Effect of neonatal and adult testosterone treatment on the cellular composition of the adult female rat anterior pituitary

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Abstract

The adult female pituitary has significantly more lactotrophs than that of the male, while the latter has a higher percent of somatotrophs. It is clear that GH and prolactin (PRL) gene expression and somatotroph and lactotroph proliferation are modulated by the postpubertal hormone environment; however, the role of the neonatal steroid environment in this process is not known. We have used *in situ* hybridization to determine the number of GH and PRL mRNA-containing cells, as well as the level of expression of these two hormones, in response to neonatal and adult testosterone treatment. Female rats exposed to testosterone during the neonatal period, adulthood or both periods, as well as normal females and males were used.

Exposure to testosterone during the neonatal period significantly increased the percentage of somatotrophs (ANOVA: $P<0.005$) and decreased that of lactotrophs in the adult female rat (ANOVA: $P<0.001$). Adult testosterone treatment had no significant effect on the percentage of somatotrophs. The percentage of lactotrophs was significantly increased by adult testosterone only in those rats

also exposed to neonatal testosterone. PRL mRNA concentrations, as reflected by silver grains/cell, were reduced by neonatal testosterone and increased by adult testosterone treatment (ANOVA: $P<0.0001$). Overall PRL mRNA levels, measured by densitometry, were also reduced by neonatal testosterone exposure, but adult testosterone had no effect (ANOVA: $P<0.001$). GH mRNA levels per cell, as reflected by silver grains/cell, were increased by adult testosterone, while neonatal testosterone treatment had no effect. Overall GH mRNA levels per unit area, determined by densitometry measurements, were increased by both neonatal and adult testosterone treatment, with the combination of these two treatments resulting in adult females having levels indistinguishable from intact males (ANOVA: $P<0.003$).

These results suggest that, in combination with postpubertal sex steroids, the neonatal gonadal steroid environment plays an important role in determining anterior pituitary hormone synthesis and cellular composition.

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Introduction

After the onset of puberty the hormonal output of the anterior pituitary differs substantially between males and females. While females produce and secrete larger quantities of prolactin (PRL), the male pituitary synthesizes significantly more growth hormone (GH) (Birge *et al.* 1967, Döhler & Wuttke 1974, Jansson *et al.* 1985, Tong *et al.* 1989, Gabriel *et al.* 1992, González-Parra *et al.* 1996a). Postpubertal sex steroids are important in generating this phenomenon, but the neonatal gonadal steroid environment is also implicated (Jansson *et al.* 1985, Jansson & Frohman 1987a). However, the role of neonatal steroids is less well understood. Development of the neuroendocrine hypothalamus, including those neurons involved in GH or PRL control, is affected by the neonatal steroid environment (Matsumoto & Arai 1976, Matsumoto 1991,

Chowen *et al.* 1992, 1993, Murray & Gilles 1993, Reznikov & Nosenko 1995) and various hypothalamic neuropeptides are also implicated in somatotroph and lactotroph development (Chatelain *et al.* 1979, Billestrup *et al.* 1986, Hoeffler & Frawley 1987, Cella *et al.* 1994, Van Bael *et al.* 1998, Dean & Porter 1999). Hence, anterior pituitary development and control could be influenced indirectly by the neonatal steroid environment via modulation of its hypothalamic input, or neonatal steroids could act directly at the level of the pituitary.

In the postpubertal animal, the anterior pituitary of the female has more lactotrophs, while that of the male has a higher percentage of somatotrophs (Takahashi & Kawashima 1982, Ho *et al.* 1986, 1988, Hoeffler & Frawley 1986, González-Parra *et al.* 1998). These two cell types are derived from a common precursor and require the presence of the transcription factor Pit-1 for their

