Androgens Increase Survival of Adult Born Neurons in the Dentate Gyrus by an Androgen Receptor Dependent Mechanism in Male Rats.

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Abstract

Gonadal steroids are potent regulators of adult neurogenesis. We previously reported that androgens, such as testosterone (T) and dihydrotestosterone (DHT), but not estradiol, increased survival of new neurons in the dentate gyrus of the male rat. These results suggest androgens regulate hippocampal neurogenesis via the androgen receptor (AR). To test this supposition, we examined the role of ARs in hippocampal neurogenesis using two different approaches. In Experiment 1, we examined neurogenesis in male rats insensitive to androgens due to a naturally occurring mutation in the gene encoding the AR (termed TFMs) compared to wild type males. In Experiment 2, we injected the AR antagonist, flutamide, into castrated male rats and compared neurogenesis levels in the dentate gyrus of DHT and oil-treated controls. In Experiment 1, chronic T increased hippocampal neurogenesis in wild type males but not in androgen-insensitive TFM males. In Experiment 2, DHT increased hippocampal neurogenesis via cell survival, an effect that was blocked by concurrent treatment with flutamide. DHT, however, did not affect cell proliferation. Interestingly, cells expressing doublecortin, a marker of newborn immature neurons, did not colabel with ARs in the DG, but ARs were robustly expressed in other regions of the hippocampus. Together, these studies provide complementary evidence that androgens regulate adult neurogenesis in the hippocampus via the AR but at a site other than the dentate gyrus. Understanding where in the brain androgens act to increase the survival of new neurons in the adult brain may have implications for neurodegenerative disorders.
Introduction

Neurogenesis in the adult is observed in the dentate gyrus (DG) in rodents (1) and humans (2). Despite its evolutionarily conserved nature, the role of adult neurogenesis remains to be fully elucidated, although studies suggest adult hippocampal neurogenesis is an important factor in learning, memory (3) and depression (4).

Neurogenesis in the hippocampus is a multistep process that begins with the proliferation of cells, differentiation into neurons, migration to the granule cell layer, survival and integration of these new neurons into the circuitry. Adult hippocampal neurogenesis can be increased via independent modulation of any one of these stages. For example, spatial learning increases neurogenesis in the hippocampus via enhancement in cell survival independent of changes in cell proliferation (5, 6). Conversely, chronic antidepressant treatment increases neurogenesis in the hippocampus via increasing cell proliferation independent of cell survival (7).

Androgens and estrogens are potent modulators of adult hippocampal neurogenesis. Chronic estradiol decreases neurogenesis in adult female rats (8). In males, however, chronic treatment with testosterone or dihydrotestosterone (DHT), but not estradiol, increases hippocampal neurogenesis via cell survival (9). Given that testosterone can be converted to estradiol via aromatase or to the non-aromatizable androgen, DHT via 5α reductase, this previous finding suggests that androgens mediate hippocampal neurogenesis via binding to the androgen receptor (AR) in male rats. However, this is somewhat equivocal as DHT can be reduced to another metabolite, 5α-androstane-3α,17β-diol (3α-5α-androstane-3α,17β-diol; 3α-Diol) (10) that may act via a non AR mediated mechanism (11, 12). Thus, it is unclear whether androgens mediate neurogenesis via ARs or through some other mechanism. Although there are no reported ARs in the DG in the majority of rat strains (13,14, but see 15) it is possible that there is transient AR expression in immature neurons which may suggest a direct mechanism for androgens to promote survival of immature neurons.

Thus, the current study examined the hypothesis that androgens increase neurogenesis via cell survival in the adult DG via ARs using genetic (Experiment 1) and pharmacological (Experiment 2)
In Experiment 1, we used male rats harboring a mutation in the gene encoding the AR, the testicular feminization mutation (TFM) rats. These chromosomal males are insensitive to androgens due to a missense mutation in the steroid-binding domain (16), producing a non-functional AR at physiological androgen concentrations (17). In Experiment 2, we block the ability of DHT to bind ARs via systemic treatment with the competitive AR antagonist, flutamide, and examine neurogenesis and AR expression in different regions of the hippocampus. We hypothesize that if androgens are enhancing neurogenesis in the DG by acting on ARs, androgens should not affect neurogenesis in TFM-affected or in flutamide-treated male rats.

Methods

Animals

In Experiment 1, 7 wild type (wt) Sprague Dawley males and 6 males carrying a mutation in the gene encoding the androgen receptor (termed testicular feminization mutation males, or TFMs) were generated at Simon Fraser University. Sprague Dawley females previously identified as carrying the TFM allele (X\textsuperscript{wt}X\textsuperscript{tfm}) were mated with males and, after weaning, pups were identified at 30 days of age as having a male (large anogenital distance, scrotal sac, penis, no nipple line) or a female (short anogenital distance, no scrotal sac, nipple line) phenotype. Because males carrying the testicular feminization mutation (X\textsuperscript{tfm}Y) cannot be distinguished phenotypically from females (18, 19, 20), TFMs were positively identified as being male and having a mutated AR via a PCR assay of genomic DNA extracted from ear tissue (see 21 for details). In Experiment 2, 23 adult male Sprague-Dawley rats were generated from our breeding colony at the University of British Columbia. Animals were housed in clear polyurethane bins with wood chip bedding, maintained on a 12hr light:12hr dark schedule in a temperature controlled room, and given access to tap water and lab chow ad libitum. All protocols were approved by the institutional animal care committee (UBC and SFU) and conformed to the regulations set by the Canadian Committee for Animal Care. All efforts were made to reduce animal suffering.

