocrinol Metab*. 1993; 77:1529–1534. [PubMed: 8263136]
- Rivera HM, Bethea CL. Ovarian steroids increase spinogenetic proteins in the macaque dorsal raphe. *Neuroscience*. 2012; 208:27–40. [PubMed: 22342969]
- Roselli CE, Resko JA. Androgens regulate brain aromatase activity in adult male rats through a receptor mechanism. *Endocrinology*. 1984; 114:2183–2189. [PubMed: 6723579]
- Roselli CE, Resko JA. Testosterone regulates aromatase activity in discrete brain areas of male rhesus macaques. *Biol Reprod*. 1989; 40:929–934. [PubMed: 2765616]
- Rostal DC, Glick BB, Eaton GG, Resko JA. Seasonality of adult male Japanese macaques (*Macaca fuscata*): androgens and behavior in a confined troop. *Horm Behav*. 1986; 20:452–462. [PubMed: 3793026]
- Salli U, Reddy AP, Salli N, Lu NZ, Kuo H-C, Pau FK-Y, Wolf DP, Bethea CL. Serotonin neurons derived from rhesus monkey embryonic stem cells: similarities to CNS serotonin neurons. *Experimental Neurology*. 2004; 188:351–364. [PubMed: 15246835]
- Sanchez MG, Morissette M, Di Paolo T. Oestradiol modulation of serotonin reuptake transporter and serotonin metabolism in the brain of monkeys. *J Neuroendocrinol*. 2013; 25:560–569. [PubMed: 23414342]
- Sanchez RL, Reddy AP, Centeno ML, Henderson JA, Bethea CL. A second tryptophan hydroxylase isoform, TPH-2 mRNA, is increased by ovarian steroids in the raphe region of macaques. *Mol Brain Res*. 2005; 135:194–203. [PubMed: 15857682]
- Satoh H, Mori K, Furuhashi K. Morphological and immunohistochemical characteristics of the heterogeneous prostate-like glands (paraurethral gland) seen in female Brown-Norway rats. *Toxicol Pathol*. 2001; 29:237–241. [PubMed: 11421491]
- Saunders PT, Millar MR, Williams K, Macpherson S, Harkiss D, Anderson RA, Orr B, Groome NP, Scobie G, Fraser HM. Differential expression of estrogen receptor- α and - β and androgen receptor in the ovaries of marmosets and humans. *Biol Reprod*. 2000; 63:1098–1105. [PubMed: 10993832]
- Sheng Z, Kawano J, Yanai A, Fujinaga R, Tanaka M, Watanabe Y, Shinoda K. Expression of estrogen receptors (α , β) and androgen receptor in serotonin neurons of the rat and mouse dorsal raphe nuclei; sex and species differences. *Neurosci Res*. 2004; 49:185–196. [PubMed: 15140561]
- Slayden OD, Brenner RM. Hormonal regulation and localization of estrogen, progesterone and androgen receptors in the endometrium of nonhuman primates: effects of progesterone receptor antagonists. *Arch Histol Cytol*. 2004; 67:393–409. [PubMed: 15781981]
- Stanley B, Molcho A, Stanley M, Winchel R, Gameroff MJ, Parsons B, Mann JJ. Association of aggressive behavior with altered serotonergic function in patients who are not suicidal. *Am J Psychiatry*. 2000; 157:609–614. [PubMed: 10739421]
- Taniuchi S, Fujishima F, Miki Y, Abe K, Nakamura Y, Sato S, Kasajima A, Fue M, Ishida K, Watanabe M, Sakakibara T, Maeda S, Suzuki T, Sasano H. Tissue concentrations of estrogens and aromatase immunolocalization in interstitial pneumonia of human lung. *Mol Cell Endocrinol*. 2014; 392:136–143. [PubMed: 24861259]
- Tokuyama Y, Ingram SL, Woodward JS, Bethea CL. Functional characterization of rhesus embryonic stem cell-derived serotonin neurons. *Exp Biol Med (Maywood)*. 2010; 235:649–657. [PubMed: 20463306]