development and hormone expression (Li *et al.* 1991). This transcription factor is differentially expressed in lactotrophs and somatotrophs both *in vivo* and *in vitro* (González-Parra *et al.* 1996a, Chowen *et al.* 1998) and its regulation by sex steroids and other factors is cell-type specific and highly correlated to changes in PRL and GH gene expression (González-Parra *et al.* 1996a, Chowen *et al.* 1998). Modulation of the adult gonadal steroid environment can shift the percentage of GH- or PRL-containing cells, or increase the number of mammosomatotrophs, so that the female anterior pituitary more closely resembles that of the male, or vice versa (Boockfor *et al.* 1985, Ho *et al.* 1988, Goth *et al.* 1996, González-Parra *et al.* 1998). However, only a relatively small percentage of these cells appear to have the ability to change their hormone production, suggesting that the phenotype of some anterior pituitary cells may be determined during an earlier stage of development and be unchangeable. Indeed, we have previously shown that a larger change in the percentage of both lactotrophs and somatotrophs occurs in the male rat when the neonatal steroid environment is modulated (González-Parra *et al.* 1998).

The *in vivo* synthesis and secretion per individual somatotroph or lactotroph also differs between adult female and male rats, with males producing higher levels of GH and females of PRL (Ho *et al.* 1986, 1988, Hoeffler & Frawley 1986, González-Parra *et al.* 1996a). Whether this is due solely to differences in the postpubertal steroid environment is controversial; however, when exposed *in vitro* to the same sex steroid environment, lactotrophs and somatotrophs of male and female animals have similar levels of PRL and GH mRNA (González-Parra *et al.* 1998). This suggests that the adult sex steroid environment may be more involved in modulating the level of hormone production, with the neonatal steroid environment playing a more important role in determining the cellular composition of the anterior pituitary.

The aims of this study were as follows. (1) To evaluate the effects of the neonatal steroid environment on GH and PRL mRNA expression in the adult female pituitary. (2) To determine if the neonatal sex steroid environment modulates the number of lactotrophs and somatotrophs in the adult female anterior pituitary. (3) To examine whether exposure to testosterone during neonatal life influences the ability of the pituitary to modulate these cell populations in response to adult testosterone treatment. (4) To correlate the changes in the number of lactotrophs and somatotrophs with changes in GH and PRL mRNA concentrations.

Materials and Methods

Animals and housing

Wistar rats from our in-house breeding colony were housed with a ratio of 12 h light:12 h darkness, with lights

on at 0600 h. They were given free access to rat chow and tap water.

Experimental design

To assess the effects of sex steroids on GH and PRL mRNA levels and the number of lactotrophs and somatotrophs, female rats were treated with testosterone during the neonatal period (day of birth), adulthood (60 days) or both periods. Neonatal steroid treatment consisted of an s.c. injection of 250 µg testosterone, a dose shown to masculinize GH secretory patterns (Jansson & Frohman 1987a), dissolved in 100 µl sesame oil. The neonatal control group received an injection of 100 µl sesame oil. The neonatal animals that received sesame oil are referred to as FNO (female neonatal oil) and those receiving sesame oil containing testosterone as FNT (female neonatal testosterone). The intact male group (IM) also received an injection of sesame oil.

At 60 days of age, the animals received the second steroid treatment. Each of the neonatal treatment groups was randomly divided into two groups, one that received an empty Silastic capsule and the other a Silastic capsule containing crystalline testosterone. These capsules were designed to deliver adult male levels of circulating testosterone and their preparation has been described previously (Steiner *et al.* 1982). These treatments resulted in the following experimental groups: FNOAO (female, neonatal oil, adult oil), FNTAO (female, neonatal testosterone, adult oil), FNOAT (female, neonatal oil, adult testosterone), FNTAT (female, neonatal testosterone, adult testosterone) and IM (intact male). To minimize fluctuation in the results for cyclic females, in the group that had estrous cycles (FNOAO) only those females exhibiting at least two consecutive 4-day cycles were used and were killed on the morning of estrus. This day was chosen since females treated with testosterone are in a state similar to constant estrus. All animals were killed between 75 and 77 days of age between 0900 and 1100 h.

To determine overall mRNA levels for each peptide, *in situ* hybridization using a radioactive probe was performed. To determine the number of either lactotrophs or somatotrophs per unit area, riboprobes labeled with digoxigenin were employed.

