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Predominant Suppression of FSH β -immunoreactivity after Long-Term Treatment of Intact and Castrate Adult Male Rats with the GnRH Agonist *Deslorelin*

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Abstract

GnRH agonists are used to treat gonadal steroid-dependent disorders in humans and contraceptive animals. These agonists are thought to work by desensitizing gonadotropes to GnRH, thereby suppressing FSH and LH secretion. It is not known whether changes occur in the cellular composition of the pituitary gland following chronic GnRH agonist exposure.

Adult male Sprague-Dawley rats were treated with a sham, deslorelin, or deslorelin plus testosterone implant for 41.0 $\pm$ 0.6 days. In a second experiment, rats were castrated and treated with deslorelin and/or testosterone. Pituitary sections were labeled immunocytochemically for FSH β and LH β , or α GSU.

Deslorelin suppressed testis weight by two thirds and reduced plasma FSH and LH in intact rats. Deslorelin decreased the percentage of gonadotropes but the effect was specific to the FSH β -ir cells. Testosterone did not reverse the deslorelin-induced reduction in the overall gonadotrope population. However, in the presence of testosterone, the proportion of gonadotropes that was FSH β -ir increased in the remaining gonadotropes. There was no effect of treatment on the total LH β -ir cell population although the loss of FSH β in bi-hormonal cells increased the proportion of mono-hormonal LH β -ir gonadotropes. The castration-induced plasma LH and FSH increases were suppressed by deslorelin, testosterone or both. Castration increased both LH-ir and FSH-ir without increasing the overall gonadotrope population; thus increasing the proportion of bi-hormonal cells. Deslorelin suppressed these increases. Testosterone increased FSH-ir in deslorelin-treated castrate rats. Deslorelin did not affect α GSU immunoreactivity, suggesting that the gonadotrope population *per se* is not eliminated by deslorelin but the ability of gonadotropes to synthesize FSH β is compromised.

We hypothesize that the FSH dominant suppression may be central to the long-term contraceptive efficacy of deslorelin in the male.

Introduction

Continuous delivery of gonadotropin-releasing hormone (GnRH) agonists suppresses sex steroid synthesis in most male mammals (e.g., mouse (1), rat (2), dog (3), monkey (4), sheep (5, 6)), although there are a few notable exceptions (possum (7), red deer (8), wallaby (9) and especially the bull (10, 11)). As GnRH agonists are extremely effective in humans, they offer a powerful alternative to surgical orchidectomy for contraception and gonadal steroid-dependent disorders, particularly prostate cancer (12-14); approximately 600 000 men received GnRH agonist therapy in 2007 (15). GnRH agonist therapy is also used in women (endometriosis (16), uterine fibroids (17), central precocious puberty (18)). The number of people receiving agonist treatment may increase significantly as this therapy is also under investigation for Alzheimer's disease (19), bowel disease (20), polycystic ovarian syndrome (21) and preservation of ovarian function during chemotherapy (22). Thus, GnRH agonist therapy may become even more commonplace.

The mechanism by which GnRH agonists suppresses the pituitary component of the reproductive axis is not fully understood. There are three aspects that may be compromised and these are not mutually exclusive. First, there is compelling evidence that the GnRH receptor is affected. After initially stimulating the reproductive axis, GnRH agonists induce internalization and down-regulation of the GnRH receptor (23, 24). However, it has been suggested for many years that GnRH receptor modification fails to explain all changes induced by GnRH agonists (25-30). Second, indirect evidence suggests that chronic exposure to GnRH agonists may uncouple the associated intracellular signaling systems (31-33). Third, there may be a change in the cellular composition of the pituitary gland. This last component, which has not been investigated, was hypothesized to explain some of the effects of chronic GnRH agonist exposure in men. Specifically, if GnRH agonist therapy lasts for several years, recovery of testosterone levels takes 18 months or more after the cessation of treatment (34-39). Experimental studies have shown that chronic GnRH agonist exposure decreases the pituitary content of gonadotropins (40-43) and *in vitro* evidence suggests that this may also occur in humans (44). Such depletion may involve an impaired ability to synthesize the gonadotropins and/or the loss of gonadotropes entirely.

Identifying potential cellular changes may help further elucidate the mechanisms through which GnRH agonists inhibit reproductive function and provide initial insight into why sustained suppression of the reproductive axis may occur after treatment ceases. Accordingly, this study investigated the effect of long-term exposure to the GnRH agonist deslorelin on the density of FSH β -, LH β - and α GSU-immunoreactive (-ir) cells within the pituitary gland of the adult male rat. To determine whether any pituitary changes were through a direct action of deslorelin or through the accompanying testosterone loss, a some rats were testosterone replaced. In a second experiment on castrate rats, we determined whether potential pituitary changes were due, at least in part, on the loss of some testicular factor other than testosterone, (e.g. inhibin).

Materials and Methods

Experiment 1: Deslorelin and the intact male rat

Adult male Sprague-Dawley rats (135.9 ± 0.2 days old) were treated for 41.0 ± 0.6 days with a sham implant (Control; $n=13$), a slow-release 1.1 mg deslorelin implant (DESL; $n=13$; Suprelorin®; Peptech Animal Health; Sydney, Australia) or deslorelin implant and a 15 mg testosterone pellet (DESL+T; $n=7$; 90-day release, Innovative Research of America, Sarasota, FL). As reported by others (45), and supported by preliminary studies (data not shown), this testosterone treatment restored total testosterone concentrations in castrated rats to the intact range. Free testosterone levels were also not different between intact (4.5 ± 1.9 pg/ml) and testosterone-treated castrate (3.3 ± 1.2 pg/ml) rats. Based on these preliminary investigations, the testosterone dose was not increased as we did not want confounding anabolic effects, if this steroid was elevated above the normal range. Deslorelin implants were produced by carefully cutting 4.7mg Suprelorin implants into quarters with a scalpel, with particular care taken to prevent implant fragmentation. Implants ($n=14$) weighed 23.1 ± 0.2 mg, equating to ~ 1.1 mg deslorelin per implant. As the *in vitro* deslorelin release rate from an implant containing 5mg deslorelin is >1 µg/day (3), we estimated the smaller implants would release ~ 250 ng/day; this is likely to be an underestimate on account of the increased surface area. Plasma deslorelin was 92.9 ± 9.3 pg/ml at the end of the study. This concentration is lower than reported for leuporelin in men: levels of 190-430 pg/ml and 930 pg/ml leuporelin were evident after 3 and 12 month depot Lupron implants, respectively (46). Similarly, a different 4 month implant produced mean serum leuporelin levels from day 3 to day 112 of treatment of between 1 ng/ml and 100 pg/ml, with individuals ranging from 50 pg/ml to 5 ng/ml (47). Deslorelin is, however, considered to be a more potent GnRH agonist than leuporelin (48).

All implants were inserted sc aseptically in the scapular region under isoflurane anesthesia. Rats were placed in individual cages with nesting supplies and a heat source for 6 days and then returned to group housing (4-5 per cage). Food and water were provided *ad libitum* and rats were maintained on a 12L/12D cycle (06:00-18:00; 21°C). Rats were weighed twice a week for the duration of the study. All studies were performed under IACUC authorization from the University of Wyoming.

Experiment 2: Deslorelin and the castrated rat

A second group of rats, run concomitantly with Experiment 1, were bilaterally castrated and treated with a sham implant (castrate; $n=8$), a deslorelin implant (castrate+D; $n=8$), a deslorelin implant and testosterone (castrate+D+T; $n=6$), or only testosterone (castrate+T; $n=8$). Implants were inserted and animals maintained as described. Rats were weighed twice a week.

