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Androgen and estrogen receptor mediated mechanisms of testosterone action in male rat pelvic autonomic ganglia

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Abstract

Although male reproductive function is primarily androgen dependent, many studies suggest that estrogens have direct actions on the male reproductive organs. Pelvic autonomic neurons provide the motor control of the internal reproductive organs and the penis and various properties of these neurons are affected by endogenous androgens. However, the possible role of estrogens at this site has not been examined. Here we have investigated the significance of estrogens produced by aromatisation of testosterone in the physiological actions of androgens on adult male rat pelvic ganglion neurons. RT-PCR studies showed that aromatase and both estrogen receptors (ER α and ER β) are expressed in these ganglia. Western blotting also showed that aromatase is expressed in male pelvic ganglia. Using immunohistochemical visualisation, ER α was predominantly expressed by nitric oxide synthase (NOS)-positive parasympathetic pelvic ganglion neurons. *In vivo* studies showed that the decrease in pelvic ganglion soma size caused by gonadectomy could be prevented by administration of testosterone (T) or dihydrotestosterone (DHT), but not 17 β -estradiol (E2), showing that this maintenance action of testosterone is mediated entirely by androgenic mechanisms. However, *in vitro* studies of cultured pelvic ganglion neurons revealed that T, DHT and E each stimulated the growth of longer and more complex neurites in both noradrenergic and cholinergic NOS-expressing neurons. The effects of T were attenuated by either androgen or estrogen receptor antagonists, or by inhibition of aromatase. Together these studies demonstrate that estrogens are likely to be synthesised in the male pelvic ganglia, produced from testosterone by local aromatase. The effects of androgens on axonal growth are likely to be at least partly mediated by estrogenic mechanisms, which may be important for understanding disease-, aging- and injury-induced plasticity in this part of the nervous system.

Keywords

Steroid; autonomic ganglion; urogenital; regeneration

Recent studies suggest that within the framework of androgen dependence of male reproductive function, endogenous estrogens produced by aromatisation of testosterone within local tissues

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are important for development and function of male reproductive organs. Many of the male reproductive organs express aromatase (a member of the cytochrome P450 family encoded by the *CYP19* gene) to provide a local source of estrogens from circulating androgens; these estrogens directly influence various aspects of reproductive physiology and pathology (Simpson et al., 2002). For several decades it has been known that establishment of sexual dimorphisms in the rodent brain that underlie gender-specific reproductive behaviours are also heavily dependent on the estrogens produced within particular brain regions by the actions of aromatase (reviewed by Cooke et al., 1998). Much less studied have been the pelvic autonomic neurons that provide a link between central control and organ function, determining the final motor outflow to the internal reproductive organs and the penis (Dail, 1993). Therefore, to fully understand how estrogens affect male reproductive behaviours, their role in pelvic autonomic function also needs to be addressed.

Many studies have demonstrated that testosterone influences development of the pelvic autonomic ganglia and their projections to urogenital targets, and their maturation at puberty (reviewed by Keast, 1999,2006). In adults, gonadectomy studies have revealed that testosterone continues to affect the structure, chemistry and function of adult pelvic autonomic neurons. This indicates the potential for considerable steroid-related plasticity of motor control in the reproductive organs, particularly during ageing, endocrine disorders or administration of steroid-related pharmaceutical agents (e.g. anti-androgens) (Keast, 1999,2006). While the effects of androgens on adult pelvic autonomic neurons are now well documented, their mechanisms have only received limited attention. Testosterone administration from the time of gonadectomy prevents the profound effects of androgen-deprivation on pelvic ganglion soma size (Melvin et al., 1988;Keast, 1999), electrophysiological properties (Kanjhan et al., 2003), target innervation density (Keast, 1999) and transmitter synthesis (Hamill et al., 1984;Hamill and Schroeder, 1990;Keast, 1999). Therefore androgen production is necessary for the maintenance of these properties. However these studies do not shed light on whether androgen or estrogen actually mediates testosterone effects. This could be determined by instead administering estradiol from the time of castration. Studies of this type are limited, but have shown that the gonadectomy-induced decrease in tyrosine hydroxylase (TH) expression in pelvic ganglia is prevented by testosterone but not estradiol administration (Hamill et al., 1984;Hamill and Schroeder, 1990).

Another clue to the mechanism of testosterone action in adult pelvic ganglia would be provided by expression of androgen receptors (ARs), estrogen receptors (ERs) or both receptor types by the ganglion neurons. In rats, ARs are expressed by many adult male pelvic ganglion neurons (Schirar et al., 1997;Keast and Saunders, 1998) and in culture these neurons respond to testosterone with soma and neurite growth (Meusburger and Keast, 2001). However if pelvic ganglion cells express aromatase and ERs, then it is possible that at least some androgen actions are mediated by estradiol. In male rats, ER α is expressed in cultured pelvic ganglion neurons (Purves-Tyson and Keast, 2004) and ER β has been reported in pelvic ganglion tissue sections (Mäkelä et al., 2000;Salmi et al., 2001). Therefore both receptor types could potentially be involved in mediating the actions of testosterone.

