Differential neonatal testosterone imprinting of GH-dependent liver proteins and genes in female mice

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Abstract

Abnormal exposure to steroid hormones within a critical developmental period elicits permanent alterations in female reproductive physiology in rodents, but the impact on the female GH axis and the underlying sexual differences in hepatic enzymes have not been described in detail. We have investigated the effect of neonatal androgenization of female mice (achieved by s.c. injection of 100 μg testosterone propionate (TP) on the day of birth: TP females) on the GHRH–somatostatin–GH axis and downstream GH targets, which included female and male predominant liver enzymes and secreted proteins. At 4 months of age, an organizational effect of neonatal testosterone was evidenced on hypothalamic *Ghrh* mRNA level but not on somatostatin (*stt*) mRNA level. *Ghrh* mRNA levels were higher in males than in females, but not in TP females. Increased expression in TP females correlated with increased pituitary GH content and somatotrope population, increased serum and liver IGF-I concentration, and ultimately higher body weight. Murine urinary proteins (MUPs) that were excreted at higher levels in male urine, and whose expression requires pulsatile occupancy of liver GH receptors, were not modified in TP females and neither was liver *Mup 1/2/6/8* mRNA expression. Furthermore, a male predominant liver gene (*Cyp2d9*) was not masculinized in TP females either, whereas two female predominant genes (*Cyp2b9* and *Cyp2a4*) were defeminized. These data support the hypothesis that neonatal steroid exposure contributes to the remodeling of the GH axis and defeminization of hepatic steroid-metabolizing enzymes, which may compromise liver physiology.

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Introduction

Abnormal exposure to steroid hormones within a critical developmental period elicits various permanent alterations in female reproductive physiology in rodents (Dorner 1981). The effects of neonatal androgens, estrogens, and endocrine disruptors on reproduction and sexual behavior have been the focus of numerous studies (Dorner 1981, Becu-Villalobos *et al.* 1997, Colciago *et al.* 2006, Monje *et al.* 2007, Wilson & Davies 2007, Zama & Uzumcu 2010), but the impact of neonatal steroid exposure on the GH axis in females and the underlying sexual differences in hepatic enzymes have not been studied in detail. Different perturbations during fetal and postnatal development may unleash endocrine adaptations that permanently alter metabolism, thus increasing the susceptibility to develop later disease.

In rodents, GH regulates the sexually dimorphic patterns of a large number of liver-expressed genes, including many receptors, signaling molecules, and enzymes of steroid and drug metabolism, especially cytochrome P450s (Cyps) (Waxman & O'Connor 2006). These sexual differences are dictated by the sexual dimorphism of plasma GH profiles, which is especially prominent in rats and mice. Plasma GH secretion is highly pulsatile in males, and elevated GH peaks occur every 3·5–4 h, which are interrupted by periods of no measurable hormone, whereas adult female rats and mice are characterized by more frequent and overlapping plasma GH peaks, resulting in a nearly constant presence of GH in circulation (MacLeod *et al.* 1991, Wehrenberg & Giustina 1992). These adult patterns of pituitary GH secretion are set during the neonatal period by exposure to gonadal steroids, which program the hypothalamic regulation of GH secretion at the onset of puberty and during adulthood (Dorner 1981, Chowen *et al.* 2004). Sexual differences in the pattern of GH secretion underlie the sexual dimorphism in liver metabolism of steroid hormones and drugs (Waxman & Holloway 2009). These differences in hepatic gene expression may be beneficial during pregnancy when the liver is exposed to high continuous levels of steroid hormones (Mode & Gustafsson 2006). Furthermore, sexual dimorphism in the liver in response to GH is involved in body growth and pheromone communication pathways (Hurst *et al.* 2001, Chamero *et al.* 2007).

The roles of GH treatment (pulsatile or continuous), hypophysectomy, and disruptive GH-activated transcription factors on the sexually dimorphic pattern of liver enzymes

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have been well established (Waxman & O'Connor 2006, Holloway *et al*. 2007, Waxman & Holloway 2009). Nevertheless, the impact of permanent organization of female mice hypothalamus by neonatal testosterone treatment has only been partially addressed in gonadectomized rats (Jansson *et al*. 1985). We therefore describe the consequences of neonatal administration of testosterone to female mice on the modulation of the GHRH–somatostatin (STT) hypothalamic system that controls GH release and downstream mechanisms, which include the sexual differentiation of female and male predominant liver enzymes and secreted proteins.