Tissue Preparation

At the end of treatment, rats were anesthetized with ip ketamine (100 mg/kg)/xylazine (10 mg/kg) and injected iv with heparin (200 units/kg). Blood samples were collected via cardiac puncture. Rats were perfused transcardially with 150 ml saline, 300 ml Zamboni's fixative (49) and 125 ml 20% sucrose in 0.01M PBS. Retention of implants was confirmed

and organs were extirpated and weighed. Pituitary glands were stored in 20% sucrose in PBS (4°C) and sagittal pituitary sections (20µm) cut on a cryostat, mounted on Silane-coated slides and stored (-80°C).

Immunocytochemistry

For immunolabeling the FSH β and LH β subunits, slides were washed (all washes 3×5 min in 0.01M PBS), incubated (48h; 4°C) in 0.01M PBS containing 10% goat serum, 0.3% Triton and rabbit anti-rat FSH β (AFP7798-1289; 1:56000; NIDDK) and guinea pig anti-rat LH β (AFP-22238790GPOLHB; 1:62000; NIDDK). Slides were washed, incubated (90 min; room temp) with Alexa Fluor 488 F(ab')₂ fragment of goat anti-rabbit IgG (1:8000; Invitrogen; Carlsbad, CA) combined with affinity purified Texas Red goat anti-guinea pig IgG F(ab')₂ (1:500; Rockland; Gilbertsville, PA), washed and coverslipped with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to DNA (Vector; Burlingame, CA).

For staining the α GSU, sections were incubated (48h; 4°C) in guinea pig anti-rat α GSU (AFP5191792; 1:2000; NIDDK), washed, incubated in the Texas Red goat anti-guinea pig IgG F(ab')₂ (1:250, 90 min; room temp), washed and coverslipped with Vectashield and DAPI. For additional evidence of the specificity of the guinea-pig anti-rat α GSU antibody, we had previously performed a colocalization study with a rabbit anti-rat α GSU antibody (AFP66P9986; 1:4000; NIDDK). Labeled cells were visualized with the Alexa Fluor 488 F(ab')₂ goat anti-rabbit IgG (1:8000; Invitrogen) combined with affinity purified Texas Red goat anti-guinea pig IgG F(ab')₂ (1:500; Rockland) as described. There was 100% colocalization between these two antisera (data not shown). On a subset of pituitary sections from control rats (n=4), we also confirmed that all cells labeled with the rabbit anti-rat FSH β antibody were also labeled with the guinea pig anti-rat α GSU antibody (data not shown).

Cell counting

Gonadotropes are randomly distributed throughout the anterior pituitary, although there is a concentration along the anterior aspect of the pars distalis (50). For analysis, two mid-sagittal sections, at least 120 µm apart, were used from each animal. Images were taken of 5 randomly selected fields (220 × 295µm) from each section using a Nikon Eclipse E800 equipped with an X-Cite™ 120 Fluorescence Illumination System (EXFO; Mississauga, Ontario, Canada) and SPOT RTke digital camera (Diagnostic Instruments; Sterling Heights, MI). The illumination parameters (e.g. brightness, gamma) were fixed for each fluorescent tag. Using Image J software (NIH, Bethesda, MD), the number of FSH β -, LH β -, FSH β + LH β -, α GSU-ir cells, and DAPI-labeled nuclei, were marked and then counted by the software in each field. Only cells exhibiting cytoplasmic immunoreactivity with a nuclear exclusion, which corresponded to a DAPI-labeled nucleus, were marked. The mean value of the 10 fields from each pituitary was used to estimate the value for each rat. We have previously determined (data not shown) that this value falls within the 95% confidence interval of 30 fields.

Hormone Assays

FSH was measured by EIA (#29-AE-R004; ALPCO Diagnostics; Salem, NH) with a sensitivity of 0.2 ng/ml and intra-assay coefficient of variation of 6%. The RIA for rLH used reagents from the NIDDK (rLH-I-8 for iodination, AFP-12066B; rLH-S-10 for antiserum, AFP-571487; rLH-RP-3 for standard, AFP-7187B) and rLH was iodinated by the chloramine-T method (51). Standards ranged from 0.4 to 6 ng/ml. Assay sensitivity (0.09 ng/ml) was calculated from the standard curve as the value that exceeded the 0 ng/ml by 2SDs. The intra-assay coefficient of variation was <5%. Total testosterone was estimated by RIA (#TKTT2; Diagnostic Products Corporation; Los Angeles, CA) with a sensitivity of 0.05 ng/ml and intra-assay coefficient of variation was 5%. Free testosterone was measured by EIA (#11-FTSHU-E01; ALPCO Diagnostics; Salem, NH) with a sensitivity 0.17 pg/ml and an intra-assay coefficient of variation of <5%.

For the extraction of deslorelin, 200 μ l plasma was diluted in 800 μ l saline and then acidified with an equal volume of 1% trifluoroacetic acid (TFA, HPLC grade). HF Bond Elut C18 OH columns (Varian, Palo Alto, CA) were equilibrated with HPLC grade acetonitrile (1 ml) and 1% TFA (9 ml). The acidified plasma was added to the columns and washed with 1% TFA (6 ml). The peptide was eluted with 6 ml of 60% acetonitrile in 1% TFA. The eluate was evaporated to about half volume in a Savant SpeedVac (Thermo Fisher Scientific, Waltham, MA), frozen, and evaporated to dryness in a freeze dryer (LabConco, Kansas City, MO). Dried extracts were stored at -20°C and then reconstituted in 150 μ l assay buffer at the time of assay. Deslorelin concentrations were determined by EIA (#S1175; Bachem, Torrance, CA) according to the manufacturer's instructions. Sensitivity was 0.014 ng/ml and the intra-assay coefficient of variation 12.6%. Recovery of deslorelin added to untreated control rat plasma averaged 93%.

Each hormone was analyzed in a single assay for that hormone and if a concentration was below the threshold of detection then the value for that sample was set to the assay sensitivity.

Data analysis

As there was no significant effect of any treatment on the density of DAPI-labeled cells in the pituitary gland (data not shown), the number of cells immunoreactive for FSH β -, LH β -, or α GSU-ir in a field was normalized to a percentage of total cells. This calculated percentage underestimates the true percentage of secretory cells as DAPI labeling does not discriminate between endocrine and non-endocrine (e.g. folliculostellate) cells. The number of mono-hormonal gonadotropes was calculated by subtracting bi-hormonal cells from the total of FSH β -ir or LH β -ir cells, respectively.

A second level analysis was performed to determine the effect of treatment on the proportion of gonadotropes that were mono-FSH β , mono-LH β or bi-hormonal. The total gonadotrope population was estimated by adding the mono-hormonal FSH β -ir, mono-hormonal LH β -ir and bi-hormonal cells.

Untreated intact (Control) rats in Experiment 1 were used in the statistical analyses of both experiments. Data were analyzed by ANOVA and Tukey's *post hoc* test to compare groups. Plasma values were log transformed before ANOVA.

