PLOS | ONE

# Effect of Testosterone on Neuronal Morphology and Neuritic Growth of Fetal Lamb Hypothalamus-Preoptic Area and Cerebral Cortex in Primary Culture

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Published: June 8, 2015 • http://dx.doi.org/10.1371/journal.pone.0129521

**Abstract**

Testosterone plays an essential role in sexual differentiation of the male sheep brain. The ovine sexually dim (oSDN), is 2 to 3 times larger in males than in females, and this sex difference is under the control of testost testosterone on oSDN volume may result from enhanced expansion of soma areas and/or dendritic fields. To cells derived from the hypothalamus-preoptic area (HPOA) and cerebral cortex (CTX) of lamb fetuses were g culture to examine the direct morphological effects of testosterone on these cellular components. We found t plating, neurons derived from both the HPOA and CTX extend neuritic processes and express androgen rec immunoreactivity. Both treated and control neurites continue to grow and branch with increasing time in cultu testosterone (10 nM) for 3 days significantly ($P < 0.05$) increased both total neurite outgrowth (35%) and son HPOA and outgrowth (21%) and number of branch points (33%) in the CTX. These findings indicate that tes somal enlargement and neurite outgrowth in fetal lamb neurons may contribute to the development of a fully brain.

**Citation:** Reddy RC, Amodei R, Estill CT, Stormshak F, Meaker M, Roselli CE (2015) Effect of Testostero Morphology and Neuritic Growth of Fetal Lamb Hypothalamus-Preoptic Area and Cerebral Cortex in Prim ONE 10(6): e0129521. doi:10.1371/journal.pone.0129521

**Academic Editor:** Martine Migaud, Institut National de la Recherche Agronomique-CNRS UMR6175, FR

**Received:** January 21, 2015; **Accepted:** May 8, 2015; **Published:** June 8, 2015

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**Data Availability:** All relevant data are within the paper.

**Funding:** This work was supported by the National Institutes of Health NIH R01OD011047 (CER). The fu study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Introduction

Exposure of the vertebrate brain to testosterone during a critical period of fetal development establishes per in reproductive physiology as well as a broad spectrum of behaviors and cognitive functions [1–3]. Sex stero responsible for sexual dimorphisms in brain regions that regulate these functions [4,5]. For example, sexual

volume of the medial preoptic area (MPOA) occur in several species, in particular the central region of the m
which has been named the sexually dimorphic nucleus of the preoptic area (SDN-POA) in rats where it was
SDN-POA of males is larger in volume than that of females. This correlates with quantitatively greater measu
behavior exhibited by males [7,8]. This difference in nuclear volume has been attributed to the presence of n
SDN-POA of males than of females [9]. Gonadal steroids determine neuron number in part by controlling the
death during brain sexual differentiation in rodents [10–12]. In addition, gonadal steroids have direct effects o
dendritic growth, which also contribute to volume differences in the SDN-POA [9,13–15].

In the sheep, a cluster of neurons exists bilaterally in the central portion of the medial preoptic area. This stru
ovine sexually dimorphic nucleus (oSDN) because it is approximately two times larger and contains more ne
sexually prefer ewes than in ewes or in rams that prefer other rams as sexual partners[16]. The oSDN devel
enlarged in genetic females by exposure to exogenous testosterone during a prenatal critical period that occ
genitalia have differentiated [17,18]. Exposure of adult sheep to exogenous testosterone does not affect oSD
that nuclear size is programmed prenatally [19]. The cellular mechanism whereby testosterone controls the o
oSDN is not yet established. In contrast to rodents, gonadal steroids do not appear to modulate cell death (a
developing oSDN [20]. Testosterone has been shown to increase the volume of neuropil in motor neurons [2
in a similar manner in oSDN.

The present study was conducted to test the hypothesis that testosterone effects neuronal soma and dendrit
could in turn contribute to the development of a fully masculine sheep brain. Thus, cells derived from the hyp
area (HPOA) and cerebral cortex (CTX) of lamb fetuses were grown in primary culture and studied to examir
testosterone on soma size, total neuronal process length, the number of neuronal processes and the numbe
points.

#### Materials and Methods

##### Ethics Statement

Animal procedures complied with the National Institutes of Medicine *Guide for the Care and Use of Laboratc*
approved by the Institutional Animal Care and Use Committee of Oregon State University (protocol number:
were carried out under general anesthesia induced with ketamine hydrochloride (4.4 mg/kg) plus diazepam (
maintained during surgery with oxygen:isoflurane mixture.