In this study we have first investigated key components of estrogenic signalling in male rat pelvic ganglia, by using RT-PCR to determine whether ER α , ER β and aromatase transcripts are present in the adult. We have also performed Western blotting analysis of aromatase and immunohistochemical analysis of ER α expression. We then carried out both *in vivo* and *in vitro* studies to determine whether estrogens are likely to mediate the actions of androgens on growth of adult male rat pelvic autonomic neurons. For our *in vivo* study we compared the effects of 17 β -estradiol (E2), testosterone (T) and dihydrotestosterone (DHT; a non-aromatisable androgen) replacement on the gonadectomy-induced decrease in soma size, a robust indicator of testosterone action on pelvic ganglion neurons (Melvin et al., 1988;Melvin

et al., 1989; Keast and Saunders, 1998). Our *in vitro* studies used cultures of pelvic ganglion neurons to compare the actions of these three steroids on neurite growth, neuronal chemistry and survival. We also used androgen- and estrogen receptor antagonists to determine the involvement of each receptor type in the testosterone response, as well as testing the effects of an aromatase inhibitor on the testosterone response.

In both *in vivo* and *in vitro* experiments we compared two chemically and physiologically distinct classes of neurons to distinguish possible differences in steroid sensitivity. Tyrosine hydroxylase (TH) immunoreactivity was used to identify noradrenergic neurons; these neurons innervate smooth muscle within the reproductive tract (Keast and de Groat, 1989; Keast, 1992b; Kepper and Keast, 1997). Nitric oxide synthase (NOS) immunoreactivity labelled cholinergic neurons that innervate the glandular tissues of the reproductive tract and cavernosal tissue of the penis (Domoto and Tsumori, 1994; Schirar et al., 1994; Dail et al., 1995).

EXPERIMENTAL PROCEDURES

Animal use

All procedures were approved by the institutional ethics committee, as required by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia). Adult male outbred Wistar rats were used for all experiments. A total of 82 animals were used for this study.

Aromatase and ER expression in pelvic ganglion

For removal of tissues for RT-PCR or Western blotting studies, animals were deeply anaesthetised with sodium pentobarbitone (48mg/kg, i.p.) and pelvic ganglia dissected. Animals were exsanguinated and for RT-PCR studies, prostate tissue and skeletal muscle (mid thigh) were also removed. For RT-PCR, tissues were homogenised with a Polytron (Kinematika AB, Luzern) in Trizol reagent (750 µl; Invitrogen, Carlsbad, CA, USA) and RNA extracted according to the manufacturers protocol. RNA (1 µg) was digested with deoxyribonuclease I (amplification grade; Sigma, Castle Hill, NSW, Australia) prior to reverse transcription with SuperScript III reverse transcriptase and Oligo(dT)20 and random hexamer primers (Invitrogen) for 1 hr at 42°C. Aromatase cDNA (102 bp product) was detected with the following primers: forward “TAT TGG AAA TGC TGA TTG CGG”; and reverse, “CGA TGT ACT TCC CAG CAC AG” adapted from (Jesmin et al., 2004; Yague et al., 2004). cDNA was subjected to 43 cycles using 56°C as the annealing temperature (Mastercycler, Eppendorf, Hamburg, Germany). PCR product was run on 2.2% agarose gels alongside a 100 bp ladder (Trackit 100bp DNA ladder, Invitrogen). GAPDH primers (Katakam et al., 2005) were used to amplify GAPDH cDNA to serve as a positive PCR control using the same PCR protocol (Forward “AGA CAG CCG CAT CTT CTT GT”; reverse “CTT GCC GTG GGT AGA GTC AT”; product 207 bp). ERα (Forward: “AAT TCT GAC AAT CGA CGC CAG”; reverse “GTG CTT CAA CAT TCT CCC TCC TC”) and ERβ (Forward: “TTC CCG GCA GCA CCA GTA ACC”; reverse “TCC CTC TTT GCG TTT GGA CTA”) cDNAs (345 bp and 262 bp respectively) were detected using the primers of (Kuiper et al., 1997) and the same PCR protocol as described above.

Western blotting was performed as previously described (Purves et al., 2001). Briefly, pelvic ganglia were removed from three adult male rats and homogenized in 500 µl chilled lysis buffer containing a standard protease inhibitor cocktail (Complete Mini; Roche, Castle Hill, NSW, Australia). After centrifugation, supernatant was decanted and aliquots containing ~10 µg protein were loaded on 10% sodium dodecyl sulfate-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, and blocked with 5% non-fat milk solution for 1 hr at room temperature. The membranes were then washed and incubated overnight at 4°C with a

primary antibody raised against a peptide mapping near the C-terminus of human CYP19 (aromatase) (1:4000; Santa Cruz Biotechnology Inc., Santa Cruz, CA) raised in goat. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-goat IgG (1:50,000; Jackson ImmunoResearch) for 1hr at room temperature. Immunoreactivity was visualized using a chemiluminescent reagent (LumiGLO; Cell Signalling, Danvers, MA).