Results

Experiment 1: Deslorelin and the intact male rat

FSH β -ir cells were most abundant (mono-FSH β and bi-hormonal cells) and deslorelin significantly suppressed immunoreactivity in both populations (Figs 1, 2A; Supplementary data Table 1). Treatments did not affect α GSU-ir cell number, although deslorelin significantly decreased the percentage of β subunit-immunoreactive gonadotropes (Fig. 2A). Deslorelin did not suppress the percentage of LH β -ir cells, although the proportion of mono-hormonal LH β -ir cells increased as a result of the loss of FSH β -ir in the bi-hormonal cells (Figs 1, 2A). The percentage of bi-hormonal gonadotropes was significantly reduced by deslorelin. Although testosterone replacement did not reduce the suppression in the overall population of β subunit-immunoreactive gonadotropes, there was an increase in FSH β -ir. Specifically, testosterone replacement did not reverse the suppression in mono-FSH β -ir cells, but it increased (or prevented the loss of) FSH β immunoreactivity in bi-hormonal cells.

These FSH changes were also evident in the plasma: deslorelin reduced plasma FSH (88%; $p<0.001$; Fig. 2B) and testosterone reduced the extent of suppression (44%; $p<0.01$). Deslorelin also suppressed plasma LH (47%; $p<0.01$) but to a smaller extent than FSH. In contrast to FSH, when testosterone was co-administered with deslorelin, plasma LH suppression was enhanced (70%; $p<0.05$ vs deslorelin alone). Deslorelin suppressed plasma testosterone.

Experiment 2: Deslorelin and the castrated rat

The percentage of α GSU-ir cells was not affected by castration, testosterone or deslorelin but was reduced in castrate rats receiving both testosterone and deslorelin. Castration did not increase the percentage of gonadotropes (Figs 3, 4A; Supplementary data Table 1). However, the proportion of bi-hormonal and mono-LH β -ir cells increased significantly with a resultant rise in the percentage of total LH β -ir cells (Fig. 4A). In contrast, the proportion of mono-FSH β -ir decreased, leading to no difference in the total FSH β -ir cell population (Fig. 4A). These changes in gonadotropin immunoreactivity were reversed by testosterone replacement.

Deslorelin suppressed FSH β - and LH β -ir below intact controls. As in the intact group, testosterone replacement in deslorelin-treated castrated rats did not reduce the suppression in the percentage of total β subunit-immunoreactive gonadotropes, but there was an increase in FSH β -ir. Together, these data suggest that the real gonadotrope population remains robust in the face of endocrine changes, although the ability to synthesize the β subunits is dramatically perturbed.

Castration increased plasma FSH 6-fold and LH 12-fold ($p<0.0001$ for both) and these increases were restored to the intact control rat range by testosterone (Fig. 4B). Deslorelin

treatment also prevented the castration-induced increases and suppressed FSH below intact control rat levels.

Pituitary, testis and epididymi weights

Testis weight was suppressed by 67% (Fig. 5). Epididymis (control: 169.3 ± 6.3 ; D: 54.1 ± 3.5 mg/100g BW) weights were suppressed by deslorelin to a third of that in the untreated rat ($p < 0.001$). Testosterone replacement partially alleviated this effect in the epididymis (D+T: 93.6 ± 7.7 mg/100g BW) but not in the testis (D+T: 263.3 ± 31.2 mg/100g BW). Spermatozoa were absent from the testes of the deslorelin-treated animals at the end of the study. Deslorelin reduced (control: 3.3 ± 0.1 ; D: 2.8 ± 0.1 mg/100g BW), whereas castration increased (4.0 ± 0.2 mg/100g BW), pituitary weight ($p < 0.05$ for both). Testosterone replacement reversed these effects in deslorelin-treated intact (3.1 ± 0.2 mg/100g BW) and castrate (2.8 ± 0.2 mg/100g BW), as well as deslorelin-treated castrate (3.4 ± 0.1 mg/100g BW), rats. Deslorelin also prevented the castration-induced increase (3.3 ± 0.1 mg/100g BW) in pituitary weight.

Discussion

This is the first study reporting on specific changes in the immunocytochemically detectable gonadotrope population of the adult male rat pituitary gland following long-term treatment with a GnRH agonist. FSH β -ir was compromised by deslorelin in intact rats and plasma FSH was suppressed as expected. The reduction in FSH β -ir cells was evident in both the mono- and bi-hormonal gonadotrope populations. Such an FSH-dominant suppression has been observed previously in response to buserelin in the ewe (52). Testosterone replacement to deslorelin-treated intact rats caused a significant increase in FSH-ir, especially in bi-hormonal cells, and a commensurate increase in plasma FSH. This indicates that deslorelin's effects are mediated, at least in part, through the loss of testosterone. Deslorelin did not affect the α GSU-ir population, suggesting that this agonist does not eliminate the gonadotrope population *per se* but that there is a reduced ability to synthesize the FSH β subunit. In marked contrast to FSH, deslorelin did not affect the density of LH β -ir cells in intact rats. LH secretion was slightly suppressed by deslorelin and this suppression was enhanced in the presence of testosterone, which is notably different to FSH. Deslorelin potently suppressed LH β -ir and plasma LH after castration.

Although GnRH agonists have been used clinically for decades as an alternative to surgical castration, their exact mechanism of action remains poorly understood. The ability of GnRH agonists to decrease pituitary gonadotropin release has been attributed to GnRH receptor internalization and down-regulation. Although receptor down-regulation has been extensively studied in short-term investigations, fewer studies have determined the effects of long-term GnRH agonist exposure. It is notable therefore that after 30 days of triptorelin treatment in rats, GnRH receptor mRNA rose from the initial short-term down-regulation, and GnRH receptor protein levels were back at pre-treatment levels (28). Also, although triptorelin suppressed GnRH-induced LH and FSH release in young girls treated chronically for precocious puberty, GnRH still stimulated α GSU secretion (53, 54). α GSU secretion was also markedly increased in patients treated for pituitary tumors with GnRH agonists,

although LH and FSH were suppressed (55). More recently, Hirsch *et al* (56) reported that α GSU release increased in response to long-term exposure to histrelin, an agonist that powerfully suppresses LH and FSH secretion. Elevated α GSU release in response to deslorelin may be mediated through increased PACAP (57, 58), which stimulates α GSU (59, 60).

Our study concurs with studies on rats showing marked reduction in FSH β mRNA and pituitary FSH content after 28 days of continuous buserelin (40, 42). Together, these studies suggest extensive and coinciding decreases in FSH β mRNA and FSH protein following treatment, leading to a substantial reduction in the number of gonadotropes synthesizing FSH β . As GnRH stimulates PACAP (57, 58), the effect of deslorelin on FSH β may be transduced through PACAP and/or follistatin. Continuous PACAP suppressed FSH β mRNA levels (61) and PACAP increased follistatin mRNA in mouse (62, 63). There is preliminary evidence that follistatin, in addition to binding activin, may affect FSH β mRNA in primate pituitary cultures (64) and L β T2 cells (65).

The decrease in FSH β -ir was consistent with the changes in plasma FSH; a ~90% reduction in circulating FSH levels in intact and castrate rats after deslorelin treatment. Continuous infusion of buserelin also suppressed plasma FSH in intact and castrate rats (42). Leuprorelin similarly reduced plasma FSH (66), whereas goserelin exerted a more modest reduction (67). Interestingly, plasma levels of FSH rose following daily injections of alarelin in rats even after 12 weeks of treatment (68), suggesting that not all agonists may work through a common mechanism. Different agonists clearly have different potencies, depending on the assay (69, 70); deslorelin is considered to be 114 times more potent than GnRH, whereas leuprorelin is only 15 times more potent (48). Thus, it is noteworthy in red deer that although buserelin infusion stimulated reproductive activity (8), leuprorelin suppressed it (71). It is possible that these two GnRH agonists affect intracellular pathways differently, at least in the long term.