Surgeries and Steroid Manipulations-
**Experiment 1:** Wild type males (n=7) and TFMs (n=7) were castrated at approximately 60 days old under aseptic conditions using isoflurane. At the time of surgery, animals within each group were given either 2 20mm Silastic (Dow Corning, Midland, MI) implants filled with Testosterone propionate (Steraloids Inc; internal diameter = 1.57mm; external diameter = 3.18mm) or 2 20mm Silastic implants filled with silicone (blanks), placed sc on the back of the neck. The testosterone propionate and blank Silastic capsules were constructed and prepared following Smith, Damassa, and Davidson (22). Berndtson et al, (23) reported serum testosterone levels in the high physiological range of males implanted with the same sized capsules containing testosterone propionate. Capsules of this size are also expected to release approximately 1.5mg testosterone propionate every 24 hours (23). Following recovery, all animals were injected with BrdU (200mg/kg) intraperitonially, prepared the same as above. This experiment was carried out at Simon Fraser University.

**Experiment 2:** Castrations were performed at approximately 60 days old and under aseptic conditions using isoflurane. Animals were monitored closely and weighed daily for one week after surgery. After recovery, all animals received a single intraperitoneal injection (ip) of 5-bromodeoxyuridine (BrdU; 200mg/kg; Sigma-Aldrich, St. Louis, MO) to label dividing cells and their progeny. BrdU was prepared fresh by dissolving 20mg/ml of BrdU in warm 0.9% saline buffered with 0.7% NaOH. One day following BrdU injection, animals received subcutaneous (sc) injections for 30 days of either: 1) vehicle (oil; n=6), 2) dihydrotestosterone (DHT; 0.25mg/day; n=5), 3) flutamide (2.5mg/day; n=6), or 4) a combination of DHT and flutamide (DHT 0.25mg/day: flutamide 2.5mg/day; n=6) delivered in a 0.6cc bolus. The dose of DHT chosen previously increased neurogenesis in castrated male rats (9). The dose of flutamide, a competitive androgen receptor antagonist, has been shown to decrease the weight of the bulbocavernosus and levator ani (BC/LA) muscles (24). This experiment was carried out at the University of British Columbia. The experimental timeline is shown in Figure 1.

**Tissue Preparation-** After 30 days of steroid treatments, all animals were given an overdose of sodium pentobarbital or CO₂ and transcardially perfused with either 0.1M phosphate buffered saline (PBS; Experiment 1) or normal saline (0.9%; Experiment 2), followed by 4% phosphate buffered
paraformaldehyde (PFA). Brains were harvested, placed in 4% paraformaldehyde for 24 hours, then placed in 30% sucrose and stored at 4°C until sectioning. Brains were sectioned on a microtome at 40µm through the entire rostro-caudal extent of the DG and divided into 10 series of sequential sections. Tissue was stored an antigen-sparing solution (0.05M tris-buffered saline, 30% ethylene glycol, and 20% glycerol). All cell counts and areas were quantified on every 10th section from the entire hippocampus. The average number of sections per series was 14.5 +/- 1.2. For Experiment 2, the BC/LA muscles, located at the base of the penis, were also harvested at the time of perfusion via bilateral cuts of the ischiocavernosus and careful dissection from the anus (see 25 for a review). Muscles were then blotted dry and weighed.

**Histology**

1. Immunocytochemistry

   A. BrdU: All rats were perfused 32 days after BrdU injection and thus the majority of BrdU-labelled cells would be 31 days old. BrdU and Ki67 were visualized in the GCL and hilus on every 10th section using the avidin-biotin peroxidase method. Free-floating sections were rinsed 3 times for 10 minutes in tris-buffered saline (TBS; pH 7.4) in between each step listed below (unless otherwise stated).

   To eliminate endogenous peroxidase activity, sections were incubated for 10 minutes in 0.6% hydrogen peroxide. To denature DNA and facilitate exposure of the BrdU antigen sites, tissue was incubated in 2 N hydrochloride acid (HCl) for 30 mins in a water bath (37°C), immediately followed by 10 minutes in 0.1 M borate buffer (pH 8.5) to neutralize the HCl. Sections were blocked in 3% normal donkey serum (NDS; Chemicon, Temecula, CA, USA) diluted in TBS for 30 minutes at room temperature and then incubated in a mouse anti-BrdU monoclonal primary antibody (1:200 in TBS, 3% NDS, and 0.1% Triton-X 100; Roche Diagnostics, Laval, Quebec, Canada) for 48 hours. Sections were then incubated in a donkey anti-mouse secondary antibody (1:100 in TBS; Vector Laboratories, Burlington, ON, Canada) for 4 hours at room temperature followed by incubation in avidin-biotin horseradish peroxidase complex for 2 hours at room temperature (ABC Elite Kit; 1:100; Vector Laboratories, Burlington, ON, Canada). The chromogen, diaminobenzidine (DAB; Sigma), was used to visualize antigen sites. Tissue was reacted for
5 minutes in 0.002% DAB diluted in TBS containing 0.003% hydrogen peroxidase and 0.03% nickel chloride for enhancement. Sections were mounted onto Super-frost/Plus (Fisher Scientific, Napean, ON, Canada) slides and allowed to dry overnight at room temperature. The following day, sections counterstained with cresyl violet and cover slipped with Permount (Fisher Scientific).