Gonadal steroids were administered only at birth in order to unravel the consequences of early steroid exposure in mice. The organizational effects of androgens are thought to be mediated by intracellular conversion of these hormones in certain brain areas to estrogens (Dorner *et al*. 1987), and estrogenic chemicals in the environment have potential adverse effects on animals and humans exposed during embryonic developmental stage (Damstra 2002). To this respect, clinical studies suggest that early hormonal imprinting may influence androgenization not only in rodents but also in women (Rubin *et al*. 1981, Collaer & Hines 1995). Therefore, our results are of significance when interpreting the developmental effect of endocrine disrupting chemicals on the growth axis and liver enzyme activity in females.

Materials and Methods

Animals

C57BL/6J mice were housed in a temperature-controlled room, with lights on at 0700 h and lights off at 1900 h, and had free access to laboratory chow and tap water. A total of 14 litters were used: 23 females, 24 testosterone propionate (TP) females, and 22 males. Animals were weighed at birth and every month, until 4 months of age. Blood samples were obtained from the facial vein at 1 and 2 months and by decapitation at 4 months of age. At decapitation, control females in diestrus and TP females in diestrus/anestrus were used, as TP females had highly irregular cycles. Sera were kept at −20 °C until RIAs were performed.

On the day of birth (designated day 0) the pups were sexed, and within 48 h after birth each pup was randomly divided into treatment groups: females were injected s.c. with 100 μg TP (Sigma) in 0·010 ml castor oil (neonatally androgenized females: TP females). This dose was chosen as it has been described to effectively androgenize neonatal brain in mice (Livne *et al*. 1992, Ingman & Robertson 2007). Females and males from the same cohort were injected with castor oil and used as controls. All experimental procedures were performed in accordance with the Division of Animal Welfare, Office for Protection from Research Risks, and the NIH (A#5072-01).

Reagents

Unless otherwise specified, all chemicals were purchased from Sigma.

RIAs

Prolactin and GH were measured by RIA, using kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK; Dr A F Parlow, National Hormone and Pituitary Program (NHPP), Torrance, CA, USA). The results were expressed in terms of mouse prolactin standard RP3 or mouse GH standard AFP-10783B. Intra- and interassay coefficients of variation were 7·2 and 12·8%, and 8·4 and 13·2% for prolactin and GH respectively.

For insulin-like growth factor-I (IGF-I) RIA, serum samples (15 μl) and IGF-I standards were subjected to the acid–ethanol cryoprecipitation method as previously described (Lacau-Mengido *et al*. 2000). IGF-I was determined using antibody (UB2-495) provided by Dr L Underwood and Dr J J Van Wyk, and distributed by the Hormone Distribution Program of the NIDDK. Recombinant human IGF-I (Chiron Corp., Emeryville, CA, USA) was used as radioligand and unlabeled ligand. The assay sensitivity was 6 pg per tube. Intra- and interassay coefficients of variation were 8·2 and 14·1% respectively.

Pituitaries (1–1·5 mg) or liver samples (50 mg) were homogenized in ice-cold PBS and centrifuged at 800 ***g*** for 5 min. Supernatant protein contents were measured with the QUBIT Fluorometer and the QUANT-IT protein Assay kit (Invitrogen). Aliquots of equal quantity of protein were used to assay pituitary GH and prolactin or liver IGF-I content.

Free testosterone levels were measured using a RIA kit (KIPI1 9000) provided by Biosource (Nivelles, Belgium) according to the manufacturer's instructions.

For all RIA measurements samples were run in duplicate.

Urine dosage of major urinary proteins

Urine was collected from 1- and 4-month-old male, female, and TP female mice between 1500 and 1700 h and centrifuged briefly for 3 min at 8800 ***g***. Five microliters of the supernatant were boiled in SDS buffer and diluted 1:3. Samples were fractionated in 12% SDS-PAGE and subsequently stained with Coomassie blue. Major urinary protein (MUP) (~20 kDa) represents the major protein component of mouse urine.