Although plasma LH was diminished in response to deslorelin in intact rats, the decrease was about half that of FSH. This suppression, possibly coupled with the more dramatic loss in FSH, was clearly sufficient to suppress testosterone. A deslorelin-mediated suppression in LH was much more evident in the castrate rats (95%), which is similar to reports on bulls (72). Indeed, castration may be essential to consistently detect a GnRH agonist-mediated suppression of LH. Chronic exposure to goserelin (67), leuprorelin (66) and buserelin (43) had no effect on plasma LH in intact rats, whereas buserelin significantly reduced plasma LH in castrate rats (43). Some of the discrepancy may also originate from the inherent pulsatility of LH secretion, which can produce a large variability within a group with a single blood sample and changes in LH are best investigated by serial blood sampling. This is illustrated in a recent study on the effect of leuprorelin on LH in the pubertal human male, where no differences in LH release were apparent before or during treatment in the first 4 hours of the night, but clear suppression was evident thereafter (73). FSH suppression occurred throughout the study (73).

Previous studies have reported reduced LH β mRNA in the intact male rat in response to triptorelin (74) and buserelin (43). Thus, the absence of an effect of deslorelin on the number

of cells immunocytochemically detectable for LH β in intact rats was unexpected. Our result must be viewed within certain caveats. First, it should be noted that immunocytochemistry is not an accurate method to determine the amount of a target substance within a cell (75, 76). Thus, if two gonadotropes differed significantly in LH β content, they may be detected equally by immunocytochemistry. However, as determined by electron microscopy, long-term leuporelin exposure in mice reduced the LH β content of individual secretory granules (77). Second, FSH is largely secreted constitutively (78), and changes in synthesis will therefore be reflected accurately immunocytochemically. In contrast, LH is stored prior to secretion (78) and, thus, in the absence of stimulation, LH may still be immunocytochemically detectable. However, the retained presence of LH β -ir in our study may also be the result of cellular changes within the gonadotrope. As noted above, GnRH induces PACAP and although continuous PACAP did not affect LH β mRNA levels, it protected the LH β mRNA from degradation and thus lengthened its half-life (61). Thus, it is possible that while LH β stores in, and secretion from, individual gonadotropes are reduced in response to deslorelin, these LH β -synthesizing cells remain immunocytochemically detectable. The specific mechanisms responsible for the deslorelin-mediated suppression of FSH β -ir, but retention of LH β -ir, warrant further attention.

It is well known that castration increases the pituitary gland gonadotropin content (e.g. (79)). Our study concurs with a recent report (80) that this effect is not through gonadotrope proliferation. The increased pituitary weight in castrated rats is likely to be the result of the increased size of gonadotropes, the so called “castration cells” (see Fig. 3), as well as increased mitosis in the non-gonadotrope pituitary cell population (80).

Deslorelin suppressed circulating testosterone by 90%, with a corresponding 70% decrease in testicular weight, which can be used as a rough index of spermatogenesis suppression (81). In previous studies using different agonists, testosterone suppression was similar (82, 83), but the impact on testicular weight was less pronounced (40, 68, 82, 83). Unlike in men (84), fertility is compromised but not eliminated in male mice with disrupted FSH systems (85, 86). Thus, it is probable that this “castration” of the testes by deslorelin is not mediated solely through the marked reduction in FSH, and the smaller LH reduction is also likely to be important. In this respect, although testosterone supplementation increased FSH, testicular weight did not increase. Also, at least part of the effect of GnRH agonists may be through a direct testicular effect. Testicular GnRH receptors have been reported in several species, including humans (87, 88). In rats, GnRH affects Ca²⁺ mobilization (89) as well as steroidogenesis in Leydig cells (90). This reported direct effect of GnRH on steroidogenesis is especially noteworthy for the current study, as although testosterone implants returned testosterone concentrations to the normal range in castrated rats, the testosterone elevation was lower in deslorelin-treated intact rats. Moreover, buserelin treatment for 5 months decreased testosterone but increased testicular levels of dihydrotestosterone and estradiol (91), suggesting increased activity of 5 α -reductase and aromatase, respectively.

The effects of testosterone replacement on plasma gonadotropins in deslorelin-treated rats were diametrically opposite: it enhanced the deslorelin-mediated suppression of LH but elevated FSH secretion. This finding concurs with studies on GnRH antagonist-treated intact and castrate male rats (92, 93) and may have been even more pronounced in our study if

testosterone concentrations had been restored to control levels. It is well established that testosterone consistently suppresses LH mRNA (94). The enhanced suppression of LH by testosterone in our deslorelin-treated rats suggests that the effects are additive, possibly synergistic. In contrast, testosterone increases FSH mRNA and secretion in rodents (95). Some of this effect of testosterone may be through increased FSH β stability (93). It is also noteworthy that testosterone suppressed pituitary follistatin mRNA (95, 96). Although testosterone regulation of PACAP in the brain has been demonstrated (97, 98), we are unaware of studies in the pituitary gland. Our study suggests that increased activin secretion is unlikely to mediate the effects of testosterone on FSH. Activin stimulates both LH β and FSH β (99), but plasma LH and LH β -ir were suppressed in the deslorelin-treated rats supplemented with testosterone. However, if the direct suppressive effect of testosterone on the LH β promoter was dominant over the inductive effect of activin on the LH β promoter, then increased activin coupled to reduced follistatin would increase FSH β , but decrease LH β synthesis. Synergism between androgens and activin signaling on the FSH β promoter has been observed in L β T2 cells (100) and androgen mediated suppression of TGF- β signaling is evident in prostate tissue (101). Clearly, more mechanistic research is indicated.