B. Ki67: Ki67 is expressed during the active phase of the cell cycle except for $G_0$ and part of $G_1$ and is thus an excellent endogenous marker of cell proliferation (see 26 for review). Free-floating sections were rinsed 3 times for 10 minutes in 0.1M phosphate buffered saline (PBS) in between each step listed below unless otherwise stated. To eliminate endogenous peroxidase activity, sections were incubated for 10 minutes in 0.6% hydrogen peroxide diluted in deionized water. Sections were incubated overnight at 4°C in a rabbit polyclonal primary directed against Ki67 (1:3000; Vector VPK 451), diluted in PBS + 0.04% Triton-x, and 3% normal goat serum (NGS). The following day, sections were rinsed 5 times for 10 minutes in PBS and then incubated overnight at 4°C in a goat anti-rabbit secondary (1:500; Vector) diluted in PBS. Sections were then washed 5 times for 10 minutes in PBS and then incubated in an avidin-biotin complex for 1 hour and 15 minutes. This was followed by washing tissue 2 times for 2 minutes in sodium acetate buffer (0.175M) before being reacted in DAB (SK-4100; prepared following manufacturer’s instructions) for 15 minutes. Sections were mounted onto glass sides, counterstained, and cover slipped as previously stated.

2. Immunofluorescence

To determine a possible site of androgen action on cell survival, we used double label immunofluorescence for doublecortin (DCX) and androgen receptor (AR). DCX is a microtubule-associated phosphoprotein that preferentially labels newborn neurons in the GCL (27). Additionally, we determined the cellular phenotype of a subset of randomly chosen newborn cells in the GCL of the DG using double label immunofluorescence for BrdU and the neuron specific marker, NeuN, to give an estimate of the total number of neurons produced in the adult hippocampus (28).

Every tenth section containing the dentate gyrus was used to visualize DCX/AR or BrdU/NeuN. In between each step listed below, tissue was rinsed 3 times for 10 minutes in TBS unless otherwise
stated. Tissue was blocked before each antibody incubation listed below for 30 minutes in 3% NDS
diluted in TBS and containing 3% Triton-X 100. Additionally, all antibodies were diluted in TBS
containing 1% normal donkey serum (NDS) and 3% Triton-X 100. To label DCX and AR, tissue was
incubated for 24 hours in both the goat anti-DCX (1:200; C18, sc8066, Santa Cruz Biotechnology, Santa
Cruz, CA, USA) and rabbit anti-AR (1:200; Epitomics, Burlingame, CA, USA) primary antibodies.
Tissue was subsequently incubated in a donkey anti-goat Alexa488 secondary (1:200; Invitrogen;
Burlington, ON, Canada) to label DCX and a donkey anti-rabbit Alexa549 secondary (1:200; Invitrogen)
to label AR. Sections were mounted on Superfrost/Plus slides and cover slipped with diazobicyclooctane
(TBS, 2.5% DABCO, 10% polyvinyl alcohol, and 20% glycerol) to prevent fading.

For BrdU and NeuN, tissue was incubated for 48 hours in a mouse anti-NeuN (1:100; Chemicon)
at 4°C. Tissue was then incubated overnight at 4°C in donkey ant-rabbit FITC secondary (1:200;
Invitrogen). Sections were fixed in 4% paraformaldehyde diluted in TBS followed by two rinses in
normal saline. Tissue was incubated in 2 N hydrochloride acid (HCl) for 30 mins in a water bath (37°C),
and immediately incubated for 10 mins in 0.1 M borate buffer (pH 8.5) and then incubated for 48 hours at
4°C in a rat anti-BrdU primary (1:250; Roche Diagnostic), followed by a donkey anti-rat secondary (Cy3;
1:200; Invitrogen). Tissue was mounted and cover slipped with the anti-fade agent, as stated above.

No immunoreactivity was observed in any of the tissue processed when the primary antibody was
omitted.

Microscopy

An experimenter blind to treatment conditions counted BrdU-labelled cells in the GCL and the
hilus at 1000x magnification on a light microscope (Nikon Eclipse 600). All BrdU-labelled cells were
counted bilaterally through the entire dentate gyrus (13-15 sections in total). BrdU-labelled cells observed
within 50µm of the inner edge of the GCL (i.e., in the subgranular zone; SGZ) were combined with
counts from the GCL. Cells in the hilus were counted separately for many reasons: 1) to account for
potential changes in the blood-brain barrier permeability by treatment; 2) new neurons in the hilus are
considered ectopic; and 3) new cells in the hilus give rise to a different population of cells than new cells
in the GCL. Ki67-expressing cells were counted on all sections containing the subgranular zone. An estimate of the total number of BrdU-labelled and Ki67-expressing cells per rat was calculated by multiplying the number of cells by 10 (9). The sampling frame consisted of the entire dentate gyrus and to avoid duplication of counting, cells in the uppermost plane of focus were not counted. Areas of each section were quantified from digitized images for both the GCL and hilus. Volume estimate was calculated by multiplying the sum of the area of each section by the section thickness (Cavalieri’s principle).

In order to determine whether androgens increase neurogenesis directly in the DG by acting on immature neurons, we examined whether DCX-expressing cells were double labeled for ARs. Fifty DCX-expressing neurons were randomly selected from the GCL of each rat. The percentage of DCX-expressing cells colabelled with AR was determined at a magnification of 600x. Doublecortin is a cytoplasmic protein expressed from day 1-21 after proliferation (29) and because of this, DCX-expressing cells display a wide range of the maturation and extent of processes. We thus examined the morphology of the processes of DCX-expressing cells and classified them into 1 of 3 categories based on criteria established by (30) and used in a number of studies (31, 32, 33, 34). Briefly, DCX-expressing cells were classified as 1) “immature” if they displayed no or very short processes (<10µm); 2) “intermediate” if they displayed longer processes than the immature cells, but the processes only reached within the GCL or touched the molecular cell layer but did not extend further or 3) “postmitotic” if a single thick dendrite extended and branched into the molecular layer or if the dendrites were fine and displayed multiple branch points within the GCL.