Tissue extraction and total RNA preparation for Ghrh*,* Stt*,* Mup 1/2/6/8*, glucokinase* (Gck)*, and* Cyp *expression by real-time PCR*

Brains were rapidly removed and placed on ice for dissection. For *Ghrh* and *Stt* analysis, an area limited anteriorly by the cephalic fissure of the optic chiasm, laterally by the

hypothalamic fissures, posteriorly by the fissure caudal to the mammilary bodies, and in-depth by the subthalamic sulcus was excised. All tissue samples (hypothalami and livers) were immediately homogenized in TRIzol reagent (Invitrogen) and stored at −70 °C until used. Total RNA was isolated from tissue homogenates by use of the TRIzol reagent method. The RNA concentration was determined on the basis of absorbance at 260 nm, its purity was evaluated by the ratio of absorbance at 260/280 nm (>1·8), and its integrity by agarose gel electrophoresis. RNAs were kept frozen at −70 °C until analyzed. After the digestion of genomic DNA by the treatment with DNase I (Invitrogen), first-strand cDNA was synthesized from 3 μg of total RNA in the presence of 10 mmol/l $MgCl_2$, 50 mmol/l Tris–HCl (pH 8·6), 75 mol/l KCl, 0·5 mM deoxy-NTPs, 1 mol/l dithiothreitol, 1 U/μl RNaseOUT (Invitrogen), 0·5 μg oligo$(dT)_{15}$ primer (Biodynamics, Buenos Aires, Argentina), and 20 U of MMLV reverse transcriptase (Epicentre Biotechnologies, Madison, WI, USA). To validate successful DNase I treatment, the reverse transcriptase was omitted in control reactions. The absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA.

Quantitative real-time PCR

Sense and antisense oligonucleotide primers were designed on the basis of the published cDNA or by the use of the PrimerExpress software (Applied Biosystems, Foster City, CA, USA). Oligonucleotides were obtained from Invitrogen. The sequences are described in Table 1.

Quantitative measurements of specific mRNA levels were performed by kinetic PCR using TAQurate GREEN Real-Time PCR MasterMix (9·4 μl, 10 mmol/l Tris–HCl, 50 mmol/l KCl, 3 mmol/l $MgCl_2$, 0·2 mmol/l deoxy-NTPs, 1·25 U Taq polymerase, Epicentre Biotechnologies), 0·4 μmol/l primers, and 150 ng cDNA in a final volume of 10·4 μl. After denaturation at 95 °C for 3 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and optical reading stage at 80 °C for 33 s. The accumulating DNA products were monitored by the ABI 7500 sequence detection system (Applied Biosystems), and the data were stored continuously during the reaction. The results were validated on the basis of the quality of dissociation curves generated at the end of the PCR runs by ramping the temperature of the samples from 60 to 95 °C, while continuously collecting fluorescence data. Product purity was confirmed by agarose gel electrophoresis. Each sample was analyzed in duplicate. Relative gene expression levels were calculated according to the comparative cycle threshold (C_T) method. Normalized target gene expression relative to *Cyclophilin* or *Gapdh* was obtained by calculating the difference in C_T values, the relative change in target transcripts being computed as $2^{-\Delta C_T}$. To validate the comparative C_T method of relative quantification, the efficiencies of each target and housekeeping gene amplification (endogenous *Cyclophilin* or *Gapdh*) were measured and shown to be approximately equal.

Immunohistochemistry

Pituitaries from 4-month-old animals fixed in formalin were embedded in paraffin (using a similar orientation in each sample for cutting the tissue), and immunohistochemistry was performed using fluorescence detection (Garcia-Tornadu *et al.* 2010). We used rabbit polyclonal antibody against mouse GH (dilution 1:750, NHPP, NIDDK-AFP-5672099). Secondary antibody was FITC goat anti-rabbit IgG (dilution 1:100; Zymed Laboratories, Inc., San Francisco, CA, USA).