##### Animals

Lamb fetuses were obtained from pregnant Polypay ewes that were bred and maintained under standard hu
the AAALAC-approved Sheep Research Facility at Oregon State University in Corvallis, OR. Eight fetuses (6
were delivered surgically from 5 dams at gestational day (GD) 53 as described previously [17]. Blood sample
the umbilical artery and fetuses were sexed by examination of their external and internal genitalia.

##### Primary Cultures and Cell Treatments

Cells obtained from the brains of GD 53 lamb fetuses were cultured separately according to sex. Cells of bra
gestation were chosen because previous studies demonstrated that high yields of neurons are obtained from
cortex between 7 and 9 weeks of age [23–25]. This constitutes the main phase of neuronal proliferation prec
cell proliferation when the fetal lamb brain is similar in maturity to those of neonatal rodents [26,27]. Brains w
calvarias into ice-cold Hibernate E media (BrainBits; Springfield, IL). The meninges were removed and the h
area (HPOA) and cerebral cortex (CTX) dissected. The HPOA was removed with iridectomy scissors as a 2
tissue that was limited rostrally by the optic chiasm, caudally by the mammillary bodies and laterally by the la
sulcus. CTX cells were derived from bilateral dissections of the frontal and parietal lobes.

Cells were cultured using an adaptation of the BrainBits online protocol (http://www.brainbitsllc.com/dissocia
plating-protocol/). In brief, glass coverslips (Carolina Biological; Burlington, NC) were pre-incubated with Pol
Aldrich; St. Louis, MO), washed thoroughly with molecular grade water (HyClone), placed into 2.3 $cm^2$ multi
plates (Corning Inc. Life Sciences, Tewksbury, MA) and incubated overnight at 37°C in phenol-free Neuroba
Life Technologies, Grand Island, NY) supplemented with serum free B-27 supplement (InVitrogen). Fetal lam
dissociated into single cells by digestion for 20 minutes at 37°C with 0.2% papain (Worthington Biochemical
and 0.1% DNase I (Invitrogen) in $Ca^{2+}$Free Hibernate E buffer (BrainBits), sequentially washed in Hibernate
B-27 followed by mechanical trituration with fire polished Pasteur pipettes. Cells were then plated on glass c
of 300,000 cells per 2.3$cm^2$ well for the cortical cultures and 400,000 cells per 2.3$cm^2$ well for the HPOA cul

condition. After 1 hr., half of the medium was replaced with fresh phenol red-free Neurobasal medium supple GlutaMAX I (Gibco). For time-course analysis, cells were maintained *in vitro* for 2–5 days. For morphometric maintained for 3 days *in vitro* (DIV) under 3 conditions: no treatment; vehicle treatment (ethanol; 1:2,000 fina testosterone treatment (10 nM final concentration; Steraloids; Newport, RI). This concentration of testosteron because it is physiological and has been shown previously to stimulate neurite outgrowth in primary hypotha from rats [15]. As described above, media was changed 1 hr. after plating. For treatments, a stock solution o was made in 100% ethanol. On the day of drug treatment, stock testosterone or ethanol vehicle were diluted media and used to replace 50% of the media resulting in treatment with testosterone (10nM final concentrati (0.05% final concentration). Cultured neurons were incubated at 37°C in an atmosphere of 95% air– 5% CO

#### Immunocytochemistry

Cells were fixed in freshly made 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS; pH 7.4) fo temperature, permeabilized with 0.2% Triton-X in PBS for 10 min, blocked in 10% bovine serum albumin (BS for 1 hr and then incubated overnight at 4°C with primary antibodies diluted in 3% BSA/PBS. Immunocytoche using the following primary antibodies: a mouse monoclonal anti-neuronal class III β-tubulin antibody (diluted TUJ1, Covance Inc.; Princeton, NJ), PG-21 rabbit polyclonal anti-androgen receptor antibodies (1:100, Upst Millipore; Billerica, MA) and rabbit polyclonal anti-aromatase antibodies (1:20, Acris Antibodies Inc, SanDieg have been shown to specifically recognize their target antigen in sheep tissue [25,28,29]. Immunoreactions v Jackson Immunological FitC anti-mouse (1:1,000 dilution) or Cy3 anti-rabbit (1:500 dilution) in 3%BSA/PBS 45 min. Cells were mounted in Prolong Gold antifade reagent with DAPI (InVitrogen) to allow for nuclear cou for all immunostainings were performed by omitting the primary antisera. Under these conditions, no staining