In addition to examining AR immunofluorescence in DCX-expressing cells, we also assessed AR immunofluorescence in the CA1 and CA3 regions of the hippocampus, the ventromedial hypothalamus (VMH), and the posterodorsal division of the medial amygdala (MePD) using a 4 point subjective rating scale (robust-+++; intermediate-++; light-+, absent-0). The distribution of AR in these areas served as a positive control and the subjective ratings of the immunofluorescence were compared to previously studies (13, 14).
To determine if BrdU-labelled cells were of a neuronal phenotype, 50 cells from each animal were randomly selected from the GCL (25 from dorsal, 25 from ventral DG) and the percentage of BrdU-labelled cells colabelled with the neuron specific marker, NeuN, was determined at 600x magnification. The total number of BrdU-labeled cells was then multiplied by the percentage of BrdU/NeuN labeled neurons, for an estimation of “total neurogenesis” (35, 36).

Statistical Analyses

For Experiment 1, total BrdU-labelled and Ki67-expressing cells, the volume of the GCL and hilus were each analyzed separately using repeated measures analysis of variance (RM-ANOVA) with brain region (GCL, hilus) as a within-subjects factor and androgen treatment (DHT, Oil) and anti-androgen treatment (flutamide, Oil) as between-subjects factors. The phenotypes of DCX-expressing cells were represented as a percent and were thus arc sine transformed to facilitate statistical analysis. A RM-ANOVA was run on the transformed values with phenotype (immature, intermediate, mature) as a within-subjects factor and androgen treatment and anti-androgen treatment as between-subjects factors. The mass of the BC/LA muscles and net neurogenesis (percent BrdU/NeuN double labeled neurons x total BrdU labeled cells) were analyzed using a univariate ANOVA with androgen treatment and anti-androgen treatment as between-subjects factors. For Experiment 2, RM-ANOVA was used to separately analyze volume and total BrdU-labelled cells with brain region (GCL, hilus) as a within subjects factor and treatment (Testosterone, blank) and genotype (wild type male, TFM) as between-subjects factors. Test statistics were considered significant if their probability value was ≤ 0.05. Where appropriate, post hoc analyses were carried out using the Neuman-Keul’s procedure.

Results

Experiment 1:

1. Androgen treatment did not affect the volume of the DG in wild type males and TFMs.

As expected, the volume of the hilus was larger compared to the volume of the GCL (main effect of region: F (1, 11)=130.3,p<0.0001). However there were no other significant main or interaction effects of androgens on the volume of the GCL or hilus (all p’s >0.26; see Table 1).
2. Testosterone implants increased cell survival in wild type males, but not in TFM.

Testosterone-treated wild type males had more BrdU-labelled cells in the GCL+SGZ compared to all other groups (interaction between region, genotype, and androgen treatment: F (1, 11)=6.13, p=0.03; all p’s < p=0.008). Androgen treatment was completely ineffective at increasing cell survival in the GCL+SGZ of TFM-affected males, as the number of BrdU-labelled cells in the testosterone-treated TFM-affected males did not differ significantly from the blank-treated TFM-affected males (p=0.59). Finally, the blank-treated wild type males did not differ from both the testosterone-implanted and blank-implanted TFM-affected males on the number of BrdU-labelled cells (all p’s > 0.8). There were no other significant main or interaction effects (all p’s > 0.07). See Figure 2).

Experiment 2:

1. There were no significant group differences in the volume of the DG.

As expected, the hilus was larger than the dentate gyrus (main effect of region: p<0.0001), however, there were no other main or interaction effects (all p’s > .36; see Table 2).

2. DHT increased BC/LA muscle mass, an effect that was blocked by flutamide.

We examined the muscle mass of the highly androgen sensitive perineal muscles (37), the BC/LA, to ensure our dose of flutamide (2.5mg/rat/day) was able to block the effects of DHT. As expected, the mass of the BC/LA muscle in the DHT treated group was significantly higher compared to all other groups (all p’s<0.0001; interaction of androgen and anti-androgen treatment (F (1, 19)=62.95, p<0.0001). Flutamide alone did not have any anabolic effects on muscle mass, as the weights did not differ significantly compared to the oil treated group (p=0.21). See Table 2.

3. DHT treatment increased the number of BrdU-labelled cells in the GCL, an effect that was blocked by co-treatment with flutamide.

We examined the total number of BrdU-labeled cells in the GCL+SGZ and hilus following 30 days of treatment of either oil, DHT, DHT + flutamide, or flutamide in order to determine whether androgens increased BrdU-labeled cells by acting directly through ARs. Systemic injections of DHT increased the total number of BrdU-labelled cells in the GCL+SGZ compared to the oil-treated castrates.
(p=0.0002) and as we hypothesized, flutamide treatment blocked the ability of DHT to increase cell survival in the GCL+SGZ (p=0.0003: interaction between region, androgen treatment, and anti androgen treatment: F (1, 19) =4.68, p=0.04). However, when administered alone, flutamide had no significant effect on the total number of BrdU-labeled cells in the GCL+SGZ (vs oil treated group; p=0.86).