For immunohistochemical analysis of ER α expression animals were deeply anaesthetized with sodium pentobarbitone (48 mg/kg, i.p.) and perfused transcardially with 4% phosphate-buffered paraformaldehyde (PFA; pH 7.2). Pelvic ganglia were removed and postfixed in 4% PFA overnight, then stored in 0.1M phosphate-buffered saline (PBS). Ganglia were cryoprotected overnight in PBS containing 30% sucrose and embedded in an inert mounting medium (OCT Tissue-Tek, Sakura, Torrance, CA, USA) and frozen at -20°C. Ganglia were cryosectioned (14 μ m) and 6-7 sections at least 60 μ m apart collected onto 1% gelatinised slides. Sections were air-dried, washed with PBS, blocked and permeabilised for 1 hr with 10% horse serum (JRH Biosciences, Brooklyn, Vic, Australia) and 0.1% triton X-100 (Sigma) in PBS. This was followed by incubation with primary antibodies overnight and fluorescence-conjugated secondary antibodies for 2 hours, both at room temperature. The antisera used were: tyrosine hydroxylase (TH) (host species mouse; 1:2000; Diasorin, Stillwater, MN, USA); nitric oxide synthase (NOS) (host species sheep; 1:5000; gift from Dr Piers Emson, Babraham, UK and host species rabbit; 1:500; Zymed Laboratories, Invitrogen, San Francisco, CA, USA) and ER α (host species rabbit; 1:250 antibody PA1-308; Affinity Bioreagents Inc., Golden, CO, USA). Primary antibody binding was visualised with fluorophore- or biotin-conjugated species-specific secondary antibodies (rabbit Cy3, 1:1500; rabbit FITC, 1:100; sheep FITC 1:200; mouse Cy2 1:200 or mouse biotin 1:200 followed by Avidin-AMCA 1:100), all purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Slides were mounted with 0.5 M bicarbonate-buffered glycerol (pH 8.6) before coverslipping.

Sections were viewed under an Olympus BX51 fluorescence microscope. Images (8-bit monochrome) were captured using an RT Spot camera (Diagnostic Instruments, Sterling Heights, MI, USA) and digitised using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Images were colourised digitally, and contrast and brightness were adjusted where necessary to best represent the immunostaining viewed under the microscope using Adobe Photoshop (Version 8).

***In vivo* studies of steroid action on pelvic ganglion structure**

Male outbred Wistar rats (7 weeks old) were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (10 mg/kg). Animals were gonadectomised and given replacement hormone by placing a subdermal silastic implant between the shoulder blades. Implants contained T, DHT, E2 (Sigma) or were empty, as described previously (Singh et al., 1995; Bianco et al., 2002). T and DHT implants were 1 cm long, internal diameter 1.47mm, outer diameter 1.95 mm and ends sealed with silastic adhesive. Each animal was given one implant. E2 implants (0.5 cm long) contained 17 β -estradiol recrystallised after dilution 1:1000 with cholesterol and each animal was given two subdermal implants. A group of intact control animals (no gonadectomy, “naïve”) were given empty implants. These implants have been characterised extensively in previous studies (Singh et al., 1995; Bianco et al., 2002) and reliably achieve reproducible circulating steroid levels. While we did not assess circulating steroid levels in the present study, reproductive organs were weighed to indirectly indicate if physiological levels were restored after gonadectomy. In ovariectomised adult female rats E2 replacement with these silastic implants maintained uterine horn weight (data not shown).

After 4 weeks animals were anaesthetised, perfused with fixative and pelvic ganglia removed as described above. Seminal vesicles were removed and an average weight of both seminal

vesicles obtained. Pelvic ganglia were cryosectioned and immunostained for TH and NOS as described above. Soma size of TH- and NOS-immunoreactive neurons was measured by outlining nucleated soma profiles manually using the computer mouse and then calculating profile area (Image Pro Plus). Between 200 and 600 somata of each neuron type (TH, NOS) were measured in each ganglion.

Pelvic ganglion cultures

Adult male outbred Wistar rats (10-12 weeks) were deeply anaesthetized with sodium pentobarbitone, (48 mg/kg, i.p.) and pelvic ganglia removed prior to euthanasia by decapitation. Ganglia were dissociated using previously described methods (Tuttle and Steers, 1992; Purves-Tyson and Keast, 2004). Ganglia were incubated in Worthington type I collagenase (0.08%; ScimaR, Templestowe, Victoria, Australia) and 0.25% trypsin in 1mM EDTA (Invitrogen) for 2 h. Neurons were dissociated and plated onto glass coverslips coated with 500 µg/ml poly-DL-ornithine (Sigma) and 5 µg/ml laminin (Sigma) and allowed to attach for 1 hour in 100 µl Neurobasal A medium with 2% B27 supplement, 100 U penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B and 200 mM Glutamax I (all from Invitrogen) and incubated at 37°C, with 95% O₂ and 5% CO₂. After 1 hour medium was replaced with 1 ml fresh medium and treatments added.

Cultures were treated overnight (24 hr) with E2, T or DHT (Sigma) and where appropriate pre-treated (40 min) with the ER antagonist, ICI182780 (10µM; Biomol, Plymouth Meeting, PA, USA) or the AR antagonist, hydroxyflutamide (10µM; gift from Schering Plough). Some testosterone-treated cultures were instead pre-treated with the aromatase inhibitor, letrozole (100nM, kindly supplied by Novartis, Basel). Cultures were then fixed with 4% PFA for 30 min and processed for fluorescence immunocytochemistry as described above except that slides were incubated with primary antibodies against TH and NOS for 1 hr. DAPI (1µg/ml; Sigma) was used as a nuclear counterstain.