Table 1 Description of primers used for real-time PCR

Gene	Strand	Primer sequence (5′–3′)	Source
Cyp2a4	Sense	AGC AGG CTA CCT TCG ACT GG	Wiwi *et al.* (2004)
	Antisense	GCT GCT GAA GGC TAT GCC AT	
Cyp2b9	Sense	CTG AGA CCA CAA GCG CCA C	Wiwi *et al.* (2004)
	Antisense	CTT GAG CAT GAG CAG GAC TCC	
Mup 1/2/6/8[a]	Sense	GAC TTT TTC TGG AGC AAA TCC ATG	Holloway *et al.* (2006)
	Antisense	GAG CAC TCT TCA TCT CTT ACA G	
Cyp2d9	Sense	AGT CTC TGG CTT AAT TCC TGA T	Wiwi *et al.* (2004)
	Antisense	CGC AAG AGT ATC GGG AAT GC	
Ghrh	Sense	GCC ATC TTC ACC ACC AA	Designed by PrimerExpress
	Antisense	CCT CCT GCT TGT TCA TGA TGT	
Stt	Sense	TCT GCA TCG TCC TGG CTT T	Designed by PrimerExpress
	Antisense	CTT GGC CAG TTC CTG TTT CC	
Gapdh	Sense	CAG AAC ATC ATC CCT GCA T	Designed by PrimerExpress
	Antisense	GTT CAG CTC TGG GAT GAC CTT	
Gck	Sense	GGG AAA CCT GAC AGG GAT GAG	Designed by PrimerExpress
	Antisense	CCG TGA TCC GGG AAG AGA A	
Cyclophilin	Sense	GTG GCA AGA TCG AAG TGG AGA AAC	Garcia-Tornadu *et al.* (2009)
	Antisense	TAA AAA TCA GGC CTG TGG AAT GTG	

[a]Mayor urinary protein (MUP) primers will potentially amplify four different *Mup* mRNAs (if expressed), i.e. *Mup1*, *Mup2*, *Mup6*, and *Mup8*.

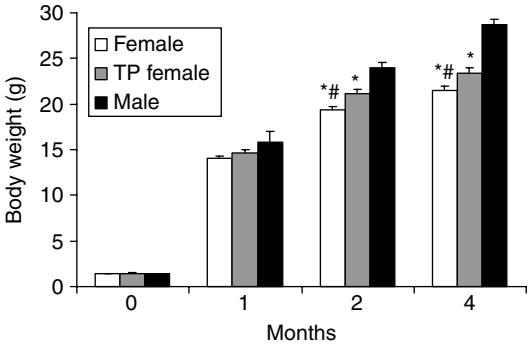

Figure 1 Body weight in females ($n=21$), males ($n=22$), and neonatally androgenized females (TP females, $n=22$). *$P<0{\cdot}05$ versus age-matched males, $^{\#}P<0{\cdot}05$ versus age-matched TP females. Averages + S.E.M. are shown.

Sections were mounted on Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent fading of the immunofluorescence reaction. Controls included substitution of primary antiserum with nonimmune serum. Four to five animals per experimental group and three pituitary sections per animal were used.

Morphometric analysis

Morphometric analysis was performed using a Carl Zeiss transmitted light microscope at a magnification of ×250 and ×400. Image analysis of pituitary sections for the calculation of tissue areas was performed by Image J, version 6.0 software. Sections were matched for morphology and the same diameter was always analyzed. The number of GH-immunoreactive cells was scored and used to calculate cell density (number of positive cells per square μm of tissue).

Statistical analysis

Data are expressed as means ± S.E.M. The differences between means were analyzed by ANOVA followed by the Newman–Keuls test or Tukey's honestly significant difference test for unequal n (for pituitary GH and prolactin content, serum IGF and prolactin, number of somatotropes per area, and hypothalamic and liver mRNA expression). Two-way ANOVA with repeated-measures design was used to analyze body weight and MUP excretion (effects of group and time) followed by the Newman–Keuls test or Tukey's honestly significant difference test for unequal n. P value $<0{\cdot}05$ was considered significant.

Results

Neonatally androgenized female mice have increased body weight and GH–IGF-I levels

Body weight was similar at birth and 1 month of age in control and neonatally androgenized (TP) females. At 2 and 4 months, body weight was increased by 9·3 and 8·8% respectively in TP compared to control age-matched females (P interaction$_{\text{sex, age (6,120)}}=0{\cdot}0021$; by *post hoc* test $P=0{\cdot}032$ and $0{\cdot}010$ for 2- and 4-month-old control versus TP females respectively; Fig. 1). Males were always heavier than the two other groups at these time points. The number of pituitary somatotropes was higher in males and TP females compared to females ($P=0{\cdot}042$ and $0{\cdot}013$ for females compared to males and TP females), and no differences were observed between males and TP females ($P=0{\cdot}43$; Fig. 2A). Pituitary GH concentration (ng/μg protein) was higher in males compared to females ($P=0{\cdot}012$) and was not different from TP females ($P=0{\cdot}095$; Fig. 2B). Increased GH secretion in TP females compared to control females was further inferred by an increase in serum IGF-I levels ($P=0{\cdot}042$; Fig. 2C), while no differences between males and TP females were observed for this growth factor ($P=0{\cdot}71$).