In summary, our study demonstrates specific changes in the cellular composition of the adult male rat pituitary gland after long-term treatment with deslorelin. The deslorelin treatment we used was far more suppressive on testis weight than previously reported using other agonists in the rat. Deslorelin compromised the FSH β -ir cell population in both intact and castrated rats and the deslorelin-induced change in the composition of gonadotropes was similar in both groups. At least some of the suppression is mediated through the loss of testosterone as the proportion of FSH-ir cells, as well as plasma FSH, increased with testosterone replacement in intact rats. As deslorelin-mediated changes on FSH were similar between intact and castrated rats, it suggests that testicular factors other than testosterone are not essential for suppression. LH may be less susceptible to the effect of deslorelin alone, although marked deslorelin-induced LH changes were evident following castration. In contrast to FSH, the addition of testosterone served to amplify the suppressive effects of deslorelin on plasma LH. We hypothesize that the strong reduction in FSH by deslorelin in intact animals may be pivotal to the efficacy of this GnRH agonist when used as a chemical castrant in the rat. Thus, from an applied perspective, it may be more important to analyze changes in FSH, than the more routinely analyzed LH, to gauge efficacy of a specific GnRH analog in steroid deprivation therapy in the male.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kher R, Kalla NR. Antifertility effects of an LHRH agonist in male mice. *Contraception*. 1996; 53:299–306. [PubMed: 8724620]
2. Labrie F, Auclair C, Cusan L, Kelly PA, Pelletier G, Ferland L. Inhibitory effect of LHRH and its agonists on testicular gonadotropin receptors and spermatogenesis in the rat. *Int J Androl Suppl*. 1978; 2:303–18.
3. Trigg TE, Wright PJ, Armour AF, Williamson PE, Junaidi A, Martin GB, Doyle AG, Walsh J. Use of a GnRH analogue implant to produce reversible long-term suppression of reproductive function in male and female domestic dogs. *J Reprod Fertil Suppl*. 2001; 57:255–61. [PubMed: 11787159]
4. Resko JA, Belanger A, Labrie F. Effect of chronic treatment with a potent luteinizing hormone releasing hormone agonist on serum luteinizing hormone and steroid levels in the male rhesus monkey. *Biol Reprod*. 1982; 26:378–84. [PubMed: 7044432]
5. Lincoln GA, Fraser HM, Abbott MP. Blockade of pulsatile LH, FSH and testosterone secretion in rams by constant infusion of an LHRH agonist. *J Reprod Fertil*. 1986; 77:587–97. [PubMed: 3090246]
6. Tilbrook AJ, Galloway DB, Williams AH, Clarke IJ. Treatment of young rams with an agonist of GnRH delays reproductive development. *Horm Behav*. 1993; 27:5–28. [PubMed: 8440517]
7. Eymann J, Herbert CA, Thomson BP, Trigg TE, Cooper DW, Eckery DC. Effects of deslorelin implants on reproduction in the common brushtail possum (*Trichosurus vulpecula*). *Reprod Fertil Dev*. 2007; 19:899–909. [PubMed: 18076822]
8. Lincoln GA. Long-term stimulatory effects of a continuous infusion of LHRH agonist on testicular function in male red deer (*Cervus elaphus*). *J Reprod Fertil*. 1987; 80:257–61. [PubMed: 3110411]
9. Herbert CA, Trigg TE, Renfree MB, Shaw G, Eckery DC, Cooper DW. Effects of a gonadotropin-releasing hormone agonist implant on reproduction in a male marsupial, *Macropus eugenii*. *Biol Reprod*. 2004; 70:1836–42. [PubMed: 14973259]
10. Aspden WJ, Rao A, Rose K, Scott PT, Clarke IJ, Trigg TE, Walsh J, D'Occhio MJ. Differential responses in anterior pituitary luteinizing hormone (LH) content and LH β - and α -subunit mRNA, and plasma concentrations of LH and testosterone, in bulls treated with the LH-releasing hormone agonist deslorelin. *Domest Anim Endocrinol*. 1997; 14:429–37. [PubMed: 9437579]
11. Aspden WJ, van Reenen N, Whyte TR, Maclellan LJ, Scott PT, Trigg TE, Walsh J, D'Occhio MJ. Increased testosterone secretion in bulls treated with a luteinizing hormone releasing hormone (LHRH) agonist requires endogenous LH but not LHRH. *Domest Anim Endocrinol*. 1997; 14:421–8. [PubMed: 9437578]
12. Labrie F, Belanger A, Luu-The V, Labrie C, Simard J, Cusan L, Gomez J, Candas B. Gonadotropin-releasing hormone agonists in the treatment of prostate cancer. *Endocr Rev*. 2005; 26:361–79. [PubMed: 15867098]
13. Shally AV. Luteinizing hormone-releasing hormone analogs: their impact on the control of tumorigenesis. *Peptides*. 1999; 20:1247–62. [PubMed: 10573298]
14. Schally AV, Redding TW, Comaru-Shally AM. Potential use of analogs of luteinizing hormone-releasing hormone in the treatment of hormone-sensitive neoplasms. *Cancer Treat Rep*. 1984; 68:281–9. [PubMed: 6362868]
15. Smith MR. Androgen deprivation therapy for prostate cancer: new concepts and concerns. *Curr Opin Endocrinol Diabetes Obes*. 2007; 14:247–54. [PubMed: 17940447]
16. Crosignani PG, Luciano A, Ray A, Bergqvist A. Subcutaneous depot medroxyprogesterone acetate versus leuprolide acetate in the treatment of endometriosis-associated pain. *Hum Reprod*. 2006; 21:248–56. [PubMed: 16176939]
17. Rackow BW, Arici A. Options for medical treatment of myomas. *Obstet Gynecol Clin North Am*. 2006; 33:97–113. [PubMed: 16504809]
18. Badaru A, Wilson DM, Bachrach LK, Fechner P, Gandrud LM, Durham E, Wintergerst K, Chi C, Klein KO, Neely EK. Sequential comparisons of one-month and three-month depot leuprolide regimens in central precocious puberty. *J Clin Endocrinol Metab*. 2006; 91:1862–7. [PubMed: 16449344]

19. Bowen RL, Verdile G, Liu T, Parlow AF, Perry G, Smith MA, Martins RN, Atwood CS. Luteinizing hormone, a reproductive regulator that modulates the processing of amyloid- β precursor protein and amyloid- β deposition. *J Biol Chem.* 2004; 279:20539–45. [PubMed: 14871891]
20. Mathias JR, Clench MH, Reeves-Darby VG. Effects of leuprolide acetate in patients with functional bowel disease. Long-term follow-up after double-blind, placebo-controlled study. *Dig Dis Sci.* 1994; 39:1163–70. [PubMed: 8200248]
21. Setji TL, Brown AJ. Comprehensive clinical management of polycystic ovary syndrome. *Minerva Med.* 2007; 98:175–89. [PubMed: 17592439]
22. Clowse ME, Behera MA, Anders CK, Copland S, Coffman CJ, Leppert PC, Bastian LA. Ovarian preservation by GnRH agonists during chemotherapy: a meta-analysis. *J Womens Health.* 2009; 18:311–9.
23. Hazum E, Cuatrecasas P, Marian J, Conn PM. Receptor-mediated internalization of fluorescent gonadotropin-releasing hormone by pituitary gonadotropes. *Proc Natl Acad Sci U S A.* 1980; 77:6692–5. [PubMed: 6256756]
24. Conn PM, Huckle WR, Andrews WV, McArdle CA. The molecular mechanism of action of gonadotropin releasing hormone (GnRH) in the pituitary. *Rec Prog Horm Res.* 1987; 43:29–68. [PubMed: 2819996]
25. Smith MA, Perrin MH, Vale WV. Desensitization of cultured pituitary cells to gonadotropin-releasing hormone: evidence for a post-receptor mechanism. *Mol Cell Endocrinol.* 1983; 30:85–96. [PubMed: 6301908]
26. Crowder ME, Herring RD, Nett TM. Rapid recovery of gonadotroph function after down-regulation of receptors for GnRH in ewes. *J Reprod Fert.* 1986; 78:577–85.
27. Counis R, Garrel G, Laverriere J, Simon V, Bleux C, Magre S, Cohen-Tannoudji J. The GnRH receptor and the response of gonadotrope cells to GnRH pulse frequency code. A story of an atypical adaptation of cell function relying on a lack of receptor homologous desensitization. *Folia Histochem Cytobiol.* 2009; 47:S81–7. [PubMed: 20067899]
28. Horvath JE, Bajo AM, Schally AV, Kovacs M, Herbert F, Groot K. Effects of long-term treatment with the luteinizing hormone-releasing hormone (LHRH) agonist Decapeptyl and the LHRH antagonist Cetrorelix on the levels of pituitary LHRH receptors and their mRNA expression in rats. *Proc Natl Acad Sci U S A.* 2002; 99:15048–53. [PubMed: 12409615]
29. Huhtaniemi I, White R, McArdle CA, Persson BE. Will GnRH antagonists improve prostate cancer treatment? *Trends Endocrinol Metab.* 2009; 20:43–50. [PubMed: 19008119]
30. Pawson AJ, Faccenda E, Maudsley S, Lu Z-L, Naor Z, Millar RP. Mammalian type I gonadotropin-releasing hormone receptors undergo slow, constitutive, agonist-independent internalization. *Endocrinology.* 2008; 149:1415–22. [PubMed: 18039780]
31. McArdle CA, Forrest-Owen W, Willars GB, Davidson J, Poch A, Kratzmeier M. Desensitization of gonadotropin-releasing hormone action in the gonadotrope-deprived alpha T3-1 cell line. *Endocrinol.* 1995; 136:4864–71.
32. Willars GB, Challiss RA, Nahorski SR. Acute regulation of the receptor-mediated phosphoinositide signal transduction pathway. *J Lipid Mediat Cell Signal.* 1996; 14:157–68. [PubMed: 8906558]
33. Willars GB, Royall JE, Nahorski SR, El-Gehani F, Everest H, McArdle CA. Rapid down-regulation of the type I inositol 1,4,5-trisphosphate receptor and desensitization of gonadotropin-releasing hormone-mediated Ca²⁺ responses in alpha T3-1 gonadotropes. *J Biol Chem.* 2001; 276:3123–9. [PubMed: 11069921]
34. Rolandi E, Franceschini R, Giberti C, Brancadoro T, Martorana G, Barreca T. Sustained impairment of pituitary and testicular function in prostatic cancer patients treated with a depot form of a GnRH agonist. *Horm Res.* 1988; 30:22–5. [PubMed: 2975631]
35. Hall MC, Fritzsche RJ, Sagalowsky AI, Ahrens A, Petty B, Roehrborn CG. Prospective determination of the hormonal response after cessation of luteinizing hormone-releasing hormone agonist treatment in patients with prostate cancer. *Urology.* 1999; 53:898–902. [PubMed: 10223480]