All analyses were performed on a minimum of three separate experiments for each manipulation (where n =number of separate experiments performed on cultures from different animals, with each culture containing pelvic neurons from both pelvic ganglia of two animals). All experiments had their own internal control. Each data point was obtained from one coverslip (8 coverslips per culture), as described above. Approximately 700 neurons were plated on each coverslip but not all neurons were assessed in each experiment (see below).

Immunohistochemical and structural analysis of cultured pelvic ganglion neurons

Slides were viewed using an Olympus BX51 microscope. Neuron viability was assessed visually and by DAPI staining of neuronal nuclei. Unhealthy or dead cells were easily distinguished from healthy cells and were excluded from the analysis. Under phase contrast dead cells were grey compared to phase-bright healthy neurons. Using DAPI, healthy neuronal nuclei are rounded, pale blue and the nucleoli can be clearly seen, whereas unhealthy or dead cells have condensed nuclei, brighter DAPI staining and the nucleoli cannot be seen. Neuron survival was determined by counting the number of healthy neurons per coverslip. The number of neurons per steroid-treated coverslip was then expressed as a percentage of neurons on the control (untreated) coverslip. Transmitter phenotype was assessed by calculating the proportion of all neurons on each coverslip expressing either TH or NOS. There were consistently <5% of neurons expressing both NOS and TH. This population did not change with treatment and was excluded from any further analysis. Initiation of neurite sprouting was determined by counting the proportion of neurons with at least one neurite that extended longer than the soma diameter (200-700 neurons were analysed per coverslip for each population). To quantify neurite extension (i.e. number of primary neurites, neurite branching and neurite length), images of 30-50 randomly selected neurons with neurites were captured per treatment,

using the methods described above. Images were inverted to allow better visualisation of fine processes, traced using Neurolucida and analysed using Neuroexplorer (MicroBrightField, VT, USA).

Statistical analysis

Effects of treatments on neurite outgrowth parameters were determined using one-way ANOVA (repeated measures) followed by either Dunnett's multiple comparison test or Student-Newman-Keuls multiple comparison test. Dunnett's test was used to compare all treatments with matched controls for dose-response studies, and Student-Newman-Keuls test was used to compare all treatments in experiments using antagonists. Paired Student's *t*-test was used to compare the effect of estradiol treatment. Effect of hormone replacement on soma size *in vivo* was determined using one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. All results are expressed as mean $\pm$ SEM. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Aromatase, ER α and ER β mRNA transcripts are present in male rat pelvic ganglion

RT-PCR analysis demonstrates the presence of a 102 bp aromatase band in pelvic ganglion extracts (Fig. 1A). As expected, prostate tissue expressed aromatase mRNA, but it was undetected in skeletal muscle (Fig. 1A). No band was amplified from RNA not subjected to reverse transcription, indicating that the band was not due to amplification of DNA. GAPDH (207 bp product) was amplified in prostate and pelvic ganglion tissue as a PCR positive control (Fig. 1B). RT-PCR analysis of ER α and ER β transcripts in tissue extracts yielded the expected bands of 345 bp and 262 bp respectively (Fig. 1B). Both were present in pelvic ganglion extracts but only ER β was evident in prostate extracts. Aromatase protein was identified in extracts of pelvic ganglia from three rats (Fig. 1C), each showing a single band at ~ 55 kD.

ER α is expressed primarily by NOS-positive neurons

Intact pelvic ganglia from naïve adult male rats ($n=9$) were immunostained with an antibody against ER α (Fig. 1D). The proportions of TH- and NOS-positive neurons expressing nuclear ER α were calculated (300 - 700 cells were analysed for each population in each ganglion). Many NOS-positive neurons ($39.5 \pm 4.4\%$) expressed ER α , whereas ER α expression was less common in TH neurons ($14.0 \pm 4.5\%$). The intensity of ER α -immunostaining varied widely between neurons (Fig. 1D). The brightest nuclear immunostaining was commonly found in NOS-immunoreactive neurons, but rarely present in TH neurons. Pale cytoplasmic ER-immunostaining was seen in a minority of neurons but was not quantified.

Testosterone (T) and dihydroxytestosterone (DHT) but not estradiol (E2) maintain pelvic ganglion soma size *in vivo*

Gonadectomy caused an 11-fold decrease in seminal vesicle size (37 ± 4 mg, $n=5$) when compared to intact naïve animals (421 ± 57 mg, $n=4$) (Fig 2A). This decrease in seminal vesicle size was prevented by T replacement (457 ± 12 mg, $n=6$) and partially prevented by DHT replacement (229 ± 37 mg, $n=5$) but was unaffected by E2 replacement (51 ± 5 mg, $n=6$).

NOS-positive (Fig. 2B) and TH-positive (Fig. 2C) somata were measured in pelvic ganglia from each of these animal groups. In naïve adult male rats NOS neurons had a mean soma area of $421 \pm 29 \mu\text{m}^2$ ($23 \pm 6 \mu\text{m}$ diameter) and TH neurons had a mean area of $861 \pm 34 \mu\text{m}^2$ ($33 \pm 7 \mu\text{m}$ diameter). After gonadectomy the somata of both groups decreased significantly in size (Figs. 2B,C). These decreases in soma size were prevented by T and DHT but not by E2 replacement (Figs. 2B,C).