Tissue preparation for *in situ* hybridization

On the day on which they were killed, animals were asphyxiated with carbon dioxide and immediately decapitated. The pituitaries were removed, rapidly frozen on dry ice, and stored intact at -80°C until processed for *in situ* hybridization histochemistry. Trunk blood was collected at the time of decapitation and separated by centrifugation. The serum was stored at -20°C until assayed for testosterone.

Before cutting, pituitaries were allowed to equilibrate in the cryostat (-17°C) and embedded in OCT (Tissue-Tek, Elkhart, IN, USA). Sections ($12\text{ }\mu\text{m}$) were thaw-mounted onto poly-L-lysine-coated slides. Tissue slices were stored at -80°C in airtight boxes until hybridization histochemistry was performed.

Riboprobe preparation

The original plasmid, pBR322-GH1, contains a 790 bp insert of a rat GH cDNA clone (Harpold *et al.* 1978, Dobner *et al.* 1981). A 451 bp Ava I/Pvu II fragment of this clone was further subcloned into a pGem3Z vector. This plasmid was kindly provided by P M Martha Jr, Baustate Medical Center, Springfield, MA, USA. The plasmids, SP65-1 and SP65-2, each containing a 780 bp fragment of the PRL cDNA, but in opposite directions, were used to transcribe antisense and sense PRL probes respectively. These plasmids were provided by R A Maurer (Gubbins *et al.* 1979, 1980).

In vitro transcription with S^{35} -UTP for radioactive probes was carried out as previously described (Chowen *et al.* 1991) and resulted in a specific activity of approximately 1.3×10^8 d.p.m./ μg for both GH and PRL. Transcription of digoxigenin probes was carried out according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). All probes were hydrolyzed to approximately 150 bases in 100 mM bicarbonate (pH 10.2) at 60°C .

In situ hybridization

After fixation in 4% paraformaldehyde, tissue sections were pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. The slides were rinsed in $2 \times \text{SSC}$ (300 mM NaCl and 30 mM Na citrate) and blotted. The probes were applied in 60 μl hybridization buffer at saturating concentrations. The slides were then covered with a strip of parafilm, sealed with rubber cement, and incubated overnight in moist chambers at 45°C . The following day, the parafilm coverslips were peeled off and the slides rinsed in $4 \times \text{SSC}$. After RNAase treatment and a series of washes in SSC of increasing stringency, the slides were dehydrated in alcohol and air-dried.

Slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA) that had been diluted 1:1 with 600 mM ammonium acetate and heated to 45°C in a water bath. They were allowed to air-dry for 15 min, then further dried in a moist chamber at room temperature for 1 h. Slides were placed in light-tight boxes containing desiccant and exposed for 4 days (PRL) or 5 days (GH) at 4°C . For all assays, slides were developed and counterstained with cresyl violet, and coverslips were then applied. (For more detail see Chowen *et al.* 1991.)

In situ hybridization assays with digoxigenin probes were performed equal to that for radioactive probes through the final wash in $0.1 \times \text{SSC}$; thereafter, instead of dehydrating the slides, they were incubated with $2 \times \text{SSC}$ containing 0.5% Triton X-100 and 2% normal sheep serum for 1 h at room temperature. The slides were then washed in buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) twice (10 min each) and incubated overnight at 4°C with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim; 1:1000 in buffer 1 containing 1% normal sheep serum and 0.4% Triton X-100). The slides were washed once in buffer 1 (10 min) and then buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl_2) twice for 10 min each. They were then incubated in 338 $\mu\text{g}/\text{ml}$ nitroblue tetrazolium-chloride, 175 $\mu\text{g}/\text{ml}$ 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) and 240 $\mu\text{g}/\text{ml}$ levamisole (Sigma Chemical Co., St Louis, MO, USA) in buffer 2 in a light-tight box overnight at room temperature. The following day the reaction was stopped in 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. The slides were rapidly dehydrated and coverslipped.

Control experiments

Control experiments, including employment of sense riboprobes and saturation curves, were performed for the riboprobes to verify their specificity and to determine the optimum concentration of probe for the quantitative assays. These results have been published previously (González-Parra *et al.* 1996a,b). As expected, specific binding of both riboprobes was seen over the anterior pituitary and not the posterior pituitary.