Sexual differences in serum prolactin levels (higher in females than males) were not affected by neonatal androgenization, even though pituitary prolactin concentration was higher in control females ($P=0{\cdot}038$) and not in TP females compared to males ($P=0{\cdot}34$; Fig. 2D and E).

Hypothalamic Ghrh *and* Stt *mRNA levels*

Hypothalamic *Ghrh* mRNA expression levels were higher in males than in females ($P=0{\cdot}028$), and female neonatal androgenization abolished this difference (males versus TP females, $P=0{\cdot}21$; Fig. 3A). On the other hand, no difference in the hypothalamic *Stt* mRNA levels was evident between the groups (Fig. 3B).

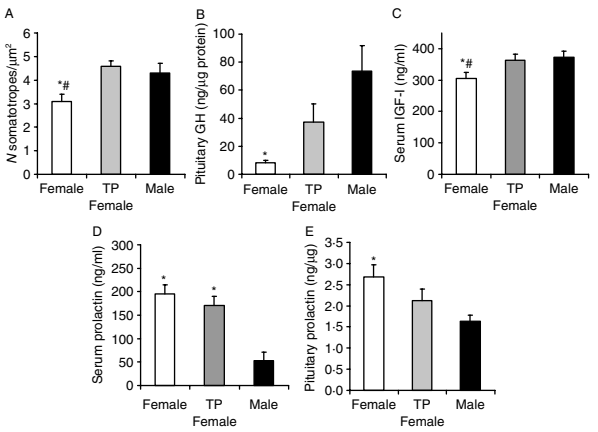

Figure 2 (A) Number of GH-immunopositive cells (somatotropes) per μm^2 of pituitary tissue in 4-month-old female, TP female, and male mice ($n=4$, 5, and 4 respectively); (B) pituitary GH concentration (ng/μg protein, $n=5$, 8, and 7 respectively); (C) serum IGF-I ($n=12$, 16, and 14 respectively); (D) serum prolactin levels ($n=12$, 17, and 15); and (E) pituitary prolactin concentration (ng/μg protein, $n=5$, 8, and 7). For all panels *$P<0{\cdot}05$ versus males, and $^{\#}P<0{\cdot}05$ versus TP females.

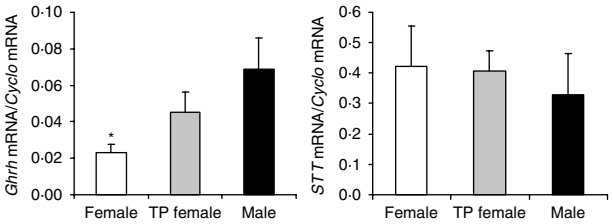

Figure 3 (A) *Ghrh* mRNA levels normalized to Cyclophylin (*Cyclo*) mRNA levels in 4-month-old females, TP females, and males ($n=6$, 6, and 5 respectively). *$P<0{\cdot}05$ versus males. (B) *Stt* mRNA levels normalized to *Cyclo* mRNA levels ($n=6$, 6, and 4 respectively).

Liver IGF-I concentration

Liver IGF-I (ng/μg protein), a downstream target of GH, was higher in samples from males in comparison to females ($P=0{\cdot}013$), but not in comparison to TP females ($P=0{\cdot}088$; Fig. 4). These results paralleled serum IGF-I levels in the three groups (see Fig. 2C).

Liver Cyp2d9, Cyp2b9, Cyp2a4, Mup 1/2/6/8, *and* Gck *mRNA expression*

Male-specific expression was confirmed for *Cyp2d9* and *Mup 1/2/6/8* mRNA levels, with male/female ratios of 6·2 and 2·4 respectively. Real-time PCR analysis of the two male predominant genes revealed that neonatal androgenization did not masculinize their expression in the female livers (Fig. 5A and B).