#### Morphometric Analysis

Images were acquired by standard epifluorescence using a 0.63X digital camera attached to a Zeiss Axiopla microscope with a 20X objective. Digitized images of 50–60 neurons were taken of each treatment in replica photographed for analysis, neurons had to lie separated from adjacent cells so that each process could be c particular neuron. At DIV3 both the cortical and hypothalamic neurons generally came to lie well scattered th dish. Any area where clustered neurons occurred was omitted from the analysis, thus eliminating a biased s analyses were performed using the MetaMorph Microscopy Automation & Image Analysis software program Devices, Inc., Sunnyvale, CA). Calibration settings were performed and measurement parameters were dete the analysis and then maintained throughout the data collection process. Measurements were taken for the t soma size, the number of stem processes per cell originating directly from the cell soma, and the number of Data were obtained from 5 independent replicate experiments resulting in a total of 250–300 analyzed cells morphological parameters were calculated and expressed per individual neuron.

#### Statistical Analysis

Morphometric comparisons between untreated and treated cells were performed using the non-parametric K analysis of variance followed by the Dunn's Multiple Comparison test. A level of $P < 0.05$ was considered to significant.

## Results

### Time course

A time course study was performed on 3 independent replicates with cells derived from the CTX and HPOA male lamb fetus. Twenty four hrs after plating cells were attached to the poly-L-lysine substrate and showed such as lamellipodia and minor processes (not shown). Neurons were fixed and stained between DIV 2 and neurites were identified by cross reactivity with neuron-specific mouse anti-β-tubulin antibody. By DIV2 neuro primary neurites with little branching. Between DIV 2 and 3 neurites increased in length and began to show b elongated and became more complex with time in culture. Immunocytochemistry for androgen receptor and positive staining of the cytoplasm and processes of both hypothalamic and cortical β-tubulin-identified neuro points examined e.g., DIV 3 shown in Fig 2.

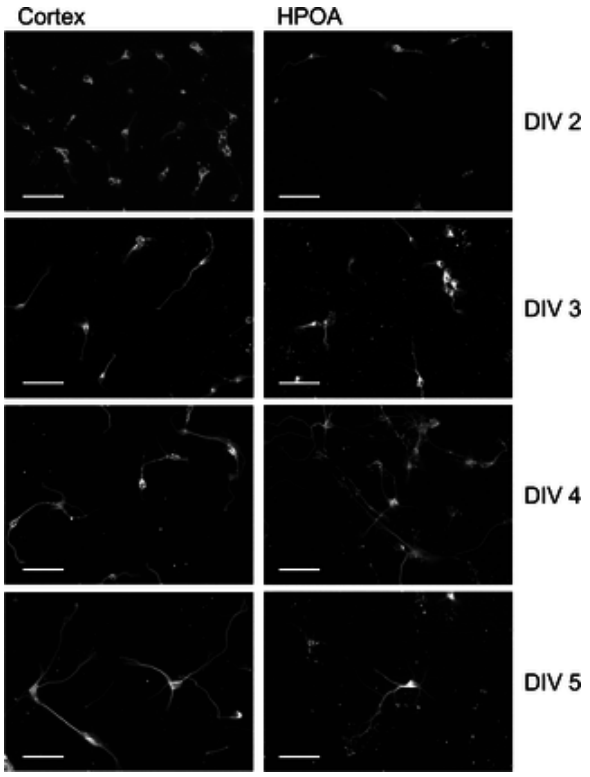


**Fig 1. Photomicrographs illustrating the growth of untreated primary neurons derived from GD53 fetal lamb cortex and h area (HPOA) maintained *in vitro* for 2 days (DIV2) to 5 days (DIV5).**
Soma and neurites were identified by immunohistochemical staining with the neuron-specific mouse anti-
Scale Bar = 50 μm.
http://dx.doi.org/10.1371/journal.pone.0129521.g001

