Growth hormone: a newly identified developmental organizer

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Abstract

The sexually dimorphic expression of cytochromes P450 (CYP) drug-metabolizing enzymes has been reported in all species examined. These sex differences are only expressed during adulthood and are solely regulated by sex differences in circulating growth hormone (GH) profiles. Once established, however, the different male- and female-dependent CYP isoform profiles are permanent and immutable, suggesting that adult CYP expression requires imprinting. As the hormone that regulates an adult function is likely the same hormone that imprints the function, we selectively blocked GH secretion in some newborn male rats, whereas others received concurrent physiologic replacement of rat GH. The results demonstrate that adult male GH activation of the signal transduction pathway regulating expression of the principal CYP2C11 isoform is obligatorily dependent on perinatal GH imprinting, without which CYP2C11 and drug metabolism would be permanently and profoundly suppressed. As there are other adult metabolic functions also regulated by GH, pediatric drug therapy known to disrupt GH secretion could unintentionally impair adult health.

Key Words

- ▶ CYP2C11
- ▶ growth hormone
- ▶ imprinting
- ▶ JAK/STAT
- ▶ miRNA

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Introduction

Hormonal imprinting refers to a biological process in which the target tissue becomes responsive to the hormone. During the initial exposure, the hormone irreversibly reprograms the development of the affected tissue so as to permanently alter some functional aspect normally responsive to the hormone and often establishing a sexual dimorphism (Csaba 2008). Moreover, the tissue is programmable for only a brief developmental period, after which time the tissue becomes permanently unresponsive to imprinting (Goldman 1970, Ohno 1979, Feder 1981, Shapiro 1985a). Imprinting alone, however, is generally insufficient to ensure the expression of the programmed function. Accordingly, expression of the affected function requires both imprinting and activation, the latter being a reversible process, but required to express the imprinted function. For example, perinatal testosterone or one of its metabolites is required to permanently 'wire' or

imprint the male brain to exclusively express masculine sexual behavior. However, the brain has to be stimulated or activated in adulthood by the same hormone to elicit the imprinted male sexual behavior. Both imprinting and activation are required for normal masculine sexual behavior (Shapiro *et al.* 1980, Feder 1981, Shapiro 1985a), which explains why perinatally castrated males and intact females, not hormonally imprinted, are unable, regardless of adult hormone treatment, to exhibit normal masculine sexual behavior (Shapiro *et al.* 1980, Feder 1981).

The cytochromes P450 (CYP) monooxygenases are an ancient family of heme enzymes that catalyze a large variety of essential metabolites, which include the synthesis and the deactivation of adrenal, gonadal and thyroid hormones, eicosanoids, bile acids and fatty acids, as well as the detoxification of innumerable drugs and environmental chemicals; hence, their ubiquitous

presence throughout all species, where hundreds of isoforms have evolved to catalyze a countless number of substrates (Nebert & Dieter 2000, Hannemann *et al.* 2007). In the case of mammals, including humans, the CYPs are most prominent in the liver, where they are expressed during adulthood as dozens of different isoforms, many of which are sexually dimorphic (sex differences in CYP expression occurs postpubertally) (Shapiro *et al.* 1995). The only endogenous factor known to regulate the expression of adult hepatic CYP is growth hormone (GH) (Legraverend *et al.* 1992, Shapiro *et al.* 1995). At puberty, males begin to secrete GH in what is referred to as an 'episodic' profile characterized by several daily bursts of hormone separated by lengthy undetectable or barely detectable GH concentrations. In contrast, the adult female GH profile is considered 'continuous,' as there are far more secretory bursts, often at lower amplitudes, of the hormone separated by briefer interpulse periods, often containing measurable levels of GH. In fact, these sex differences in the circulating GH profile are solely responsible for the sex difference in CYP expression and resulting drug metabolism observed during adulthood (Legraverend *et al.* 1992, Shapiro *et al.* 1995). That is, the masculine 'episodic' GH profile induces the expression of the male-specific CYP isoforms, whereas the feminine 'continuous' GH profile induces the expression of the female-specific CYP isoforms. In this regard, we previously reported that adult male rats and men cannot be induced (regardless of GH treatment) to express the normal female profile of hepatic CYPs (Pampori & Shapiro 1999, Thangavel *et al.* 2004, 2011, Dhir *et al.* 2006, Thangavel & Shapiro 2008) nor can adult female rats or women be induced to fully express the masculine CYP profile (Dhir *et al.* 2006, 2007, Thangavel *et al.* 2006, Thangavel & Shapiro 2007). The response of each of the dozen or so sex-dependent rat CYP isoforms to GH regulation has been described (Thangavel & Shapiro 2008, Das *et al.* 2013a, Banerjee *et al.* 2015). If CYP enzymes were not imprinted, then irrespective of sex, the same adult treatment should produce the same CYP expression levels in males and females. As this is not the case, we have concluded that the sex differences in adult expression profiles of CYPs are permanent and irreversible, a condition demonstrating imprinting. Because an adult tissue/function is apt to be responsive to the same hormone by which it was imprinted, we have proposed, for the first time, that like androgens, perinatal GH is a developmental organizer required for the normal expression of adult CYP-dependent drug metabolism.