Cyp2a4 and *Cyp2b9* mRNA levels were both expressed predominantly in female mice, and female/male ratios obtained were 7·7 and 11·6 respectively. Neonatal androgenization of females induced a defeminization of both genes in the female livers, leading to a loss of sex-specific expression in the case of *Cyp2a4* ($P=0{\cdot}0034$ and 0·41, for males versus females and TP females respectively; Fig. 5C). Defeminization of *Cyp2b9* mRNA expression was partial, as TP females were different from males and females ($P=0{\cdot}0068$ and 0·026, TP females versus females and males respectively; Fig. 5D).

On the other hand, no effect of neonatal androgenization was evidenced on liver mRNA levels of glucokinase, a sex-independent mouse liver enzyme (Fig. 5E).

MUPs excreted in urine

MUPs were similar in the three groups in 1-month-old mice, and increased significantly only in males at 4 months (P interaction $_{\text{sex, age}\ (2,95)}=0{\cdot}00054$; $P<0{\cdot}0001$ for 1- vs 4-month-old males). At 4 months, sexual differences were well established ($P<0{\cdot}0001$ for males versus females and TP females; Fig. 6), and no effect of neonatal testosterone was evidenced in MUP excretion in adult females. As MUP levels are also susceptible to serum testosterone levels, we measured free testosterone levels in 4-month-old mice and found higher levels in males and low levels in both females and TP females (pg/ml ±S.E.M.: 14·30±6·10, 0·77±0·24, and 0·45±0·21 for males, females, and TP females respectively; $P<0{\cdot}05$ for males versus females and TP females, and differences between females and TP females were not significant).

Discussion

Gonadal hormones exert an organizational influence on the developing central nervous system during very restricted or critical periods of neural differentiation (Arnold & Gorski 1984, Becu-Villalobos & Libertun 1995, Becu-Villalobos *et al.* 1997). Exposure of the developing central nervous system to the presence or absence of androgen or estrogen results in the differentiation of a broad spectrum of responses that are congruent with the genotype. This process, referred to as sexual differentiation, assures adequate behavioral and neuroendocrine responses in males and females, which ultimately tend toward reproductive success. Abnormal exposure to steroid hormones or endocrine disrupting chemicals during this critical period may result in anomalies in fitness and reproductive success (Gore 2008, Fernandez *et al.* 2009). Although most studies have concentrated on the disruption of the reproductive axis we demonstrate that the GHRH–GH axis is also perturbed by neonatal steroids.

GH secretion is sexually differentiated in many species including rats, mice, and humans (Muller *et al.* 1999, Wehrenberg & Giustina 2000). Sexually dimorphic plasma GH profiles first emerge at puberty but are set and ultimately regulated by gonadal steroid imprinting during the neonatal period (Jansson & Frohman 1987). This dimorphic pattern of GH secretion controls the sex-dependent expression of a large number of hepatic genes.

A group of liver proteins that increase in direct relation to the male pattern of GH secretion are those encoded by the

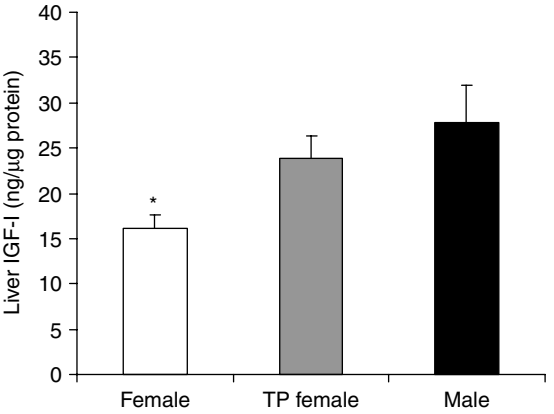

Figure 4 Liver IGF-I concentration (ng/μg protein) in 4-month-old females, TP females, and males ($n=12$, 16, and 14 respectively). *$P<0{\cdot}05$ versus males.