36. Kaku H, Saika T, Tsushima T, Ebara S, Senoh T, Yamato T, Nasu Y, Kumon H. Time course of serum testosterone and luteinizing hormone levels after cessation of long-term luteinizing hormone-releasing hormone agonist treatment in patients with prostate cancer. *Prostate*. 2006; 66:439–44. [PubMed: 16329145]
37. Nejat RJ, Rashid HH, Bagiella E, Katz AE, Benson MC. A prospective analysis of time to normalization of serum testosterone after withdrawal of androgen deprivation therapy. *J Urol*. 2000; 164:1891–4. [PubMed: 11061874]
38. Bolla M, Collette L, Blank L, Warde P, Dubois JB, Mirimanoff RO, Storme G, Bernier J, Kuten A, Sternberg C, Mattelaer J, Lopez Torecilla J, Pfeffer JR, Lino Cutajar C, Zurlo A, Pierart M. Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate cancer (an EORTC study): a phase III randomised trial. *Lancet*. 2002; 360:103–6. [PubMed: 12126818]
39. Oefelein MG. Time to normalization of serum testosterone after 3-month luteinizing hormone-releasing hormone agonist administered in the neoadjuvant setting: implications for dosing schedule and neoadjuvant study consideration. *J Urol*. 1998; 160:1685–8. [PubMed: 9783932]
40. Sandow J, von Rechenberg W, Baeder C, Engelbart K. Antifertility effects of an LH-RH analogue in male rats and dogs. *Int J Fertil*. 1980; 25:213–21. [PubMed: 6108932]
41. McNeilly JR, Brown P, Clark AJ, McNeilly AS. Gonadotrophin-releasing hormone modulation of gonadotrophins in the ewe: evidence for differential effects on gene expression and hormone secretion. *J Mol Endocrinol*. 1991; 7:35–43. [PubMed: 1716438]
42. Rodin DA, Lalloz MRA, CLayton RN. Gonadotropin-releasing hormone regulates follicle-stimulating hormone beta-subunit gene expression in the male rat. *Endocrinology*. 1989; 125:1282–9. [PubMed: 2503366]
43. Lalloz MRA, Detta A, CLayton RN. Gonadotropin-releasing hormone desensitization preferentially inhibits expression of the luteinizing hormone β -subunit gene in vivo. *Endocrinology*. 1988; 122:1689–94. [PubMed: 3278895]
44. Wormald PJ, Abrahamson MJ, Millar RP. Culture of functionally active human pituitary cells: investigation of gonadotropin regulation. *J Clin Endocrinol Metab*. 1988; 66:1272–7. [PubMed: 3131382]
45. Song J, Kost CK Jr, Martin DS. Androgens potentiate renal vascular responses to angiotensin II via amplification of the Rho kinase signaling pathway. *Cardiovasc Res*. 2006; 72:456–63. [PubMed: 17049502]
46. Periti P, Mazzei T, Mini E. Clinical pharmacokinetics of depot leuporelin. *Clin Pharmacokinet*. 2002; 41:485–504. [PubMed: 12083977]
47. Sartor O, Dineen MK, Perez-Marreno R, Chu FM, Carron GJ, Tyler RC. An eight-month clinical study of LA-2575 30.0 mg: a new 4-month, subcutaneous delivery system for leuprolide acetate in the treatment of prostate cancer. *Urology*. 2003; 62:319–23. [PubMed: 12893343]
48. Conn PM, Crowley WF Jr. Gonadotropin-releasing hormone and its analogues. *N Engl J Med*. 1991; 324:93–103. [PubMed: 1984190]
49. Stefanini M, De Martino C, Zamboni L. Fixation of ejaculated spermatozoa for electron microscopy. *Nature*. 1967; 216:173–4. [PubMed: 4862079]
50. Dada MO, Campbell GT, Blake CA. The localization of gonadotrophs in normal adult male and female rats. *Endocrinology*. 1984; 114:397–406. [PubMed: 6197294]
51. Hunter WM, Greenwood FC. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature*. 1962; 194:495–6. [PubMed: 14450081]
52. Taragnat C, Bernier A, Fontaine J. Gonadotrophin storage patterns in the ewe during the oestrous cycle or after long-term treatment with a GnRH agonist. *J Endocrinol*. 1998; 156:149–57. [PubMed: 9496244]
53. Hirsch HJ, Gillis D, Strich D, Chertin B, Farkas A, Lindenberg T, Gelber H, Spitz IM. The histrelin implant: a novel treatment for central precocious puberty. *Pediatrics*. 2005; 116:e798–802. [PubMed: 16322137]
54. Lahlou N, Carel JC, Chaussain JL, Roger M. Pharmacokinetics and pharmacodynamics of GnRH agonists: clinical implications in pediatrics. *J Pediatr Endocrinol Metab*. 2000; 13(Suppl 1):723–37. [PubMed: 10969915]