Materials and methods

Animal experimentation

Rats were housed in the University of Pennsylvania Laboratory Animal Resources facility under the supervision of certified laboratory animal medicine veterinarians and were treated according to a research protocol approved by the Institutional Animal Care and Use Committee of the University. Housing conditions, as well as breeding and treatment protocols, were reported previously (Shapiro *et al.* 1989, Pampori *et al.* 1991b). Basically, neonatal male rat pups were injected, sc, with either of 2 of 4 possible treatments. (1) Monosodium glutamate (MSG), 4mg/gbw on alternate days, starting within 24 h of birth for a total of 5 injections. (2) An equivalent amount of the MSG NaCl diluent. (3) Rat growth hormone (GH), 20 µg/100 gbw, twice/day for the first 12 days of life. (4) An equivalent amount of the GH bicarbonate diluent. Accordingly, control pups received treatments 2 and 4; MSG pups received treatments 1 and 4; GH pups received treatments 2 and 3 and MSG + GH pups received treatments 1 and 3. Body weights, Lee indices and hexobarbital sleep times were determined as previously reported (Shapiro *et al.* 1989, Pampori *et al.* 1991b).

Growth hormone determinations

Plasma GH concentrations were determined by a sensitive sandwich ELISA method as described previously (Das *et al.* 2013a). Procedures for collecting unstressed postnatal plasma as well as adult serial blood sampling for GH determinations have been reported (Pampori *et al.* 1991a, Banerjee *et al.* 2015).

Primary hepatocyte cultures

Preparation of hepatocytes from 18-week-old male rats was performed by *in situ* perfusion of collagenase

Table 1 Source of antibodies.

Name of antibodies	Source
CYP2C11	Detroit R&D Inc., Franklin, MI, USA
STAT5b	Santa Cruz Biotechnology
Anti-phosphotyrosine	EMD Millipore
CIS	Santa Cruz Biotechnology
SOCS2	Santa Cruz Biotechnology
β-Actin	Sigma Chemical

Table 2 Primer sequences used for qRT-PCR.

mRNA	Forward (5'→3')	Reverse (5'→3')
<i>Cis</i>	AGCCATGCAGCCCTTAC	CTAGCACCTTCGGTTCATTCT
<i>Socs2</i>	TTAAAAGAGGCGCCAGAAGGA	GCCCGGCTGATGTCTTAACA
<i>Socs3</i>	CCTCCAGCATCTTTGTCGGAAGAC	TACTGGTCCAGGAACCTCCGAATG
<i>Cyclophilin A</i>	CGACTGTGGACAGCTCTAAT	CCTGAGCTACAGAAGGAATG

through the portal vein of anesthetized rats. The viability of the initial cell suspension of hepatocytes was typically between 80% and 90% with trypan blue. The hepatocytes were plated at a density of 3×10^6 viable cells per well in 6-well plates previously coated with Matrigel. Medium, cell isolation and culture conditions were reported previously (Thangavel *et al.* 2006, Thangavel & Shapiro 2007).

Hormonal conditions

To replicate the masculine episodic GH profile, hepatocytes were exposed to recombinant rat GH (0.2 ng/mL; National Hormone and Peptide Program, Torrance, CA, USA) for 30 min, followed by 2 careful washings with GH-free medium that remained in the wells for 11.5 h, at which time the cells were again washed and exposed to the next 30 min pulse of GH. On the 6th day, cells were harvested 30 min after the addition of the last GH pulse. Following the same treatment schedule, control cells were exposed to the GH vehicle alone (Thangavel *et al.* 2006, Thangavel & Shapiro 2007).