Androgen or anti androgen treatment did not affect the total number of BrdU-labelled cells in the hilus compared to oil controls (all p’s >0.9) (see Figure 3A and 3D). We have also analyzed the average number of BrdU-labeled cells per section, and the pattern of results is the same as for the analysis of the total number of cells. That is, DHT increased the average number of BrdU-labeled cells per section and flutamide blocked this androgenic increase (data not shown).

4. Flutamide, but not DHT exposure, reduced the percentage of BrdU-labeled cells that were colabelled with NeuN.

We examined the colabelling of BrdU and NeuN (BrdU/NeuN) in the GCL to determine how many BrdU-labeled cells were neurons. Approximately 79-84% were colabelled with the neuron specific marker, NeuN, suggesting that the majority of BrdU-labelled cells were neurons (see Table 3). Flutamide treatment reduced the percentage of BrdU/NeuN labeled cells compared to oil treated controls (main effect of anti-androgen treatment: F (1)=9.1, p=0.007). However, there were no other significant effects (all p’s>0.15) and DHT treatment did not affect the percentage of BrdU/NeuN labeled cells (p=0.29; see Table 3).

5. DHT treatment increased the number of neurons in the GCL, but this effect was blocked by coadministration with flutamide.

To obtain an estimation of the overall number of neurons produced in the adult GCL (i.e., “net neurogenesis”) we multiplied the total number of BrdU-labeled cells by the proportion of BrdU-labeled cells that also expressed the neuron specific marker, NeuN (35, 36). There were more new neurons (BrdU/NeuN-labeled) in the DHT treated males compared to the DHT + flutamide, flutamide, and oil treated groups (all p’s <0.01; Table 3; interaction between androgen and anti-androgen treatment: F (1,19)=5.6, p=0.029).
30. Thirty days of DHT treatment did not significantly affect cell proliferation in the DG.

In order to determine whether systemic DHT administration affected cell proliferation, we examined the total number of Ki67-expressing cells in the GCL+SGZ and the hilus. In contrast to the effects of androgen treatment on cell survival, there were no significant main or interaction effects on the total number of Ki67-expressing cells (all p’s >0.4; Figure 3B, 3D).

7. DHT administration did not significantly affect the proportion of doublecortin-expressing immature neurons displaying immature, intermediate or mature processes in the GCL.

We examined the morphology of the processes of DCX-expressing neurons in the GCL in order to determine whether DHT treatment affected the dendritic maturation of these neurons. The proportion of DCX-expressing neurons displaying the mature phenotype (~49%) was significantly higher compared to the immature (~31%; p= 0.013) and intermediate (~20%; p= 0.0006) phenotypes (main effect of phenotype: F (2, 36)= 8.66, p= 0.0008, all other main or interaction effects (all p’s> 0.13). Figure 4 (A-F, K-M) depict examples of DCX-expressing immature neurons in the GCL. Figure 4N contains a histogram of the percentages of DCX phenotypes expressed by each treatment group.

8. No ARs were observed in DCX-expressing neurons in the DG.

In order to determine whether androgens increase neurogenesis in the DG by acting directly upon immature neurons in the adult hippocampus, we examined the colocalization of DCX and AR in the GCL. None of the DCX-expressing neurons displayed nuclear AR immunofluorescence (see Figure 4G-J). In fact, there were no nuclear AR expressing cells throughout the entire extent of the DG. However, DHT-treated males displayed robust AR immunofluorescence in the CA1 (see Figure 5B) of the hippocampus and the ventromedial hypothalamus (VMH). While AR immunofluorescence was also observed in the CA3 (see Figure 5D that depicts AR immunofluorescence in the CA3 of a representative animal treated with DHT) and in the medial amygdala, it was not as robust compared to the CA1 and VMH. Conversely, androgen receptor expression was completely absent in these regions of oil-, flutamide-, and DHT+ flutamide-treated groups (see Figure 5E that depicts a lack of AR immunofluorescence in the CA3 of a representative animal treated with DHT+ flutamide).
Discussion

This is the first study to provide direct evidence that androgens affect survival of newborn neurons in the hippocampus of adult male rats by acting directly through the AR using both genetic and pharmacological methods. First, we replicate a previous report showing that systemic DHT treatment increased the survival of new neurons in the DG (9). Second, we extend these results by showing that the survival promoting effects of DHT on neurogenesis can be blocked via systemic injection of the AR specific antagonist, flutamide. We further demonstrate that a competent AR is necessary for androgen-induced neurogenesis in the hippocampus, as chromosomal males carrying a mutation in the gene encoding the androgen receptor (TFM-affected males) do not show an androgen-induced increase in neurogenesis compared to wild type (wt) males. Finally, given our observation that ARs are not expressed by newborn neurons in the DG, the data support the conclusion that androgens mediate the survival of new neurons by acting on ARs somewhere other than the DG.

Androgens increase the survival of new neurons in the DG

In the current report, androgen treatment (both DHT and T) in adult wildtype male rats increased the survival of new neurons in the DG. In Experiment 2 BrdU was injected 24 h prior to hormone treatment, thus BrdU-labeled cells would have been daughter cells at the time of the first androgen treatment as the length of the cell cycle is approximately 24 h (28). There was no effect of castration or DHT treatment after 30 days on cell proliferation (Ki67-expressing cells) in the DG. This is consistent with previous reports that showed short- or long-term castration did not significantly affect cell proliferation in the hippocampus (9, 38, 39). DHT treatment also did not alter the phenotype of DCX expressing cells or the percentage of cells double-labeled for BrdU and NeuN. Together these data suggest androgens increase adult neurogenesis in the DG by affecting neuron survival independent of affecting cell proliferation or differentiation of new neurons.