Image analysis system

Eight tissue sections throughout the entire anterior pituitary from each animal were included in the analysis of mRNA concentration. Slides selected for analysis were assigned a random three-letter code and analyzed in alphabetical, hence random, order by an operator unaware of the experimental group to which the animal belonged. The automated image-processing system consists of a Pixel Grabber video acquisition board (Perceptics Corp., Knoxville, TN, USA) attached to a Macintosh IICI computer. The software, Image, supplied by the National Institutes of Health, was used for densitometric analysis of tissue sections and Grains, written by D K Clifton at the University of Washington, Seattle, WA, USA, was used to determine grains/cell. Video images were obtained by a Sony camera (Sony Corporation, Japan) attached to a Zeiss Axioplan Photomicroscope.

To determine the relative level of specific mRNA per cell, the number of silver grains/individual cell was analyzed in approximately 100 cells per animal. A background reading was taken with each individual cell analysis and automatically subtracted. One difficulty with

this analysis was the close proximity of the anterior pituitary cells, which limits the availability of some cells for analysis. Only cells that could be individually isolated were analyzed with this system, which could bias the data. This caveat should be taken into consideration.

For densitometric analysis, darkfield illumination with a $\times 20$ magnification objective was used. In each tissue section, approximately ten different fields were selected and analyzed (approximately 80 readings/animal). Each field included only the anterior pituitary and had a clear signal free of artifacts. Background readings were taken in each section by measuring the non-specific signal over the posterior pituitary. The specific signal in each section was then calculated by subtracting the mean density obtained in the posterior pituitary from that obtained in the anterior pituitary. The non-specific signal over the posterior pituitary did not differ significantly between the experimental groups or within a group and did not exceed 10% of the specific signal.

The relationship between increasing concentrations of radioactivity and densitometry readings in this assay system has been reported previously, as well as the relationship between the signals received with *in situ* hybridization and protection assays (Chowen *et al.* 1991). Furthermore, it was verified that with increasing amounts of radioactive probe applied to the sections an increase in signal was detected with all probes until the available binding sites were saturated. All assays were run at saturating concentrations. Therefore, the densitometry readings reported reflect relative levels of specific mRNA signal and are reported as densitometry units.

The number of GH mRNA- or PRL mRNA-containing cells was determined by analyzing six tissue sections/animal dispersed throughout the entire anterior pituitary. Using a $\times 100$ objective, the operator counted the number of positive cells in 12 different fields in each section (12 fields/section, 6 tissue sections or 72 fields per pituitary). A cell was determined to be positive if the intensity of its labeling was at least 5% higher than the mean background reading taken over cells of the posterior pituitary. The mean number of unlabeled cells per area was also determined so that the percentage of labeled cells could be determined.

Serum testosterone assay

Trunk blood was collected when the animals were killed and allowed to clot. It was then centrifuged and the serum separated and stored at -20°C until assayed. Using an antibody and standard supplied by the WHO Matched Reagent programme, testosterone levels were measured by RIA, as previously described (Matsumoto *et al.* 1983). The sensitivity of this assay was 0.17 nmol/l in a $100\text{ }\mu\text{l}$ sample. The intra- and inter-assay coefficients of variations were 4.0% and 10% respectively.

Statistical analysis

For both GH and PRL, approximately 100 cells/animal were analyzed to obtain the mean grains/cell for each animal. The mean number of densitometry units for each animal was determined from the analysis of approximately 80 fields of view per animal. The mean number of cells per unit area was calculated from the analysis of approximately 72 fields per animal. The mean for each animal was then used to determine the means $\pm$ S.E.M. for each experimental group, with the n in all statistical analyses referring to the number of animals in the group ($n=5$ per group). To determine whether there was an interaction between neonatal steroid treatment and adult steroid treatment on either cell number or overall mRNA levels, a two-way analysis of variance (ANOVA) was performed. A one-way ANOVA followed by a Scheffé F test was performed to determine whether differences between the various groups existed. The significance level was chosen as $P<0.05$.