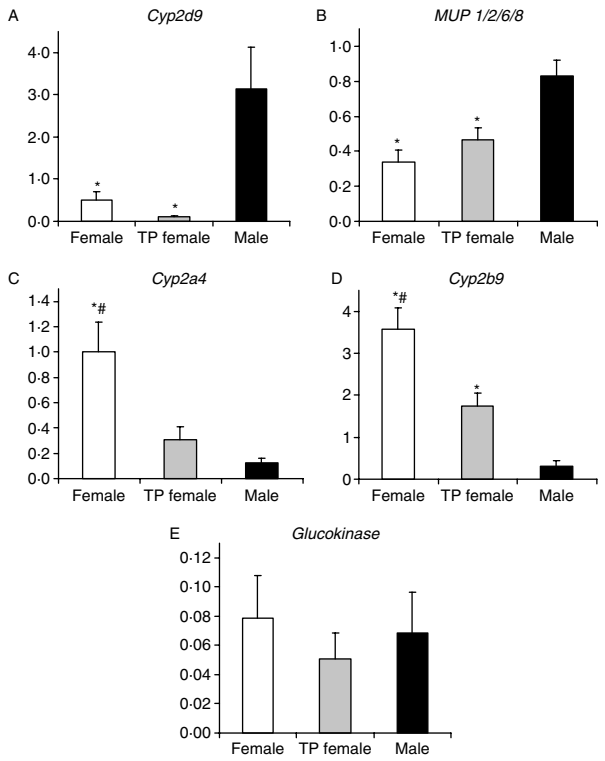

Figure 5 Liver genes normalized to *Gapdh* mRNA levels expressed in arbitrary units, in 4-month-old females, TP females, and males. (A) *Cyp2d9* mRNA levels ($n=9$, 11, and 9 respectively); (B) liver *Mup 1/2/6/8* mRNA levels ($n=11$, 9, and 12 respectively); (C) *Cyp2a4* mRNA levels ($n=10$, 11, and 11 respectively); (D) *Cyp2b9* ($n=13$, 12, and 12); (E) *Gck* mRNA levels ($n=11$, 11, and 10 respectively). For all panels $^{*}P<0{\cdot}05$ versus males, and $^{\#}P<0{\cdot}05$ versus TP females.

MUP gene family. MUPs are proteins, excreted in the urine, which are used by male adults to scent mark and countermark territories (Hurst *et al.* 2001, Hurst & Beynon 2004, Chamero *et al.* 2007). Although originally viewed as inert proteins with caging capacity to slow-release volatile pheromones secreted in the urine (Bacchini *et al.* 1992, Hurst & Beynon 2004), recent studies have demonstrated that adult male MUPs are able to directly promote territorial aggression between males (Chamero *et al.* 2007). Furthermore, sexually dimorphic GH secretion regulates certain liver *Cyp* genes, and sexual differences in hepatic steroid metabolism may support a pregnant state when the liver is exposed to high levels of steroid hormones (Waxman & O'Connor 2006). Therefore, apart from its well-known action to promote long bone growth, GH is important in the regulation of social rank determination and steroid metabolism.

In the present work, we have determined whether neonatal testosterone administration in female mice impacts the expression of sex-dependent liver gene expression, as well as IGF-I liver content and MUP excretion in intact mice. If testosterone is present in the blood during an early critical period, female traits will be permanently masculinized or defeminized. Masculinization refers to the organization of male qualities, and defeminization refers to the lack of development of feminine characteristics (Arnold & Gorski 1984).

Testosterone had an organizational effect on hypothalamic *Ghrh* mRNA level but not on *Stt* mRNA level. Increased *Ghrh* mRNA expression in TP females may underlie the higher somatotrope population found in this group compared to females, and ultimately increased serum and liver IGF-I and body weight. GHRH and STT release is primary in the regulation of the differences in the pattern of GH secretion found in males and females. In rats, hypothalamic *Ghrh* mRNA levels and pituitary GH content were greater in male versus female and increased after neonatal testosterone treatment (Wehrenberg & Giustina 2000, Chowen *et al.* 2004), as it is described here in mice. Hence, neonatal sex steroid administration masculinizes GHRH–GH system and this may partially determine the ability of the pituitary gland to secrete GH throughout life. The lack of sexual difference on *Stt* expression that we found has also been shown using whole hypothalamus (Werner *et al.* 1988, Bouyer *et al.* 2006, Luque & Kineman 2007), while others demonstrated increased STT in the periventricular nucleus of the male hypothalamus (Argente *et al.* 1991, Chowen *et al.* 1993, Murray *et al.* 1999). The differences encountered may be explained by STT anatomical distribution within the hypothalamus. On the other hand, we also describe partial androgenization of pituitary prolactin content. The decrease in pituitary prolactin concentration in TP females may be related to the increase in somatotrope population, and may condition maternal behavior or reproductive success in TP females (Guerra & Hancke 1982, Stern & Strait 1983, Bridges & Ronsheim 1990).