The duration of androgen administration is an important factor in promoting the survival of new neurons. We found that 30 days of systemic androgen treatment increased cell survival in the DG of adult male rats (current study, and 9). However, a shorter duration (15 days) of androgen treatment did not
enhance DG neurogenesis (40), suggesting that the optimal time period for androgens to increase cell survival is between 16-30 days, and given the time frame involved, likely via a genomic mechanism. 

Androgens Increase Neurogenesis via the Androgen Receptor

Flutamide, a competitive AR antagonist, blocked the ability of DHT to increase both the total number of BrdU-labeled cells and the overall number of neurons generated in the GCL (i.e., “net neurogenesis”). Given that flutamide prevented the DHT induced increase in the mass of the BC/LA muscles, and blocked the expression of ARs in the hippocampus, amygdala, and ventromedial hypothalamus, all which are AR mediated effects (14, 41, 42, 43) suggests our dose of flutamide was effective in antagonizing ARs systemically. Thus, data from Experiment 2 support the conclusion that androgens mediate adult neurogenesis in the GCL via the AR and not through the transformation of DHT to a metabolite that acts via GABAa or progesterone receptors.

Repeated estradiol administration in adulthood results in a decrease in cell survival in female, but not in male, rats (8), suggesting a sex difference in the neurogenic response to estradiol. Given the lack of an effect of chronic estradiol on hippocampal neurogenesis in adult males in adulthood strongly suggests androgens may have an organizational (i.e., developmental) effect in determining the response to estradiol. Further, given that the androgen receptor plays an important role in the masculinization of sexually dimorphic structures in the nervous system, and that TFM males do not have a functional androgen receptor throughout their entire life, one possibility that could account for the results in Experiment 1 is that TFM-affected males may have responded in a feminine manner to estradiol. In the current study, we used capsules filled with testosterone propionate, and thus estradiol would have been formed in both males and TFMs despite a non-functional AR protein (see 44). If TFMs are feminine in their response to estradiol, then the overall lower levels of neurogenesis observed in the TFMs compared to wildtype males may have been due to a decrease in cell survival due to a lack of AR stimulation as we have proposed or the effects of estradiol to decrease neurogenesis in TFMs. It has previously been reported that certain regions of the TFM nervous system are not fully masculine (45), however, some aspects are fully masculinized despite the mutation in the AR gene (reviewed in 46). What is more, there
are no published reports suggesting the dentate gyrus in TFM is feminine and thus we have no reason to suspect a feminine response to estradiol. Finally, given that the results in Experiment 1 and Experiment 2 are similar despite the different androgens used, we conclude androgens mediate new neuron survival in the DG via the androgen receptor.

Adult Born Immature Neurons Do Not Express Androgen Receptors.

In the current report, we observed nuclear AR immunoreactivity in the medial amygdala, VMH, CA1 and CA3 regions but not within the DG. This is consistent with a variety of published reports showing that both nuclear AR mRNA and protein are expressed in the CA1/CA3 regions of the hippocampus, the amygdala, and hypothalamus, but not in the DG (13, 14, 47, 48). To our knowledge, this is the first study to examine whether immature neurons express ARs in the DG. We did not find evidence of DCX and AR colabelling indicating that immature neurons do not express ARs. While it is possible that ARs are transiently expressed within immature neurons, given that DCX is expressed anywhere from 1-21 days after birth of the neuron, we believe that this is not the case. Furthermore we did not observe AR immunofluorescence independently of DCX in the SGZ, thus, we are confident ARs are not expressed within the DG.

Given that we did not observe AR expression in immature neurons or elsewhere in the DG leads to the parsimonious conclusion that androgens act in a non cell-autonomous manner, and in another region to affect neuron survival. The CA3 region is of interest as newborn granule neurons extend axons to CA3 pyramidal cells within 4 days after mitosis (49), CA3 pyramidal cells contain ARs, and destruction of this region decreases survival, but not proliferation, of new neurons in the DG (50). All of these findings suggest that the CA3 is an important region regulating adult hippocampal neurogenesis.

Further, terminals from granule neurons synapse on to specialized structures of CA3 pyramidal neurons, termed thorny excrescences (51). The induction of thorns is dependent upon AR as an AR antagonist (hydroxyflutamide) blocks the ability of androgens to induce production of thorns (51). Thus, androgens appear to be coordinating the necessary structural changes in CA3 that may lead to increased survival of newborn neurons.
How do androgens increase new neuron survival in the DG via a non cell autonomous mechanism? We propose that androgens act upon neurons in the CA3 region of the hippocampus, which then send an as yet unidentified retrograde survival factor to newborn neurons in the DG. A similar proposal of non cell-autonomous action has been put forth for the androgen dependent survival of motor neurons in the spinal nucleus of the bulbocavernosus (SNB), and thus may be a common mechanism of androgen action in the nervous system. Androgens act on the target muscles, BC/LA, to spare SNB motor neurons from programmed cell death during development, possibly via ciliary neurotrophic factor (52).