- Tsutsui K. Neurosteroid biosynthesis and action during cerebellar development. *Cerebellum*. 2012; 11:414–415. [PubMed: 22198872]
- Vanderhorst VG, Terasawa E, Ralston HJ 3rd. Estrogen receptor-alpha immunoreactive neurons in the brainstem and spinal cord of the female rhesus monkey: species-specific characteristics. *Neuroscience*. 2009; 158:798–810. [PubMed: 18996446]
- Vija L, Boukari K, Loosfelt H, Meduri G, Viengchareun S, Binart N, Young J, Lombes M. Ligand-dependent stabilization of androgen receptor in a novel mouse ST38c Sertoli cell line. *Mol Cell Endocrinol*. 2014; 384:32–42. [PubMed: 24440575]
- Yamaguchi N, Yuri K. Changes in oestrogen receptor-b mRNA expression in male rat brain with age. *J Neuroendocrinol*. 2011; 24:310–318. [PubMed: 21981044]

Highlights

Nuclear AR are not in male macaque serotonin neurons.

Nuclear ER α and ER β are in male macaque serotonin neurons.

Male macaques and mice have similar receptors in serotonin neurons.

Male and female macaques have different receptors in serotonin neurons.

Figure 1. Photomicrographs of ER α in the endometrial glands of a female macaque (top, arrows) and ER β in the prostate gland of a male macaque (bottom, arrows)

Top panels. Nuclear ER α was observed in an isolated endometrial gland (left), and in a gland that is surrounded by endometrial stromal tissue (right) with the commercial antibody used in this study (C1355, Millipore).

Bottom panels. The prostate gland exhibits robust expression of nuclear ER β with the commercial antibody used in this study of the brain (GTX70174, GeneTex).

Black arrows point to positively stained nuclei.

Figure 2. Photomicrographs of AR, ER α and ER β in the dorsal raphe of male monkeys AR immunostaining at different magnifications is shown in panels A, B, and C. ER α immunostaining at different magnifications is shown in panels D, E, and F. ER β immunostaining at different magnifications is shown in panels G, H and I. The insert (to panel I) shows the different types of cellular staining patterns observed for ER β . Panels A, D, and G illustrate the widespread and dense receptor staining observed in the dorsal raphe region.

Black arrowheads point to positively stained nuclei.

White arrowhead points to a neuron with largely cytoplasmic staining for ER β .

Figure 3. Quantitation of AR immunostaining in the dorsal raphe of male macaques
AR-positive pixel area and the number of AR-positive neurons are illustrated in the top and bottom panels, respectively. AR-positive pixel area and the number of AR-positive neurons were significantly increased in the T and DHT+ATD groups compared to placebo or FLUT+ATD.

* significantly increased with Newman-Keuls posthoc comparison

Figure 4. Quantitation of ER α immunostaining in the dorsal raphe of male macaques ER α -positive pixel area and the number of ER α -positive neurons are illustrated in the top and bottom panels, respectively. There was no difference in ER α -positive pixel area or -positive neurons between the groups.

Figure 5. Quantitation of ER β immunostaining in the dorsal raphe of male macaques ER β -positive pixel area and the number of ER β -positive neurons are illustrated in the top and bottom panels, respectively. There was no difference in ER β -positive pixel area or -positive neurons between the groups.

Figure 6. Double immunolabelling for each steroid receptor and serotonin neurons using TPH as a specific marker

Panels A – B. AR + TPH staining is illustrated at different magnifications. Asterisks indicate TPH-positive neurons with an overlapping neuron containing an AR-positive nucleus.

Panels C – D. ER α + TPH staining is illustrated at different magnifications

Panels E – F. ER β + TPH staining is illustrated at different magnifications.

Black arrowheads point to steroid receptor positive nuclei that are not in serotonin neurons.

Black arrows point to neurons that are double labeled for TPH and a steroid receptor.

White arrows point to neurons that are single labeled for TPH.

Sex and species differences in reproductive steroid receptor expression in the dorsal raphe nucleus (**DRN**) and co-localization within serotonin neurons (**5HT**).

Table 1

	AR		ERα		ERβ	
	DRN	5HT	DRN	5HT	DRN	5HT
<i>Rhesus macaque</i>						
Male	+	-	+	+	+	+
Female	-	-	-	-	+	+
<i>Mice</i>						
Male	+	-	+	+	+	+
Female	-	-	+	+	+	+
<i>Rat</i>						
Male	+	-	~	-	+	+
Female	-	-	~	-	+	+

+ yes - no ~sparse