55. Klibanski A, Jameson JL, Biller BM, Crowley WF Jr, Zervas NT, Rivier J, Vale WW, Bikkal H. Gonadotropin and α -subunit responses to chronic gonadotropin-releasing hormone analog administration in patients with glycoprotein hormone-secreting pituitary tumors. *J Clin Endocrinol Metab.* 1989; 68:81–6. [PubMed: 2535852]
56. Hirsch HJ, Lahlou N, Gillis D, Strich D, Rosenberg-Hagen B, Chertin B, Farkas A, Hartman H, Spitz IM. Free α -subunit is the most sensitive marker of gonadotropin recovery after treatment of central precocious puberty with the histrelin implant. *J Clin Endocrinol Metab.* 2010 In Press.
57. Kanasaki H, Purwana IN, Mijiddorj T, Oride A, Miyazaki K. Possible involvement of PACAP and PACAP type 1 receptor in GnRH-induced FSH beta-subunit gene expression. *Regul Pept.* 2011
58. Grafer CM, Thomas R, Lambrakos L, Montoya I, White S, Halvorson LM. GnRH stimulates expression of PACAP in the pituitary gonadotropes via both the PKA and PKC signaling systems. *Mol Endocrinol.* 2009; 23:1022–32. [PubMed: 19342443]
59. Hart GR, Gowing H, Burrin JM. Effects of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, on pituitary hormone release in rats. *J Endocrinol.* 1992; 134:33–41. [PubMed: 1380057]
60. Culler MD, Paschall CS. Pituitary adenylate cyclase-activating polypeptide (PACAP) potentiates the gonadotropin-releasing activity of luteinizing hormone-releasing hormone. *Endocrinology.* 1991; 129:2260–2. [PubMed: 1915106]
61. Winters SJ, Tsujii T, Attardi B. Effects of GnRH and PACAP on gonadotropin secretion and subunit messenger RNAs. *Ann N Y Acad Sci.* 1996; 805:343–52. [PubMed: 8993415]
62. Winters SJ, Dalkin AC, Tsujii T. Evidence that pituitary adenylate cyclase activating polypeptide suppresses follicle-stimulating hormone-beta messenger ribonucleic acid levels by stimulating follistatin gene transcription. *Endocrinology.* 1997; 138:4324–9. [PubMed: 9322946]
63. Winters S, Ghooray D, Fujii Y, Moore J, Nevitt J, Kakar S. Transcriptional regulation of follistatin expression by GnRH in mouse gonadotroph cell lines: Evidence for a role for cAMP signaling. *Mol Cell Endo.* 2007; 271:45–54.
64. Kawakami S, Fujii Y, Okada Y, Winters SJ. Paracrine regulation of FSH by follistatin in folliculostellate cell-enriched primate pituitary cell cultures. *Endocrinology.* 2002; 143:2250–8. [PubMed: 12021189]
65. Oride A, Kanasaki H, Purwana IN, Mutiara S, Miyazaki K. Follistatin induced by thyrotropin-releasing hormone (TRH), plays no role in prolactin expression but affects gonadotropin FSH β expression as a paracrine factor in pituitary somatolactotroph GH3 cells. *Regul Pept.* 2009; 156:65–71. [PubMed: 19446581]
66. Meistrich ML, Wilson DM, Porter KL, Huhtaniemi I, Shetty G, Shuttlesworth GA. Restoration of spermatogenesis in dibromochloropropane (DBCP)-treated rats by hormone suppression. *Toxicol Sci.* 2003; 76:418–26.
67. Kangasniemi M, Wilson G, Huhtaniemi I, Meistrich ML. Protection against procarbazine-induced testicular damage by GnRH-agonist and antiandrogen treatment in the rat. *Endocrinol.* 1995; 136:3677–80.
68. Cusan L, Auclair C, Belanger A, Ferland L, Kelly PA, Seguin C, Labrie F. Inhibitory effects of long term treatment with a luteinizing hormone-releasing hormone agonist on the pituitary-gonadal axis in male and female rats. *Endocrinology.* 1979; 104:1369–76. [PubMed: 220032]
69. Padula AM. GnRH analogues-agonists and antagonists. *Anim Reprod Sci.* 2005; 88:115–26. [PubMed: 15955640]
70. Davidson JS, McArdle CA, Davies P, Elario R, Flanagan CA, Millar RP. Asn¹⁰² of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycnamide. *J Biol Chem.* 1996; 271:15510–4. [PubMed: 8663086]
71. Barrell GK, Schaafsma SCT, Ridgway MJ, Wellby M, Miller LA. Effects of a synthetic gonadotrophin-releasing hormone agonist, leuprolide, on rut-associated events in male red deer. *Anim Prod Sci.* 2009; 49:1120–4.
72. Aspden WJ, Rao A, Scott PT, Clarke IJ, Trigg TE, Walsh J, D'Occhio MJ. Direct actions of the luteinizing hormone-releasing hormone agonist, deslorelin, on anterior pituitary contents of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), LH and FSH subunit

messenger ribonucleic acid, and plasma concentrations of LH and FSH in castrated male cattle. *Biol Reprod.* 1996; 55:386–92. [PubMed: 8828844]

73. van der Kaay DC, de Jong FH, Rose SR, Odink RJ, Bakker-van Waarde WM, Sulkers EJ, Hokken-Koelega AC. Overnight levels of luteinizing hormone, follicle-stimulating hormone and growth hormone before and during gonadotropin-releasing hormone analogue treatment in short boys born small for gestational age. *Horm Res.* 2009; 71:260–7. [PubMed: 19339790]
74. Lerrant Y, Kottler M, Bergametti M, Moumni M, Blumberg-Tick J, Counis R. Expression of gonadotropin-releasing hormone (GnRH) receptor gene is altered by GnRH agonist desensitization in a manner similar to that of gonadotropin β -subunit genes in normal and castrated rat pituitary. *Endocrinology.* 1995; 136:2803–8. [PubMed: 7789305]
75. Nadji M. Quantitative immunohistochemistry of estrogen receptor in breast cancer: “much ado about nothing! ”. *Appl Immunohistochem Mol Morphol.* 2008; 16:105–7. [PubMed: 18227735]
76. Leong AS. Quantitation in immunohistology: fact or fiction? A discussion of variables that influence results. *Appl Immunohistochem Mol Morphol.* 2004; 12:1–7. [PubMed: 15163011]
77. Kitahara K, Sakai Y, Hosaka M, Hira Y, Kakizaki H, Watanabe T. Effects of a depot formulation of the GnRH agonist leuporelin on the ultrastructure of male rat pituitary gonadotropes. *Arch Histol Cytol.* 2007; 70:79–93. [PubMed: 17827666]
78. McNeilly AS, Crawford JL, Taragnat C, Nicol L, McNeilly JR. The differential secretion of FSH and LH: regulation through genes, feedback and packaging. *Reprod Suppl.* 2003; 61:463–76. [PubMed: 14635955]
79. Abbot SD, Docherty K, Roberts JL, Tepper MA, Chin WW, Clayton RN. Castration increases luteinizing hormone subunit messenger RNA levels in male rat pituitaries. *J Endocrinol.* 1985; 107:R1–4. [PubMed: 2415651]
80. Nolan LA, Levy A. A population of non-luteinising hormone/non-adrenocorticotrophic hormone-positive cells in the male rat anterior pituitary responds mitotically to both gonadectomy and adrenalectomy. *J Neuroendocrinol.* 2006; 18:655–61. [PubMed: 16879164]
81. Sun YT, Irby DC, Robertson DM, de Kretser DM. The effects of exogenously administered testosterone on spermatogenesis in intact and hypophysectomized rats. *Endocrinology.* 1989; 125:1000–10. [PubMed: 2502373]
82. Pelletier G, Cusan L, Auclair C, Kelly PA, Déry L, Labrie F. Inhibition of spermatogenesis in the rat by treatment with [D-Ala⁶,Des-Gly-NH²10] LHRH ethylamide. *Endocrinology.* 1978; 103:641–43. [PubMed: 369841]
83. Ward JA, Furr BJ, Valcaccia B, Curry B, Bardin CW, Gunsalus GL, Morris ID. Prolonged suppression of rat testis function by a depot formulation of Zoladex, a GnRH agonist. *J Androl.* 1989; 10:478–86. [PubMed: 2533593]
84. Lamminen T, Jokinen P, Jiang M, Pakarinen P, Simonsen H, Huhtaniemi I. Human FSH β subunit gene is highly conserved. *Mol Hum Reprod.* 2005; 11:601–5. [PubMed: 16100240]
85. Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet.* 1997; 15:201–4. [PubMed: 9020850]
86. Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology.* 2000; 141:1795–803. [PubMed: 10803590]
87. Bahk JY, Hyun JS, Chung SH, Lee H, Kim MO, Lee BH, Choi WS. Stage specific identification of the expression of GnRH mRNA and localization of the GnRH receptor in mature rat and adult human testis. *J Urol.* 1995; 154:1958–61. [PubMed: 7563392]
88. Clayton RN, Katikineni M, Chan V, Dufau ML, Catt KJ. Direct inhibition of testicular function by gonadotropin-releasing hormone: mediation by specific gonadotropin-releasing hormone receptors in interstitial cells. *Proc Natl Acad Sci U S A.* 1980; 77:4459–63. [PubMed: 6254027]
89. Stojilkovic, SS. Calcium signalling systems. In: Conn, PM., editor. *Handbook of Physiology: The Endocrine System, Cellular Endocrinology.* New York: Oxford University Press; 1988. p. 177-224.
90. Yao B, Liu HY, Gu YC, Shi SS, Tao XQ, Li XJ, Ge YF, Cui YX, Yang GB. Gonadotropin-releasing hormone positively regulates steroidogenesis via extracellular signal-regulated kinase in rat Leydig cells. *Asian J Androl.* 2011; 13:438–45. [PubMed: 21441942]