Results

Number of GH mRNA-containing cells

Figure 1 is a representative section through the anterior pituitary of a normal female (Fig. 1A) and a female treated with testosterone both as a neonate and adult (Fig. 1B). The darkly labeled cells represent those that are GH mRNA positive. The number of labeled cells and non-labeled cells per field was assessed. The total number of cells per view was not significantly different in any of the experimental groups.

The mean percentage of GH mRNA-positive cells per unit area was significantly lower in female rats as compared with males (Fig. 1C; ANOVA: $P<0.005$). Those animals exposed to testosterone during the neonatal period had a significantly higher percentage of GH mRNA-containing cells compared with those not exposed to testosterone during this time-period (FNTAO vs FNOAO). Adult testosterone treatment had no significant effect.

Number of PRL mRNA-containing cells

Figure 2A is a section through the anterior pituitary of a normal female and Fig. 2B that of a female treated with testosterone both as a neonate and as an adult. The darkly labeled cells represent those that are PRL mRNA positive. The number of labeled cells and nonlabeled cells per field was assessed. The total number of cells per view was not significantly different in any of the experimental groups.

Normal female rats had significantly more PRL mRNA-containing cells per unit area when compared with males (Fig. 2C; ANOVA: $P<0.001$). Exposure to testosterone during the neonatal period significantly decreased the number of PRL mRNA-containing cells (FNTAO vs FNOAO and FNTAT vs FNOAT). Adult

Figure 1 Brightfield photomicrographs of sections through the anterior pituitaries of a normal adult female rat (A) and a female treated with testosterone on the day of birth and during adulthood (B) on which *in situ* hybridization to detect GH mRNA was performed. The darkly stained cell bodies represent GH mRNA-containing cells. (C) Mean percentage ($\pm$ S.E.M.) of GH mRNA-containing cells/unit area in the anterior pituitary. FNOAO=normal female, FNOAT=female receiving testosterone during adulthood, FNTAO=female receiving testosterone as a neonate, FNTAT=female receiving testosterone both as a neonate and an adult, IM=intact male. * $P<0.005$: ANOVA; $n=5$ in each group.

steroid treatment increased the percentage of PRL mRNA-containing cells, but this was only significant in those animals that were also exposed to testosterone during the neonatal period (FNTAO vs FNTAT).

GH mRNA levels

GH mRNA levels per individual cell, as represented by grains/cell, were significantly higher in intact males compared with normal estrous females (Fig. 3A). Neonatal testosterone treatment did not significantly affect GH mRNA levels per individual cell. Adult testosterone treatment increased GH mRNA levels and this response was not affected by neonatal testosterone treatment.

The overall GH mRNA density level was significantly lower in normal females compared with males (Fig. 3B). Both neonatal and adult testosterone treatments significantly increased GH mRNA density levels (ANOVA:

$P<0.003$). Furthermore, exposure to testosterone during the neonatal period significantly increased the response to adult testosterone treatment (two-way ANOVA: $P<0.01$). The effect of neonatal and adult testosterone was additive, resulting in females exposed to testosterone during both periods having GH mRNA levels similar to those found in intact males.

In Fig. 4 the difference in GH mRNA signal density between a normal female (Fig. 4A) and a female treated with testosterone during both the neonatal and adult period (Fig. 4B) can be appreciated.

PRL mRNA levels

Mean PRL mRNA concentration per cell was significantly higher in normal females compared with males (Fig. 5A; ANOVA: $P<0.0001$). Neonatal testosterone treatment significantly reduced PRL mRNA

Figure 2 Brightfield photomicrographs of sections through the anterior pituitaries of a normal female rat (A) and a female treated with testosterone both as a neonate and an adult (B) on which *in situ* hybridization to detect PRL mRNA was performed. The darkly stained cell bodies represent PRL mRNA-containing cells. (C) Mean percentage ($\pm$ S.E.M.) of PRL mRNA-containing cells/unit area in the anterior pituitary. FNOAO=normal female, FNOAT=female receiving testosterone during adulthood, FNTAO=female receiving testosterone as a neonate, FNTAT=female receiving testosterone as a neonate and adult, IM=intact male. * $P<0.001$: ANOVA; $n=5$ in each group.

concentration/cell in the adult female rat. Adult testosterone had no effect on female rats not exposed to neonatal testosterone; however, it significantly increased PRL mRNA levels in those females treated with neonatal testosterone. Hence, the neonatal sex steroid environment modulated the response of lactotrophs in the adult animal to testosterone (two-way ANOVA: $P<0.0001$).