Western blot and immunoprecipitation

Whole-cell lysates and nuclear fractions (Thangavel & Shapiro 2007, Banerjee *et al.* 2015) were extracted from freshly isolated preplated hepatocytes as well as cultured primary hepatocytes 30 min after the last GH pulse. Whole-cell lysate protein was electrophoresed and electroblotted onto nitrocellulose membranes for immunoblotting. Accordingly, the blots were probed with antibodies against CYP2C11 and anti-suppressors of cytokine signaling (CIS and SOCS2). Nuclear fractions were immune precipitated with signal transducers and activators of transcription 5b (STAT5b) antibodies as described previously (Thangavel & Shapiro 2007, Banerjee *et al.* 2015). Next, the immunoprecipitates were probed with anti-phosphotyrosine. The protein signals were scanned and the densitometric units were obtained as integrated density values. Antibodies and their vendors are listed in Table 1.

Quantitative real-time PCR (qRT-PCR)

Cyp2c11, *Cis*, *Socs1*, *Socs2*, *Socs3* and *Cyclophilin A* (housekeeping gene) expression were determined by qRT-PCR according to our previously report procedures (Das *et al.* 2013b, Banerjee *et al.* 2015). The primer sequences used to measure gene expression are listed in Table 2 or reported previously (Waxman & Frank 2000, Das *et al.* 2013a).

Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed to determine the intensity of STAT5b binding to the *Cyp2c11* promoter on cultured hepatocytes 30 min after the last episodic GH pulse on the 6th day in culture as previously described (Thangavel & Shapiro 2007, Banerjee *et al.* 2015).

MicroRNA (miRNA)

Due to their possible association with CYP2C11 and GH expression and/or action, we measured the expression levels of miRNA-141-3p, miRNA-130a-3p, miRNA-381-3p and miRNA-29b-3p in freshly isolated hepatocytes as well as cultured hepatocytes exposed to the masculine-like GH profile according to our previous methods (Banerjee *et al.* 2015). The primers for the miRNAs and procedural controls are presented in Table 3.

Statistics

All data were subjected to ANOVA. Significant differences were determined with *t* statistics and the Bonferroni procedure for multiple comparisons.

Table 3 Source of miRNA primers^a.

miRNA	Source
miR-141-3p	Rn_miR-141_1 miScript primer assay
miR-130a-3p	Rn_miR-130a_1 miScript primer assay
miR-381-3p	Rn_miR-381_2 primer assay
miR-29b-3p	Rn_miR-29b_1 miScript primer assay
Reverse transcriptase control	Ctrl_miRTC_1 miScript primer assay
PCR control	Hs_SNORD96A_11 miScript primer assay

^aFrom Qiagen Sciences, Frederick, MD, USA.

Figure 1

Plasma growth hormone (GH) of male rat pups treated with diluents (CONTROL), MSG and/or GH. Neonatally treated pups were injected with either MSG (4 mg/g bwt) or an equivalent dose of diluent on postnatal days 1, 3, 5, 7 and 9; rat GH (20 µg/100 g bwt) and/or the GH diluent, twice/d for the first 12 days of life. Results are presented as the means ± s.d. of at least 7 pups/group. * $P < 0.01$ vs CONTROL; † $P < 0.01$ vs MSG treatment alone.

Results

Immediate and long-term suppression of GH secretion by neonatal MSG

In agreement with previous (Oliver *et al.* 1982, Kacsoh *et al.* 1989) as well as our own report (Banerjee *et al.* 2015), plasma GH levels in control groups were maximum the first few postpartum days of life, thereafter declining ~50% by 5 days of age to 75–80% from 8 through 15 days of age (Fig. 1). GH levels of the MSG-treated neonates at