MUPs are excreted in male mouse urine at levels that are threefold higher than in female urine (Norstedt & Palmiter 1984), and their expression requires pulsatile occupancy of liver GH receptors, a characteristic of males. In mice, repeated

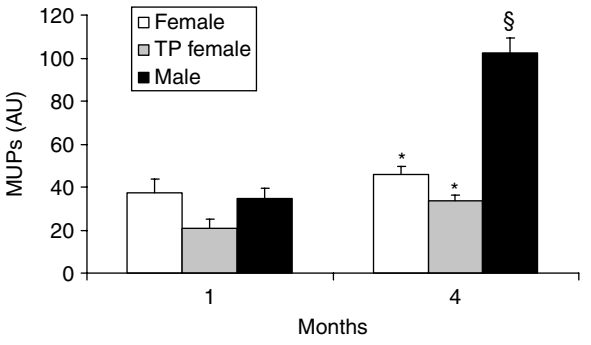

Figure 6 Murine urinary protein (MUP) excretion in 1- and 4-month-old females ($n=8$ and 22), TP females ($n=8$ and 21), and males ($n=6$ and 22). $^{*}P<0{\cdot}05$ versus age-matched males, and $^{\S}P<0{\cdot}05$ versus sex-matched 1-month-old group. AU, arbitrary units.

measures to determine GH pulsatility is technically difficult, and MUP excretion has been used as an indicator of GH pulsatile secretion (Norstedt & Palmiter 1984). Unexpectedly, even though we found higher levels of *Ghrh*, pituitary GH concentration, and serum IGF-I levels in neonatally androgenized female mice, MUP excretion was not increased. Furthermore, liver *Mup 1/2/6/8* mRNA levels were not increased in TP females. Therefore, neonatal androgenization *per se* may not be paramount in determining MUP sexual differences, and gonadal hormone milieu in the adulthood may be more directly related. In accordance, we found similar free testosterone levels in females and TP females.

In rodents, distinct male and female patterns of hepatic gene expression occur for several Cyps that are involved in steroid and drug metabolism, as well as for some proteins that function in reproduction directly via maintenance of pregnancy or indirectly via pheromone communication pathways (Roy & Chatterjee 1983). Sexual dimorphism in liver gene expression is dependent on gonadal steroids and sex-specific patterns of GH release. In the present experiments, the effect of neonatal androgen imprinting in females was divergent for different GH-dependent and sex-specific genes. Male predominant genes (*Cyp2d9* and *Mup 1/2/6/8*) were not masculinized in TP females, whereas two female predominant genes (*Cyp2b9* and *Cyp2a4*) were defeminized; on the contrary, no effect was seen in *Gck*, a sex-independent liver gene. It has been described that in neonatally androgenized and gonadectomized female rats, 16α hydrolase activity (equivalent to mouse Cyp2d9) increased to masculine levels particularly if animals were treated with testosterone during adult life, and 5α reductase activity decreased to levels seen in intact male rats (Jansson *et al.* 1985). The present experiments performed in mice indicate that the neonatal imprinting of the GHRH–GH system participates in the defeminization of some enzymes, but that masculinization of other enzymes is not fully achieved. This suggests that other factors may contribute to the observed sexual differences, or that, as previously suggested, using dwarf rats, low levels of GH are sufficient to regulate the expression of some liver-steroid-metabolizing enzymes (Bullock *et al.* 1991). On the other hand, the results on liver gene expression response to neonatal testosterone may also reflect incomplete masculinization of circulating GH profiles, as suggested by MUP excretion levels. To this respect, a distinct susceptibility to neonatal imprinting of sex-related systems has been described, and higher levels of neonatal steroids are needed to defeminize copulatory behavior than to disrupt gonadotropin secretion (Arnold & Gorski 1984).

We conclude that neonatal steroid exposure may contribute to the remodeling of the GH axis and defeminizes hepatic-steroid-metabolizing enzymes, events that may compromise liver physiology. These results should be highlighted in the study of early exposure to endocrine disrupting chemicals in females, and sexual differences in drug and xenobiotic metabolism.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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