Overall PRL mRNA density levels were significantly higher in females compared with males (Fig. 5B, ANOVA: $P<0.001$). Treatment of female animals with testosterone during the neonatal period resulted in a significant reduction in overall PRL mRNA levels, while adult testosterone treatment had no significant effect.

In Fig. 6 the difference in PRL mRNA signal density between a normal female (Fig. 6A) and a male (Fig. 6B) can be appreciated.

Testosterone levels

The testosterone levels are shown in Table 1. The serum testosterone levels of the intact male and female rats were in the normal physiological range. Neonatal treatment with testosterone did not affect adult steroid levels. Animals treated with Silastic capsules containing testosterone had levels within the physiological range for intact adult males.

Discussion

The early postnatal hormone environment can have dramatic effects on the anterior pituitary since this gland is relatively immature at birth and continues to develop throughout neonatal life (Smets *et al.* 1989, Simmons

Figure 3 Comparison of relative mean concentrations of GH mRNA in the anterior pituitary of normal females (FNOAO), females receiving testosterone as adults (FNOAT), females receiving testosterone during neonatal life (FNTAO), females receiving testosterone both as neonates and adults (FNTAT) and intact males (IM). $n=5$ in each group. (A) GH mRNA concentrations/cell, as determined by grains/cell, $*P<0.0001$: ANOVA. (B) GH mRNA concentrations per unit area as determined by densitometry, $*P<0.003$: ANOVA.

1990). During adulthood, this gland remains under a dynamic state of cellular turnover with apparently fully differentiated cells undergoing mitotic division, with the majority of these proliferating cells expressing either GH or PRL (Dada *et al.* 1984, Carbajo-Pérez & Watanabe 1990, Oishi *et al.* 1993). Although there is active cellular turnover, the phenotypic make-up of the anterior pituitary is maintained unless the surrounding milieu is changed. Indeed, if the gonadal steroid environment is modulated the percentage of lactotrophs and somatotrophs can be shifted. However, in both adult males (González-Parra *et al.* 1998) and females, as shown here, the percentage of cells capable of changing their phenotype may be limited.

The cellular composition and size of the pituitary begin to differ between the sexes after the onset of puberty when the proliferation of anterior pituitary cells in the female starts to exceed that in males (Takahashi & Kawashima

1982, Hoeffler & Frawley 1987, Carbajo-Pérez & Watanabe 1990, Oishi *et al.* 1993). Postpubertal females have larger anterior pituitaries (Hoeffler & Frawley 1987, Carbajo-Pérez & Watanabe 1990) with a higher percentage of lactotrophs, while pituitaries of males contain a higher percentage of somatotrophs (Takahashi & Kawashima 1982, Ho *et al.* 1986, 1988, Hoeffler & Frawley 1986). Our results are in agreement with these findings and the proportion of each cell type reported here is similar to that found previously by using protein content or secretion for identification of cell type (Takahashi & Kawashima 1982, Ho *et al.* 1986, 1988, Hoeffler & Frawley 1986, Goth *et al.* 1996). Hence, *in situ* hybridization is at least as sensitive as these other techniques in detecting lactotrophs and somatotrophs and the differences reported here are not due to our inability to detect cells producing low levels of the hormone in question.

Testosterone treatment during only the adult period increased the number of lactotrophs in female rats by less than 5% and the overall percentage of somatotrophs by approximately 10%. These changes are similar to those reported previously in males (Ho *et al.* 1988, González-Parra *et al.* 1998) and suggest that the percentage of cells in the normal adult female pituitary that can be recruited to shift between GH or PRL expression is also limited. In contrast, adult testosterone treatment increased lactotrophs by approximately 30% in females exposed to testosterone neonatally. Hence, the neonatal environment may be important for determining the later responsiveness of these cells to changes in the steroid environment.