It should be noted that other hormones affect neurogenesis in the adult dentate gyrus via regulation of neuron survival. Dehydroepiandrosterone (DHEA) can bind to ARs (53) and can increase neurogenesis by affecting survival, similar to androgens, but can also affect proliferation, unlike androgens (54). In male mice, 3 days of injections of progesterone increases the survival of 7, 28 and 56 day old neurons (55), while an escalating dose of progesterone can also stimulate cell proliferation in male rats (56). Finally, chronic estradiol (15 days) increases new neuron survival in the hippocampus in female, but not male, rats (57), although 5 days of exposure to estradiol can increase neurogenesis in male meadow voles when administered 6-10 days after BrdU (58). Interestingly, a percentage of newborn neurons can express estrogen receptors suggesting estradiol may regulate survival directly (59). Intriguingly there is a sex difference in the percentage of new neurons that express estrogen receptors (59, 60). For a more detailed review the reader is directed to Galea et al (61).

Conclusions

We provide evidence that androgens promote neurogenesis in the adult hippocampus by increasing the survival of newborn neurons via an androgen receptor dependent mechanism. However, androgens did not significantly affect cell proliferation, the morphology of immature neurons or the percentage of new cells differentiating into neurons in the adult hippocampus. The data support the conclusion that androgens do not promote neurogenesis by acting directly on adult born neurons, but instead act via a cell non-autonomous process that is accomplished outside of the dentate gyrus. Determining how, why and where androgens are working to promote adult neurogenesis in the
hippocampus may prove important in developing new therapeutic treatments for neurodegenerative disease.
Acknowledgements

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44. Roselli CE, Salisbury RL, Resko JA. Genetic evidence for androgen-dependent and independent


54. Karishma KK, Herbert J (2002) Dehydroepiandrosterone (DHEA) stimulates neurogenesis in the hippocampus of the rat, promotes survival of newly formed neurons and prevents corticosterone-induced


Figure Captions

**Figure 1.** Graphical depiction of the protocol used in Experiment 2.

Males were castrated at 60 days of age and allowed to recover for 1 week. Following recovery, animals were injected with bromodeoxyuridine (BrdU), and then treated with either oil, flutamide, dihydrotestosterone, or dihydrotestosterone and flutamide for 30 days. Animals were perfused and brains and bulbocavernosus/levator ani muscles were harvested at day 100.

**Figure 2.** Testosterone implants increased the total number of BrdU labeling in wild type males only.

Mean total BrdU-labelled cells in wild type males (Male) implanted with either testosterone propionate (TP) or blank capsules (Bl) and chromosomal males carrying the testicular feminization mutation (TFMs) implanted with either TP or Bl capsules. TP was only effective in increasing cell survival in the granule cell layer (GCL) of males; there was no effect of TP on survival in TFMs. *= significantly different compared to Bl males, TP TFM, and Bl TFM.

**Figure 3.** Antagonism of the androgen receptor (AR) by flutamide decreased the survival of cells in the granule cell layer.

A) Mean total BrdU-labelling in the dentate gyrus and subgranular zone (DG + SGZ) of animals treated with oil, flutamide (Flut), dihydrotestosterone (DHT), or dihydrotestosterone and flutamide (DHT + Flut). DHT treatment increased the number of BrdU-labelled cells compared to all other groups (compared to oil-treated group (p<0.0002), DHT+Flut (p<0.0003), Flut (p<.0003)). The ability of DHT to increase the number of BrdU-labelled cells was blocked by co-treatment with flutamide. *= significantly different compared to all other groups. B) Mean total number of Ki67 labeled cells in the granule cell layer (GCL) and hilus. There were no significant effects of DHT or flutamide treatment on cell proliferation in the subgranular zone (SGZ) or hilus. C) The colocalization of the neuron specific marker, NeuN (arrow), with bromodeoxyuridine (BrdU; arrow) in the same cell from a representative animal suggests this is a new adult born neuron. Scale bar = 20μm. D) Representative examples of BrdU (arrow in first panel) and Ki67 (arrow in second panel) immunoreactivity in the GCL and SGZ, respectively. Scale bar = 20μm.
Figure 4. Dihydrotestosterone treatment did not affect the ratio of immature, intermediate, or mature doublecortin expressing neurons. Panels A and B- Low power (using a 10X objective) photomicrograph of representative DCX immunofluorescence in a male treated with dihydrotestosterone (A) and a male treated with dihydrotestosterone and flutamide (B; scale bars = 200um). Arrows (A) and arrowheads (B) point to examples of DCX expressing neurons in the GCL. Panels C, D, E, and F- the same DCX expressing neurons from A and B magnified 400x. Scale bar = 20um. Panels G, H, I, and J- no AR immunofluorescence was found in any of the exemplar DCX expressing neurons. Scale bar = 20um.

Panels K, L, M- representative examples of neurons used in classifying DCX expressing neurons as (K) “immature”, (L) “intermediate”, and (M) “mature”. Panel N Doublecortin morphology of immature neurons in the granule cell layer (GCL) of males treated with Oil, flutamide (Flut), Dihydrotestosterone (DHT), and DHT and flutamide (DHT+Flut). There were no statistically significant differences between the groups in the percentage of cells displaying the immature, intermediate, or mature phenotypes. Note: K and L were taken using a 40X objective and M was taken using a 100X oil immersion objective.