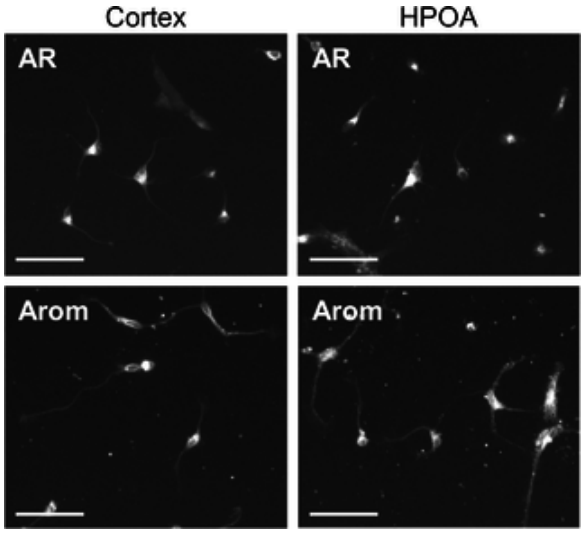


**Fig 2. Immunohistochemical staining of untreated cortical and HPOA neurons in cultures grown for 3 days (DIV3).**
Shown is immunofluorescence labeling for androgen receptor (AR) and aromatase (Arom). Scale Bar = 5
http://dx.doi.org/10.1371/journal.pone.0129521.g002

#### Effects of testosterone treatment

Cells derived from CTX and HPOA of 5 male lamb fetuses were used in independent replicate experiments t
testosterone treatment (10 nM) on neuronal differentiation *in vitro* (Fig 3). In order to control for possible vari
experimental replicates, untreated cells were evaluated together with control and testosterone-treated cells.
with testosterone for 3 days significantly changed morphological parameters related to neuronal differentiatic
HPOA cells (Table 1). Total neurite outgrowth was significantly increased ($P < 0.05$) by testosterone treatmer
and hypothalamic (35%) neurons compared to vehicle controls. Testosterone significantly ($P < 0.01$) increas
hypothalamic neurons (8%), but not in cortical neurons. The number of branch points was significantly ($P <$
testosterone treated cortical neurons, but only showed a trend for hypothalamic neurons ($P<0.07$). The numl
not significantly altered by testosterone in cells derived from either brain region.

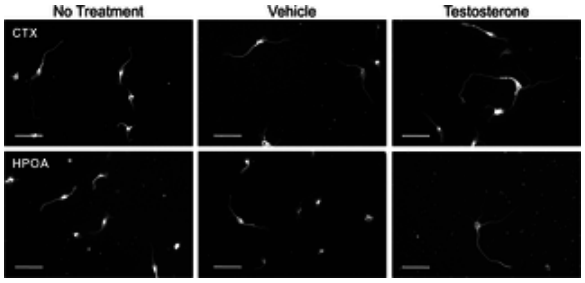


**Fig 3. Photomicrographs illustrating the effects of testosterone (10 nM) treatment for 3 days on the morphology of cortic immunolabeled for neuron-specific anti-β-tubulin.**

Morphometric analysis was carried out on randomly selected neurons from each subgroup. In order to co
variations between experimental replicates, untreated cells (No treatment) were evaluated together with c
testosterone-treated cells. Scale Bar = 50 μm.

http://dx.doi.org/10.1371/journal.pone.0129521.g003

| Tissue | Cortex | | | | MPOA | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | No Tx | Veh | T | P* | No Tx | Veh | T | P |
| Total Outgrowth (um) | 43.3±2.3 | 51.2±2.8 | 61.8±3.4 | <0.05 | 30.3±1.9 | 34.0±2.6 | 45.9±3.5 | 0.001 |
| Soma Size (um2) | 62.5±1.3 | 63.4±1.3 | 65.7±1.4 | ns | 60.3±1.0 | 63.0±1.2 | 68.3±1.3 | <0.01 |
| # of Processes | 2.8±.09 | 2.8±.08 | 3.0±.09 | ns | 2.4±.08 | 2.6±.09 | 2.8±.1 | ns |
| # of Branch Pts | 1.0±.11 | 1.2±.14 | 1.6±.16 | <0.05 | .68±.08 | .71±.10 | 1.1±.17 | ns(0.067) |

Data represent mean ± SEM obtained from 5 independent experiments using cells cultured for 3 days (DIV 3) that were derived from 5 separate GD53 male fetuses. Morphometric comparisons between untreated (No Tx), vehicle- (Veh) and testosterone (T)-treated (10 nM) cells were performed using the non-parametric Kruskal-Wallis one-way analysis of variance followed by the Dunn's Multiple Comparison test. A level of $P < 0.05$ was considered to be statistically significant. No significant differences were observed between the No Tx and Veh groups.