Modulation of the neonatal steroid environment has a dramatic effect on the cellular composition of the anterior pituitary. Exposure to testosterone during only the neonatal period reduced the number of lactotrophs by over 40% and increased the number of somatotrophs by approximately 20%. This resulted in the pituitary composition being more similar to that of an adult male and further demonstrates that the neonatal environment is involved in determining the cellular composition of the anterior pituitary gland.

Hypothalamic factors are undoubtedly important for development of the anterior pituitary (Chatelain *et al.* 1979, Hoeffler & Frawley 1987, Smets *et al.* 1989, Cella *et al.* 1994, Van Bael *et al.* 1998, Dean & Porter 1999) and whether the observed effects of sex steroids are mediated directly on the pituitary or via the hypothalamus remains to be determined. Expression of the transcription factor Pit-1 correlates temporally to when the developing pituitary and hypothalamus become physically connected and production of Pit-1 protein is followed by GH and PRL expression (Smets *et al.* 1989, Dollé *et al.* 1990). Pit-1 is involved in the expression of the GH-releasing hormone (GHRH) receptor in the pituitary (Lin *et al.* 1992) and GHRH stimulates somatotroph proliferation *in vitro* (Billestrup *et al.* 1986). Transient lack of GHRH during development causes a decrease in GH production in

Figure 4 Darkfield photomicrograph of a section through the pituitary of an estrous female (A) and a female treated with testosterone on the day of birth and during adulthood (B) on which *in situ* hybridization was performed to detect GH mRNA. The white grain clusters represent GH mRNA-positive cells.

the adult, as well as a reduced response to GHRH (Cella *et al.* 1994). Furthermore, when hypothalamic input is lacking, more lactotrophs develop (Shiino *et al.* 1977). These results could suggest that GHRH stimulates somatotroph proliferation and may be important for stopping cell development at this level, impeding the progression to lactotrophs. Other factors, such as estrogens, may function to shift the development towards lactotrophs. Indeed, activation of the estrogen receptor in conjunction with Pit-1 is necessary for lactotroph development

(Simmons *et al.* 1990) and estrogen decreases GHRH receptor mRNA levels in the pituitary (Lam *et al.* 1996), which could decrease GH expression. In contrast, expression of GHRH is increased by both neonatal and post-pubertal testosterone (Zeitler *et al.* 1992, Chowen *et al.* 1993) and is sexually dimorphic during the neonatal period (Argente *et al.* 1991) when the anterior pituitary is in a highly active phase of proliferation (Carbajo-Pérez & Watanabe 1990). Hence higher GHRH levels in male animals (Jansson *et al.* 1985, Argente *et al.* 1991)

Figure 5 Comparison of relative mean levels of PRL mRNA in the anterior pituitary of normal female rats (FNOAO), females receiving testosterone as adults (FNOAT), females receiving testosterone during neonatal life (FNTAO), females receiving testosterone both as neonates and adults (FNTAT) and intact males (IM). $n=5$ in each group. (A) PRL mRNA concentrations/cell, as determined by grains/cell, $*P<0.0001$; ANOVA. (B) PRL mRNA concentrations per unit area as determined by densitometry, $*P<0.001$; ANOVA.

may induce the development of a higher percentage of somatotrophs and fewer lactotrophs.

Both androgen and estrogen receptors are found in the developing and adult pituitary gland (Keefer & Holdeegger 1985, Babichev *et al.* 1990, Burgess & Handa 1993, Scully *et al.* 1997, Wilson *et al.* 1998) and both classes of steroids may be involved in determining the cellular composition of the anterior pituitary. Disruption of the estrogen receptor- α results in a decrease in lactotroph number and PRL production, but an increase in GH production (Scully *et al.* 1997), suggesting that the effects on lactotrophs may be via the estrogen receptor with little or no estrogen effect on somatotroph number. Neonatal castration of male rats reduces androgen receptors in the adult pituitary (Babichev *et al.* 1990), which may be due to a decrease in the number of cells expressing this receptor or the number of receptors per cell. *In vitro*, lactotrophs and somatotrophs from male and female rats have similar

changes in mean GH, PRL and Pit-1 mRNA levels per cell in response to sex steroids (Chowen *et al.* 1998), suggesting that individual cells from the two sexes have a similar responsiveness to gonadal steroids and, hence, may express similar levels of the receptors for these steroids.