2 days of age, which was often less than 24 h after the first MSG injection, were normal. Thereafter, circulating GH concentrations declined 90–95% (postnatal day 2 through 11 days of age), to undetectable (15 days of age). In spite of the administration of exogenous GH during the first 12 days of life, circulating levels of the hormone were the same as controls. This seemingly unexpected finding could be explained by the presence of a functional hypothalamic–pituitary GH feedback loop in which the administered GH suppressed the secretion of endogenous hormone (Oliver *et al.* 1982, Wehrenberg & Giustina 1992) and/or by the fact that blood collections occurred 12 h after the last GH injection at which time the exogenous hormone could have cleared from the circulation (Brownborg *et al.* 1993). Concomitant administration of MSG and GH resulted in essentially normal-like levels of circulating hormone until the GH was withdrawn, and like the MSG treatment alone, plasma GH concentrations were now undetectable in the pups. (The ~30% above-control levels of plasma GH in the 2-day-old pups treated with MSG+GH could be due to a combination of the injected hormone and any transient residual GH from the MSG treatment).

Adult plasma GH profiles are presented as schematic representations of the actual circulating profiles (Fig. 2). The control rats secreted the typical adult masculine profile (Jansson *et al.* 1985a, Agrawal *et al.* 1995, Shapiro *et al.* 1995) characterized by episodic pulses containing amplitudes of ~200 ng/mL with durations of ~1 h, separated by interpulse periods of 2–3 h devoid of measurable hormone. In contrast, none of the adult neonatally MSG-treated rats had any measurable plasma

Figure 2

Adult plasma growth hormone (GH) profiles in growth hormone-depleted neonates. Plasma levels of circulating GH were obtained from individual, undisturbed catheterized adult rats neonatally treated with diluents (CONTROL), MSG and/or GH for 8 continuous hours at 15-min intervals. Similar results were obtained from 4 to 5 additional animals in each treatment group. Figure 1 for details of neonatal treatment.

GH (>0.125 ng/mL) during the 8 continuous hours of serial sampling. Adult rats, administered GH for the first 12 days of life, secreted the typical masculine episodic GH profile with the exception of pulse amplitudes $\sim 30\%$ ($P < 0.01$) lower than controls. The combined neonatal treatment of MSG and GH resulted in adult GH profiles identical to MSG alone, i.e., no detectable plasma GH.

Neonatal GH mitigates neonatal MSG-induced adult obesity

The Lee index is a measure of obesity; the higher the value, the greater the obesity. At all ages measured

Figure 3 Growth hormone (GH) imprinting affects post-weaning maturational Lee index profiles (A) and body weight gains (B). Results are presented as the means $\pm$ s.d. of at least 15 rats/group. CONTROL, MSG and/or GH treatments are described in detail in Fig. 1. * $P < 0.01$ vs CONTROL; $^{\dagger}P < 0.01$ vs MSG treatment alone.

(5–18 weeks of age), the Lee index of the neonatally MSG-treated rats was significantly ($P < 0.01$) greater than age-matched controls (Fig. 3A); this is not likely a result of overeating (Olney 1969), but rather a consequence of thermoregulation of a lower than normal body temperature and a general lethargy (Tokuyama & Himms-Hagen 1986) preventing normal body weight gain. The Lee indices of the GH-alone-treated rats were no different from controls. In spite of the absence of any detectable plasma GH, adult rats neonatally exposed to both MSG and GH exhibited no measurable obesity at 5, 7 and 9 weeks of age; thereafter (11 and 18 weeks of age), their Lee indices were intermediate ($P < 0.01$) between controls and MSG alone, suggesting that the hormone imprints the 'obesity set point' attenuating the effects of both neonatal and adult GH depletion observed in MSG-alone-treated rats.

In contrast to its effect on obesity, neonatal GH was unable to moderate the inhibitory action of MSG on body weight gain, i.e. an index of general (e.g. musculoskeletal) body growth (Fig. 3B). Consequently, adult body weight gain of male rats neonatally treated with either MSG or both MSG and GH were no different, but significantly ($P < 0.01$ at all ages) less than controls which in turn were less than the GH alone-treated rats, the latter treatment producing the greatest body weight gain.

Neonatal GH imprints adult responsiveness of hepatic CYP2C11 to episodic GH regulation

Hexobarbital-induced sleep time is an *in vivo* measure of drug metabolism competence. When given the same dose of hexobarbital per kg of b wt, adult rats neonatally treated with MSG slept 3 times as long as control rats (Fig. 4A1), indicating a considerable deficiency in CYP isoforms, particularly CYP2C11 (Ryan & Levin 1990, Pampori *et al.* 1991b), which greatly contributes to hexobarbital metabolism, i.e., hydroxylation. Neonatal treatment of MSG and GH resulted in adult sleeping times that were about twice as long as controls but still significantly briefer than the MSG alone treatment, indicating the expression of some level of hexobarbital hydroxylase capacity, e.g., CYP2C11.