*P values indicate measures that are significantly different between the Veh- and T-treated groups. ns = not significantly different (P>0.05).

doi:10.1371/journal.pone.0129521.t001

**Table 1. Morphometric Cell Measurements.**

http://dx.doi.org/10.1371/journal.pone.0129521.t001

#### Discussion

The present experiments demonstrate that androgens are capable of promoting neurite outgrowth *in vitro* in
hypothalamic neurons derived from fetal lamb brains. Testosterone enhanced soma area in hypothalamic ne
branching in cortical neurons. Greater neurite arborization implies the potential for a larger dendritic field anc
whereas soma size relates inversely to firing threshold and recruitment potential. The subtle differences in re
between hypothalamic and cortical neurons suggest that there is regional specificity in the morphogenic pote
neuronal differentiation. More generally, our results support the conclusion that androgens have the potentia
morphological and functional differentiation of the sheep hypothalamus and cortex.

We found that both androgen receptor and aromatase-immunoreactivity was detectable in cortical and hypot
These results indicate that testosterone or its estrogenic metabolite could act directly to influence neuronal n
or in addition to indirect effects on their afferent inputs, efferent targets or glial interactions. The presence of
and aromatase in the cultures agrees with their expression in the same brain regions of sheep during compa
*in vivo* [29–31] and indicates that the culture model has validity for studying steroid hormone action on sheep

The predominant localization of androgen receptor to the cytoplasm may be related to the lack of testosteror medium during the time course study. Cytoplasmic localization of androgen receptor and aromatase protein previously in untreated cultured motor neurons [32]. Study of androgen receptor trafficking *in vitro* using a gr protein-androgen receptor fusion protein demonstrates that the receptor is predominantly cytoplasmic in the and rapidly translocates to the nucleus after testosterone treatment [33].

Our results are generally consistent with previous studies that used primary neuronal cultures derived from r studies found that testosterone promotes neurite outgrowth and other aspects of neuronal maturation in hyp less so in cortical neurons. The more robust effect of testosterone on cortical neurons derived from sheep in might reflect species differences in neuron maturity or steroid sensitivity at the developmental stages examir also differs methodologically from earlier rodent experiments in the use of a physiological testosterone conce length of treatment that began at plating. The observation that effects were apparent by three days in culture testosterone directly accelerates processes involved in the initiation of dendrite growth and arborization.

The structural effects of testosterone on the developing rodent brain are largely dependent on aromatization estrogens. Recent studies have elucidated diverse cellular mechanisms by which estradiol masculinizes the However, the effects of androgen and estrogen are intimately entwined and often complimentary. For instanc cultures, androgen receptors and aromatase co-exist in the same neurons and testosterone specifically supp aromatase-positive hypothalamic neurons, but not cortical or hypothalamic GABAergic neurons [15]. Thus te substrate for aromatase, but also acts to promote the development of estrogen-producing neurons. Furtherm effects of testosterone depend on the androgen receptor since they are blocked by the antagonist flutamide on *in vitro* PC-12 cell lines transfected with steroid receptor genes suggest that androgen and estrogen act i overlapping ways to promote neuronal maturation [41]. Our recent study suggests that the androgen recepto masculinization of the oSDN in sheep [42]. However in addition to androgen receptors, neurons within the de hypothalamus also express abundant levels of aromatase and estrogen-alpha receptors [17,30,31]. The exte may be involved in brain masculinization in sheep is not currently known. The higher circulating testosterone fetuses provide the substrates and ligands needed to activate both androgen and estrogen signaling pathwa lead to sex-specific circuit formation. Moreover, differences in cellular expression of steroid metabolic enzym hormone receptor compliment could lead to developmental and regional differences in the effects of gonadal morphology and contribute to neuroanatomical and functional sex dimorphisms. Future studies that employ s agonists and antagonists are needed to determine the roles and morphogenic potentials that can be attribute of androgens, estrogens or their combination on fetal sheep neurons in culture.

In summary, the present study demonstrates that testosterone stimulates morphological differentiation of cul neurons derived from the cerebral cortex and hypothalamus by increasing neurite outgrowth and soma size. that testosterone acts as a morphogenic signal for developing sheep neurons and by so doing contributes to fully masculine brain. The results extend previous findings obtained in rodents, a species in which sexual dif is completed after birth, to sheep, a long gestation species whose brain is sexually differentiated prior to birth

#### Acknowledgments

We thank the Oregon State University students who cared for the sheep used in this study. Hormonal analys Technology and Support Core was supported by the Oregon National Primate Research Center Core Grant

#### Author Contributions

Conceived and designed the experiments: RCR CER FS. Performed the experiments: RCR RA CTE MM. An RA CER. Contributed reagents/materials/analysis tools: CER FS. Wrote the paper: RCR RA CTE FS MM CE

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