The number of GH-expressing cells/area is not significantly modified by adult testosterone treatment, but GH mRNA levels/cell are increased and this is also reflected in an increase in GH mRNA density levels. This increase in GH expression/cell might augment our ability to detect low expressing cells and explain the slight increase in the percentage of GH mRNA-containing cells after adult testosterone treatment and not reflect a true increase in somatotrophs/area. Neonatal testosterone increases the percentage of GH mRNA-containing cells/area and GH mRNA levels/area, but has no significant effect on GH mRNA levels/cell. Hence, both neonatal and adult testosterone treatments increase GH mRNA density, but through different mechanisms. In the adult animal, the *in vivo* effects of testosterone on GH mRNA levels per individual cell are most likely via modulation of GHRH and somatostatin (Chowen-Breed *et al.* 1989, Argente *et al.* 1990, Zeitler *et al.* 1992), since GH gene expression is not modulated *in vitro* by this steroid (González-Parra *et al.* 1996b, Chowen *et al.* 1998).

When female rats were exposed to testosterone during the neonatal period, PRL mRNA levels/unit area, grains/cell and the percentage of lactotrophs decreased significantly. Therefore, neonatal exposure to testosterone decreases both the number of PRL-producing cells and their level of synthesis. Adult testosterone treatment stimulates the percentage of lactotrophs and PRL mRNA levels/cell, but only in animals exposed to neonatal testosterone. In normal females, adult testosterone treatment had no significant effect on either the number of lactotrophs or PRL mRNA levels. This result was unexpected since testosterone, via aromatization to estradiol and acting at the level of the pituitary, stimulates PRL mRNA levels (Shull *et al.* 1987, Waterman *et al.* 1988, Chowen *et al.* 1998, González-Parra *et al.* 1996b). Estrogen also stimulates the proliferation of lactotrophs (Pérez *et al.* 1986, Ho *et al.* 1988, Ellerkmann *et al.* 1991). It is possible that longer exposure to testosterone, in this case 2 weeks, does not have the same effect on lactotrophs from female animals as does acute exposure.

The effects of neonatal and adult testosterone treatment in female animals were very similar to those observed previously in male rats (González-Parra *et al.* 1998). One striking difference was observed in overall PRL mRNA levels. Treatment with testosterone during only the adult period significantly increased PRL mRNA levels in males while in the female there was no significant effect. On the other hand, neonatal testosterone significantly reduced PRL mRNA levels in females and slightly increased them in males. One caveat that must be taken into consideration is that these females were not ovariectomized and

Figure 6 Darkfield photomicrograph of a section through the pituitary of an estrous female rat (A) and a female rat treated with testosterone during the neonatal period (B) on which *in situ* hybridization to detect PRL mRNA was performed. The white grain clusters represent PRL mRNA-positive cells.

Table 1 Mean levels of serum tesotsterone (nmol/l). Values are means $\pm$ S.E.M.

FNOAO	FNOAT	FNTAO	FNTAT	IM
0.8 $\pm$ 0.09	10.0 $\pm$ 0.6	1.4 $\pm$ 0.1	10.6 $\pm$ 0.6	10.3 $\pm$ 2.7

See *Experimental design* for explanation of the various gropus

secretions by the ovaries may be inhibitory to or modulate some of these processes (Jansson & Frohman 1987*b*).

We have shown that both the neonatal and adult steroid environments are important in determining GH and PRL expression levels in the adult female rat and this is partially due to influences on the cellular composition of the anterior pituitary. Although it is well accepted that gonadal

steroids can modulate the number of somatotrophs and lactotrophs in the anterior pituitary of the adult rat, we suggest that the extent of this phenomenon may be limited and the final percentage of a specific cell type is more dependent on the neonatal steroid environment to which the animal is exposed.

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