T, DHT and E2 do not effect neuron survival, transmitter phenotype or neurite initiation *in vitro*

Neuron survival, transmitter phenotype and neurite initiation were measured in cultured pelvic ganglion neurons treated overnight with increasing concentrations of T or DHT, or E2 (10 nM). The number of neurons per steroid-treated coverslip was expressed as a percentage of neurons on the control (untreated) coverslip, as a measure of neuron survival. Steroid hormones had no significant effect on the number of NOS or TH neurons growing in each culture overnight (Table 1A), indicating that steroid hormones had no effect on neuron survival (T, n=4; DHT, n=3; E2, n=4). Analysis of the transmitter phenotype of cultured neurons, indicated by TH- and NOS-immunoreactivity, showed that the proportion of each type did not change with steroid treatment (Table 1B). Neuron initiation was measured by counting the number of neurons with one or more neurites longer than the soma diameter and this was not influenced by androgen (n=3 for both DHT and T) or by E2 (n=4) in either NOS or TH neurons (Table 1C).

T, DHT and E2 influence neurite complexity *in vitro*

Neurite length, neurite branching and number of primary neurites were measured in cultured pelvic ganglion neurons treated overnight with increasing concentrations of T (n=4) or DHT (n=4), or E2 (10 nM, n=5). Figure 3 shows control NOS-positive and TH-positive neurons and examples of neurons treated overnight with steroid (E2, T or DHT), illustrating the increased complexity of the neurite arbour in the presence of androgen or estrogen. In NOS neurons, T and DHT each caused an increase in neurite length and branching (Fig. 4A). Although DHT (100 nM) significantly increased the number of primary neurites T had no effect on this parameter. E2 (10 nM) caused a significant increase in neurite branching but had no effect on other neurite parameters (Fig. 4A). In TH neurons, T and DHT caused an increase in neurite length and neurite branching but only DHT (10 nM) significantly increased the number of primary neurites. E2 (10 nM) significantly increased both neurite length and neurite branching but had no effect on the number of primary neurites.

Steroid receptor antagonists were used to determine the role of androgen and estrogen receptors in neurite outgrowth responses. In NOS-positive neurons pre-treatment with the ER antagonist, ICI182780 (ICI, 10 μ M; n=4), or the AR antagonist, hydroxyflutamide (HF, 10 μ M; n=4), blocked testosterone-induced neurite length and neurite branching (Fig. 5A). Testosterone significantly increased the number of primary neurites of NOS-positive neurons, but while this appeared to be reduced by both antagonists, this inhibition did not reach statistical significance (Fig. 5A). In TH-positive neurons the testosterone-induced increases in neurite length and number were blocked by HF (Fig. 5B). A small effect of T on neurite branching appeared to occur but this was not statistically significant compared with controls; however, an effect of T on this parameter was indicated by the difference between T and T/HF groups ($P<0.001$). Effects of estradiol on neurite length and branching in NOS and TH neurons were inhibited by ICI182780 (Fig. 5C, D).

The effects of testosterone were probed further using the aromatase inhibitor, letrozole (100nM) to block any potential metabolism to estrogens in the cultures (Fig. 6). The results showed that letrozole attenuated many of the effects of testosterone and that this attenuation was greater in the TH neurons. In NOS neurons letrozole appeared to reduce the effects of testosterone on neurite length and branching but this did not reach statistical significance. In both NOS and TH neurons, the effects of DHT were unchanged by letrozole.

DISCUSSION

In this study we have demonstrated that in male rats many pelvic ganglion neurons are likely to be targets for estrogens, potentially produced within the ganglion by aromatase. This is supported by the presence of aromatase, ER α and ER β mRNA transcripts and ER α expression in the pelvic ganglion *in vivo*, as well as aromatase expression in pelvic ganglia. Moreover, estradiol stimulated growth of longer and more complex neurites in both sympathetic and parasympathetic pelvic ganglion neurons *in vitro*, and aromatase inhibition partially blocked the actions of testosterone. The similarity of androgen and estrogen actions on neurite outgrowth raises the possibility of both substances being involved in regulation of axonal branching and target innervation *in vivo*, or in regenerative processes following injury of pelvic autonomic axons. In contrast, we found no evidence for estrogens maintaining soma size of sympathetic and parasympathetic ganglion neurons, which appears to be a purely androgenic mechanism. This could be due to a direct action of androgens on pelvic ganglion neurons (Meusburger and Keast, 2001), or an indirect action via androgen-driven production of target-derived neurotrophic factors (Katoh-Semba et al., 1994; Forger et al., 1998; Xu et al., 2001). This contrasts with a recent study where the dendritic tree of spinal bulbocavernosus motoneurons was maintained by target-dependent estrogenic mechanisms (Nowacek and Sengelaub, 2006).

The potential production of estrogens by aromatase and the presence of ERs within male pelvic ganglia parallels the situation in some of the male reproductive organs, which also express aromatase and one or both ERs (Hess et al., 2001; Weihua et al., 2001; Bianco et al., 2002; Jesmin et al., 2002). Immunohistochemical analysis of ER α distribution showed a more prominent expression in NOS- than TH-positive neurons, with higher proportions of NOS neurons expressing ER α and brighter staining being more common in NOS neurons. Most of the NOS-positive neurons express vasoactive intestinal peptide and innervate reproductive organs (Keast, 1992a; Ceccatelli et al., 1994; Alm et al., 1995), but our studies did not determine which particular organs or tissues were innervated by ER α -positive neurons.