91. Lefebvre F, Belanger A, Pelletier G, Labrie F. Recovery of gonadal functions in the adult male rat following cessation of five-month daily treatment with an LHRH agonist. *J Androl.* 1984; 5:181–92. [PubMed: 6086556]
92. Wierman ME, Wang C. Androgen selectively stimulates follicle-stimulating hormone-beta mRNA levels after gonadotropin-releasing hormone antagonist administration. *Biol Reprod.* 1990; 42:563–71. [PubMed: 2111188]
93. Paul SJ, Ortolano GA, Haisenleder DJ, Stewart JM, Shupnik MA, Marshall JC. Gonadotropin subunit messenger RNA concentrations after blockade of gonadotropin-releasing hormone action: testosterone selectively increases follicle-stimulating hormone β -subunit messenger RNA by posttranscriptional mechanisms. *Mol Endocrinol.* 1990; 4:1943–55. [PubMed: 1707128]
94. Winters SJ, Ishizaka K, Kitahara S, Troen P, Attardi B. Effects of testosterone on gonadotropin subunit messenger ribonucleic acids in the presence or absence of gonadotropin-releasing hormone. *Endocrinology.* 1992; 130:726–34. [PubMed: 1370794]
95. Leal AM, Blount AL, Donaldson CJ, Bilezikjian LM, Vale WW. Regulation of follicle-stimulating hormone secretion by the interactions of activin-A, dexamethasone and testosterone in anterior pituitary cell cultures of male rats. *Neuroendocrinology.* 2003; 77:298–304. [PubMed: 12806175]
96. Bilezikjian LM, Corrigan AZ, Blount AL, Vale WW. Pituitary follistatin and inhibin subunit messenger ribonucleic acid levels are differentially regulated by local and hormonal factors. *Endocrinology.* 1996; 137:4277–84. [PubMed: 8828487]
97. Sharma N, Marzo SJ, Jones KJ, Foecking EM. Electrical stimulation and testosterone differentially enhance expression of regeneration-associated genes. *Exp Neurol.* 2010; 223:183–91. [PubMed: 19427307]
98. Shuto Y, Somogyvari-Vigh A, Onda H, Arimura A. Effect of hypophysectomy on pituitary adenylate cyclase activating polypeptide gene expression in the rat hypothalamus. *Peptides.* 1995; 16:407–13. [PubMed: 7651892]
99. Thackray VG, Mellon PL, Coss D. Hormones in synergy: regulation of the pituitary gonadotropin genes. *Mol Cell Endocrinol.* 2010; 314:192–203. [PubMed: 19747958]
100. Thackray VG, Mellon PL. Synergistic induction of follicle-stimulating hormone β -subunit gene expression by gonadal steroid hormone receptors and Smad proteins. *Endocrinology.* 2008; 149:1091–102. [PubMed: 18079204]
101. Song K, Wang H, Krebs TL, Wang B, Kelley TJ, Danielpour D. DHT selectively reverses Smad3-mediated/TGF- β -induced responses through transcriptional down-regulation of Smad3 in prostate epithelial cells. *Mol Endocrinol.* 2010; 24:2019–29. [PubMed: 20739403]

Figure 1.

Representative photomicrographs of β subunit immunoreactive gonadotropes from control animals and rats treated with deslorelin, or both deslorelin and testosterone. Treatment with deslorelin reduced the proportion of FSH β -ir cells independently of testosterone replacement. In contrast, there was no change in the percentage of LH-ir cells. Bar = 50 μ m (10 μ m for inset).

Figure 2.

Effect of the GnRH agonist, deslorelin, on intact adult male rats. A) cells immunoreactive for the α subunit and gonadotrope β subunits (mean $\pm$ SEM) in the pituitary gland. The percentage of cells immunoreactive for α GSU (white bar) was not affected by deslorelin. In contrast, the total β subunit gonadotrope (mono-LH (red) + mono-FSH (green) + bi-hormonal (yellow)) population was significantly suppressed by deslorelin. Although testosterone replacement did not reverse this suppression, the relative expression of β subunits was changed. B) Effect of deslorelin on plasma FSH, LH and testosterone. *Control vs Deslorelin or Deslorelin+T*: ** $p<0.01$, *** $p<0.001$; *Deslorelin vs Deslorelin+T*: † $p<0.05$, †† $p<0.01$, ††† $p<0.001$; n noted below total gonadotrope and plasma columns

Figure 3.

Representative photomicrographs of β subunit immunoreactive gonadotropes from castrate rats treated with deslorelin, testosterone or both deslorelin and testosterone. Treatment with deslorelin reduced the proportion of FSH β - and LH β -ir cells independently of testosterone. Note the increased size of the gonadotropes (c.f. Controls in Figure 1). Bar = 50 μ m (10 μ m for inset)

Figure 4.

Effect of deslorelin on castrated adult male rats. A) cells immunoreactive for the α subunit and gonadotrope β subunits (mean $\pm$ SEM) in the pituitary gland. Castration increased LH β subunit expression in mono-FSH β immunoreactive cells, resulting in no significant increase in the total β subunit gonadotrope (mono-LH (red) + mono-FSH (green) + bi-hormonal (yellow)) population. The percentage of cells immunoreactive for α GSU (white bar) only decreased when both deslorelin and testosterone were co-administered. In contrast, the total β subunit gonadotrope population was significantly suppressed by deslorelin and, as for intact rats, testosterone replacement did not reverse this suppression, although the relative expression of β subunits was changed. B) Effect of treatments on plasma FSH, LH and testosterone. Control vs castrate, castrate+ deslorelin or castrate+ deslorelin+ testosterone: *

$p < 0.01$, ** $p < 0.01$, *** $p < 0.001$; *castrate vs castrate+ deslorelin or castrate+ deslorelin +testosterone*: † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$; *n* noted below total gonadotrope and plasma columns

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Figure 5.

A) Testes of control and deslorelin-treated rats. Deslorelin decreased testis weight by two thirds. B) Cross section through seminiferous tubules of control and deslorelin-treated rats. Bar A = 5mm; B = 100 μ m