Figure 5. Androgen receptor (AR) immunofluorescence in the CA1 and CA3 regions of the hippocampus. Panels A, B, and C- Representative low power photomicrograph of doublecortin (DCX; A) and androgen receptor (AR; B) immunofluorescence in the hippocampus. The arrow in A points to neurons in the granule cell layer (GCL) expressing DCX from a male treated with dihydrotestosterone (DHT). The arrowheads in B point to nuclear AR immunofluorescence localized to the pyramidal cell layer of the CA1/CA3 region of the hippocampus in the same DHT treated male. Panel C is the overlay of DCX and AR immunofluorescence. Scale bar = 500um. Panels D and E- representative nuclear AR immunofluorescence in pyramidal cells of CA3 in a male treated with DHT (D) and a lack of AR immunofluorescence in a male treated with both DHT and flutamide (E). Arrowheads in E point to presumptive CA3 region. Scale bar = 200um.
Table 1. Volume of the granule cell layer (GCL) and Hilus of animals in Experiment 1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>GCL (mm$^3$)</th>
<th>Hilus (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Testosterone</td>
<td>2.9±0.27</td>
<td>7.0±0.87</td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>3.3±0.31</td>
<td>9.2±0.90</td>
</tr>
<tr>
<td>TFM</td>
<td>Testosterone</td>
<td>3.1±0.27</td>
<td>8.1±0.93</td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>3.1±0.31</td>
<td>8.5±1.10</td>
</tr>
</tbody>
</table>

There were no statistically significant differences in the volume of both the GCL and hilus among groups. As expected, the volume of the hilus was larger than the GCL.
Table 2. Volume (mean+/-S.E.M.) of the granule cell layer (GCL), Hilus of animals, and weight (mean+/-S.E.M.) of the bulbocavernosus/levator ani muscle (BC/LA) in Experiment 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GCL (mm$^3$)</th>
<th>Hilus (mm$^3$)</th>
<th>BC/LA (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil (n=6)</td>
<td>2.6±0.17</td>
<td>7.7±0.55</td>
<td>0.36±0.03</td>
</tr>
<tr>
<td>Flut (n=6)</td>
<td>2.4±0.17</td>
<td>6.8±0.55</td>
<td>0.44±0.04</td>
</tr>
<tr>
<td>DHT (n=5)</td>
<td>2.6±0.19</td>
<td>6.9±0.60</td>
<td>1.05±0.05 *</td>
</tr>
<tr>
<td>DHT+Flut (n=6)</td>
<td>2.3±0.17</td>
<td>6.6±0.55</td>
<td>0.46±0.04</td>
</tr>
</tbody>
</table>

There were no statistically significant differences in the volume of both the GCL and hilus among the groups in Experiment 2, however, the volume of the hilus was larger than the GCL. The mass of the BC/LA muscle (in grams; gm) was higher in the DHT treated group compared to the oil treated group. Flutamide alone did not affect the mass of the BC/LA weight, however, flutamide blocked the anabolic effects of DHT on BC/LA muscle mass. Statistical analysis revealed the mass of the BC/LA was highest in the DHT treated males compared to all other groups. The mass of the BC/LA did not differ between the oil-, flutamide-, and DHT + flutamide groups. Note: * = p<0.001 for all post hoc comparisons.
Table 3. Percentage (mean +/- S.E.M.) of BrdU-labeled cells colabeled with NeuN and Net Neurogenesis (mean +/- S.E.M.) in the granule cell layer of the dentate gyrus.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BrdU/NeuN (%)</th>
<th>Net Neurogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil (n=6)</td>
<td>84.33 +/- 0.01</td>
<td>3044.55 +/- 387.07</td>
</tr>
<tr>
<td>Flutamide (n=6)</td>
<td>82.67 +/- 0.01</td>
<td>2811.90 +/- 408.10</td>
</tr>
<tr>
<td>DHT (n=5)</td>
<td>84.80 +/- 0.01</td>
<td>4881.21 +/- 571.24 *</td>
</tr>
<tr>
<td>DHT + Flutamide (N=6)</td>
<td>79.43 +/- 0.01</td>
<td>2753.05 +/- 224.36</td>
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</table>

The average number of neurons (i.e., BrdU/NeuN labeled) reaching survival was lowest in the groups that received flutamide. Overall, the number of neurons produced (percent BrdU/NeuN x total BrdU labeled cells = net neurogenesis) was highest in the animals that received DHT compared to the other groups. Note: * = p < 0.001 for all post hoc comparisons regarding “Net Neurogenesis”.
Figure 1

- GDX: day 60
- BrdU: day 67
- Treatment: days 68 to 69
- Perfusion: days 99 to 100
Figure 2

Mean Total BrdU-labelled Cells

- **GCL+SGZ**
- **Hilus**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
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<tbody>
<tr>
<td>TP Male</td>
<td>3,000</td>
</tr>
<tr>
<td>BI Male</td>
<td>1,500</td>
</tr>
<tr>
<td>TP TFM</td>
<td>1,500</td>
</tr>
<tr>
<td>BI TFM</td>
<td>1,000</td>
</tr>
</tbody>
</table>

*Indicates a significant difference.
Figure 3

A

Mean Total BrdU-labelled cells

<table>
<thead>
<tr>
<th></th>
<th>Oil</th>
<th>Flut</th>
<th>DHT</th>
<th>DHT+Flut</th>
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</thead>
<tbody>
<tr>
<td>GCL+SGZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hilus</td>
<td></td>
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</tbody>
</table>

B

Mean Total Ki67-expressing cells

<table>
<thead>
<tr>
<th></th>
<th>Oil</th>
<th>Flut</th>
<th>DHT</th>
<th>DHT+Flut</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCL+SGZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hilus</td>
<td></td>
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</tr>
</tbody>
</table>

C

NeuN

BrdU

Overlay

D

hilus

GCL

hilus

GCL
Figure 5

A. DCX
B. AR
C. DCX + AR
D. AR
E. CA3