In this study, each of the three steroids consistently increased neurite length and branching but did not consistently change the number of primary neurites. None of the steroids had any effect on neurite initiation. This is similar to the previously reported action of estradiol on neurite growth in adult nociceptor neurons (Blacklock et al., 2004) and androgen actions on PC12 cells transfected with AR (Lustig et al., 1994) or immortalised motoneurons (Marron et al., 2005). However, it is quite different from the actions of neurotrophic factors on pelvic autonomic neurons, where nerve growth factor (Meusburger and Keast, 2001) and neurturin (Wanigasekara and Keast, 2005) stimulated both neurite initiation and increased neurite complexity (length, branching and primary neurites) in sympathetic and parasympathetic pelvic neurons, respectively. This leads us to the possibility that gonadal steroids are involved in determining axonal branching within target organs (either during development or axon regeneration) but would be unlikely to initiate growth of new axons. Previous *in vivo* studies on facial nerve regeneration in hamsters have shown that application of estradiol, testosterone or dihydrotestosterone are each able to promote regeneration, if administered within the first few hours following axotomy (Tanzer and Jones, 1997; Tanzer and Jones, 2004). Our studies raise the possibility that each of these steroids has potential pro-regenerative effects on both sympathetic and parasympathetic components of the male pelvic autonomic nervous system.

The similar actions of E, T and DHT raise the possibility of ER and AR convergence on a common mechanism of neurite growth. However the ability of both androgen and estrogen receptor antagonists to prevent the action of testosterone in NOS-positive neurons is unexpected. The testosterone effect on neurite growth is inhibited by the AR antagonist, hydroxyflutamide, implying that most or all of the response is AR-mediated, as appears to be

the case in noradrenergic neurons. However, in NOS-positive neurons testosterone action is also inhibited by the ER antagonist, ICI182780, which indicates that much of the exogenous androgen effect is attributable to aromatisation to estradiol. Therefore it is clear that both NOS- and TH-positive male pelvic neurons can respond to estrogenic signals, but that in NOS-positive neurons there is potential direct interaction between ER and AR or interplay between ER- and AR-activated signalling pathways. Our results could be explained by the presence of an AR/ER heterodimer, the transcriptional activity of which would be blocked by either an AR or an ER antagonist. There is a precedent in the steroid receptor literature for the formation of heterodimers between nuclear hormone receptor family members (Forman and Samuels, 1990; Forman et al., 1995). While no studies have been performed to examine ER and AR interaction in peripheral neurons, this concept has been raised by a study using yeast and mammalian two-hybrid systems (Panet-Raymond et al., 2000). These authors reported evidence of inhibitory interactions between AR and ER α but not between AR and ER β . Further, there was some steroid-dependence to these interactions, consistent with a need for ligand-induced conformational change in at least one receptor type prior to the interaction occurring. Inhibitory effects of AR activation on ER function have also been suggested (Buchanan et al., 2005). Irrespective of the mechanisms underlying our results, it is clear that male pelvic ganglion cells are both androgen- and estrogen-sensitive, and that more sophisticated technical approaches will be required to understand the interactions between these two signalling pathways.

Our studies using the aromatase inhibitor, letrozole, also support the involvement of estrogens in mediating at least some of the actions of testosterone on neurite growth of male pelvic neurons. Aromatase inhibition attenuated some but not all of the actions of testosterone but had no effect on the actions of the non-aromatisable androgen, dihydrotestosterone. However it is puzzling that the effects of letrozole were greatest on TH neurons, because there was little effect of the estrogen receptor antagonist on the testosterone response in these neurons. The potential complexity of ER and AR interactions may also impact on the interpretation of this group of experiments.

The mechanism of androgen and estrogen action on neurite growth in male pelvic ganglia is not known, but our antagonist studies imply that it is likely to include activation of AR and ER. This is consistent with our observation that ER α is strongly expressed by NOS-positive parasympathetic pelvic ganglion neurons, which have previously been shown to also express AR (Schirar et al., 1997; Keast and Saunders, 1998). Sympathetic noradrenergic pelvic neurons do not express ER α as strongly (i.e. fewer neurons show ER α expression and even in positively stained neurons, the staining is not as bright as in NOS neurons), yet are estrogen-sensitive in the context of neurite outgrowth. Therefore, either the levels of ER α are sufficient for mediating this response, or another ER is responsible. Unfortunately we were unable to obtain reliable ER β immunostaining for this study, but our RT-PCR data shows that this receptor is present. The mechanism of DHT action also requires further investigation in this neuron group, as a previous study reported that no AR was expressed by TH-positive pelvic ganglion neurons (Keast and Saunders, 1998). It is possible that this earlier study was unable to detect low levels of AR that may be sufficient to mediate neurite outgrowth in our current study. Despite the evidence supporting ER- and AR-mediated neurite growth, this does not discount the possible involvement of non-genomic mechanisms such as second messenger signalling pathways (McEwen, 2001; Lösel and Wehling, 2003), although in rat pelvic ganglia these are unlikely to require activation of CREB (Purves-Tyson and Keast, 2004). The role of non-genomic mechanisms in neurite growth has been suggested by experiments in neuroblastoma cells, where testosterone stimulates oscillations in intracellular calcium levels, especially in neurite tips (Estrada et al., 2006).

Our *in vivo* studies exploited a well-established indicator of genomic steroid action, namely maintenance of soma size (e.g. Keast and Saunders, 1998; Melvin et al., 1989). In contrast to the neurite outgrowth experiments, testosterone-driven maintenance of soma size was entirely due to androgenic mechanisms. This mechanism parallels that in developing male reproductive organs, where the effects of neonatal androgens in the development of the prostate gland require androgens but cannot be replaced by estrogens (Singh and Handelsman, 1999). Our results also relate closely to studies that examined a different property of rat pelvic ganglion neurons, where gonadectomy-induced decrease in TH expression was prevented by administration of testosterone but not estradiol (Hamill et al., 1984; Hamill and Schroeder, 1990). Together our results indicate that testosterone can have multiple actions on adult male pelvic ganglion neurons, some of which are mediated by AR, and some by a mixture of AR and ER. The challenge will be to understand how these mechanisms combine *in vivo* to produce physiological changes, either to normal metabolism and synaptic communication, or during regenerative events.

In conclusion, estrogenic mechanisms mediate some but not all actions of androgens in adult male rat pelvic ganglia. The involvement of this additional signalling pathway may provide new strategies for manipulating the structure and function of pelvic neurons during aging or disease, and allow the development of new targets for promoting their regeneration following injury.

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Abbreviations

AR, androgen receptor; DHT, dihydrotestosterone; E2, 17 β -estradiol; ER, estrogen receptor; NOS, nitric oxide synthase; PBS, phosphate buffered saline; PFA, phosphate-buffered paraformaldehyde; TH, tyrosine hydroxylase.

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Figure 1. Aromatase and estrogen receptors in male rat pelvic ganglion

- A.** Aromatase RT-PCR yielded a 102 bp band in prostate and pelvic ganglion but not skeletal muscle; lane 1, 100 bp ladder; 2, blank; 3, prostate; 4, pelvic ganglion; 5, skeletal muscle.
- B.** ER RT-PCR yielded a 345 bp band for ERα and a 262 bp for ERβ in pelvic ganglion but only the ERβ transcript was present in prostate; lane 1, 100 bp ladder; 2, pelvic ganglion GAPDH (207 bp); 3, pelvic ganglion ERβ (262 bp); 4, prostate ERβ; 5, pelvic ganglion ERα (345 bp); 6, prostate ERα; 7 blank, ERα primers; 8, prostate GAPDH. For all primer sets, experiments were repeated twice.

- C.** Aromatase protein was identified in extracts of pelvic ganglia from three rats, each showing a single band at ~55 kD.
- D.** Triple staining immunofluorescence showing examples of ER α expression in noradrenergic (TH-positive) and nitrergic (NOS-positive) pelvic ganglion neurons. Arrows show examples of ER α expression in NOS-positive, TH-negative neurons, and arrowheads show examples of weaker ER α expression in TH-positive, NOS-negative neurons. In merged colour images, ER α is red, NOS is green and TH is blue. Calibration in D represents 20 μ m.

A seminal vesicle weights **B NOS neurons** **C TH neurons**

Figure 2. Effects of gonadectomy and steroid replacement on seminal vesicle weight and size of pelvic ganglion somata

- A.** Seminal vesicles (mean of paired seminal vesicles in each animal) were much smaller after gonadectomy (n=5, “control”) compared to naïve intact animals (n=4) and this effect was completely prevented by testosterone (T, n=6), partly prevented by dihydrotestosterone (DHT, n=5) but unchanged by estradiol (E2, n=6). *, significantly different from control and from E2, $P < 0.001$; not indicated, but DHT vs. naïve or T, $P < 0.001$.

- B.** Size of NOS-immunoreactive somata decreased after gonadectomy (“control”) and this effect was completely prevented by T or DHT but not by E2. Data is from the same animals as shown in A. *, significantly different from control and from E2, $P<0.05$.
- C.** Size of TH-immunoreactive somata decreased after gonadectomy (“control”) and this effect was completely prevented by T or DHT but not by E2. Data is from the same animals as shown in A. *, significantly different from control and from E2, $P<0.001$.

Figure 3. Effects of estradiol (E2), testosterone (T) and dihydrotestosterone (DHT) on neurite outgrowth *in vitro*

Typical examples of NOS- or TH-positive pelvic ganglion neurons cultured overnight in the absence of steroid (control) or steroid (E2, 10 nM; T and DHT, 100 nM). Each steroid caused an increase in neurite complexity. Images have been inverted to show neurite structure more clearly.

Figure 4. Effects of testosterone (T), dihydrotestosterone (DHT) and estradiol (E2) on neurite complexity *in vitro*

- A.** In NOS neurons, all three steroids increased neurite branching, both androgens increased neurite length and only DHT increased primary neurite number.
- B.** In TH neurons, all three steroids increased neurite length and branching but only DHT increased primary neurite number.

Groups significantly different from control are indicated: * $P < 0.05$, ** $P < 0.01$

Figure 5. Testosterone (T)-stimulated neurite growth involves both androgen and estrogen receptors

- A.** In NOS neurons, T (10 nM) caused an increase in neurite length ($P < 0.01$), branching ($P < 0.001$) and number ($P < 0.05$) compared with untreated controls. The effects on length and branching were blocked by the AR blocker, hydroxyflutamide (HF, 10 μM ; T vs. T/HF, $P < 0.01$ and $P < 0.001$ for length and branching respectively) and by the ER blocker, ICI182780 (ICI, 10 μM ; T vs. T/ICI, $P < 0.01$ for both parameters). The modest effects of T on neurite number appeared to be reduced by each blocker but neither effect reached statistical significance. Groups significantly different from T are indicated, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
- B.** In TH neurons, T (10 nM) caused an increase in neurite length ($P < 0.05$), and number ($P < 0.05$) compared with untreated controls. A small effect of T on neurite branching appeared to occur but this was not statistically significant compared with controls;

however, an effect of T on this parameter was indicated by the difference between T and T/HF groups ($P<0.001$). The effects on length and number were blocked by the AR inhibitor, hydroxyflutamide (HF, 10 μ M; T vs. T/HF, $P<0.001$ for length and $P<0.05$ for number). The ER blocker, ICI182780 (ICI, 10 μ M) had no significant effect on the T-induced increase in neurite length and branching. Groups significantly different from T are indicated, * $P<0.05$, *** $P<0.001$.

C, D. Estradiol (E2, 10 nM) actions on neurite growth were blocked by ICI182780 (ICI, 10 μ M). E2 caused a significant increase in neurite length and neurite branching in both NOS (C) and TH (D) neurons. Both effects in both neuron types were abolished with ICI182780. E2 did not affect the number of primary neurites. Groups significantly different from E2 are indicated, *, $P<0.05$, **, $P<0.01$.

A NOS **B TH** **Figure 6. Effects of aromatase inhibition on testosterone responses**

- A.** In NOS neurons, both T and DHT (each 10 nM) stimulated neurite length and branching. The aromatase inhibitor letrozole (L, 100 nM) appeared to partially reduce the effects of testosterone (10 nM) on neurite length and branching but T/L was not significantly different to either control or T alone. Letrozole had no effect on the response to DHT (10 nM). In this group of experiments, androgens had weak or no effects on the number of primary neurites, but it should be noted that the controls showed a relatively high number of primary neurites compared with experiments shown in Figure 5. Groups significantly different from control are indicated, * $P < 0.05$, ** $P < 0.01$.
- B.** In TH neurons, the effects of T on neurite length and number of primary neurites were abolished by letrozole (L, 100 nM) (T vs T/L, $P < 0.05$), whereas the effect on neurite branching appeared to be only slightly reduced, i.e. T/L not significantly different to either control or T alone. The effects of DHT were unchanged by letrozole. Groups significantly different from control are indicated, * $P < 0.05$, ** $P < 0.01$. Groups significantly different from T are indicated, # $P < 0.05$.

Table 1Neuron survival, transmitter phenotype and neurite initiation *in vitro* after steroid treatment.

A. Neuron survival*				
Steroid / marker	1 nM	10 nM	100 nM	
T / TH	96.8±14.9	109±7.9	120.3±11.1	
T / NOS	105.7±13.8	99.3±13.5	117.5±9.7	
DHT / TH	113.8±26.6	95.7±17.8	106.9±10.4	
DHT / NOS	81.2±12.8	111.4±15.2	108.5±3.3	
E2 / TH	-	97.12±15.9	-	
E2 / NOS	-	120.5±17.2	-	
B. Transmitter phenotype**				
Steroid / marker	No steroid	1 nM	10 nM	100 nM
T / TH	37.0±3.2	34.9±4.0	40.2±6.4	37.5±2.3
T / NOS	61.9±2.4	63.9±3.2	58.8±5.7	61.7±1.8
DHT / TH	37.6±2.4	45.2±5.5	33.9±2.4	37.2±3.9
DHT / NOS	62.4±2.4	54.8±5.6	66.1±2.4	62.8±3.9
E2 / TH	39.9±3.3	-	34.9±3.9	-
E2 / NOS	60.1±3.3	-	67.2±4.5	-
C. Neurite initiation***				
Steroid / marker	No steroid	1 nM	10 nM	100 nM
T / TH	58.6±3.4	58.8±2.7	62.7±4.4	64.8±5.3
T / NOS	66.1±4.4	68.1±11.7	64.9±5.9	70.3±7.1
DHT / TH	53.0±12.6	54.2±3.8	57.0±8.1	64.0±13.1
DHT / NOS	56.4±8.2	57.4±4.9	61.1±7.9	66.9±12.0
E2 / TH	66.5±5.9	-	76.8±5.7	-
E2 / NOS	80.6±3.5	-	68.2±8.5	-

Data from A-C are from the same cultures for each steroid (n=3 or 4 for each measurement). No steroid had any statistically significant effects on any parameter. T, testosterone; DHT, dihydrotestosterone; E2, estradiol.

* Values are expressed as a percentage of neurons growing on untreated coverslips in the same experiment (mean ± SE).

** Values are expressed as the percentage of all DAPI-stained neurons growing on the same coverslip expressing NOS or TH (mean ± SE).

*** Values are expressed as the proportion of all neurons on the coverslip that have one or more neurites longer than the soma diameter (mean ± SE).