CYP2C11 is the principal male-dependent form, comprising $>50\%$ of the total CYPs in adult male rat liver (Morgan *et al.* 1985), but undetectable in female liver, explaining its designation as male specific (Legraverend *et al.* 1992). (CYP2C11 is expressed only in adulthood and its full expression is solely dependent on the circulating masculine GH profile whose secretion

Figure 4

Neonatal growth hormone (GH) imprints adult hepatic CYP2C11 expression. (A1) CYP2C11-dependent hexobarbital-induced sleeping times in adult rats neonatally administered diluents (CONTROL), MSG and/or GH determined following an i.p. injection of hexobarbital (150 mg/kg bw). Results are presented as the means $\pm$ s.d. of at least 10 rats/group. * $P < 0.01$ vs CONTROL; † $P < 0.01$ vs MSG treatment alone. (B1) Relative expression levels (mRNA and protein) of the paramount male-specific CYP2C11 isoform in freshly isolated preplated hepatocytes from adult male rats neonatally administered diluent (CONTROL), MSG and/or GH. Results are presented as a percentage of mRNA or protein in hepatocytes from CONTROL rats arbitrarily designated as 100%. Values are means $\pm$ s.d. of at least 6 rats/data point. * $P < 0.01$ vs CONTROL; † $P < 0.01$ vs MSG treatment alone. (B2) Hepatocytes from the same rats as (B1) above were exposed to either the episodic male-like GH profile or the hormone's vehicle for 6 days in culture. Results are presented as a percentage of mRNA or protein in hepatocytes exposed to vehicle arbitrarily designated 100%. Values are means $\pm$ s.d. of at least 6 rats/data point. * $P < 0.01$ vs hepatocytes exposed to the episodic vehicle profile from rats of the same neonatal treatment, i.e. CONTROL, MSG, GH or MSG+GH as described in Fig. 1.

occurs only postpubertally (Legraverend *et al.* 1992, Shapiro *et al.* 1995)).

As freshly isolated hepatocytes, CYP levels *in vitro* reflect *in vivo* concentrations (Thangavel *et al.* 2006). Accordingly, expression of episodic GH-dependent CYP2C11 was profoundly reduced in freshly isolated hepatocytes from GH-devoid, neonatally MSG-treated adult male rats (Fig. 4B1). In contrast, neonatal administration of both MSG and GH resulted in substantial expression levels of CYP2C11 (~40% of control values) in spite of the same total absence of circulating GH as observed in the MSG-treated rats. In this regard, a minimal baseline level of the hepatic CYP2C11 (~20–40% of normal) can be expressed in the absence of GH, whereas full induction of the isoform requires exposure to the masculine episodic GH profile (Waxman *et al.* 1991, Agrawal & Shapiro 2000). Consequently, these findings indicate that neonatal GH programs developmental mechanisms responsible for expression of GH-independent, baseline CYP2C11 expression. Neonatal GH treatment alone, however, had no effect on *in vivo*-like adult expression of CYP2C11.

After 6 days of exposure to the male-like episodic GH profile, CYP2C11 expression levels in the primary hepatocytes derived from control rats doubled above vehicle-treated baseline concentrations (Fig. 4B2). The inability of the masculine GH profile to induce CYP2C11 expression in hepatocytes from MSG-alone-treated males indicates an irreversible repression of the enzyme. In contrast, hepatocytes derived from adult males neonatally

treated with both MSG and GH responded to the episodic GH profile with a ≥ 2.5 -fold increase in CYP2C11 expression. The only difference between the MSG-alone group and the MSG plus GH group was the presence in the latter of GH during the critical postnatal developmental period, demonstrating the imprinting action of postnatal GH on adult function. Recall, both groups were lacking GH during their immature, peripubertal and adult lives. (The difference between CYP2C11 induction levels in the control and the combined MSG and GH treatment group is not as dramatic as it appears. Induction levels are based on vehicle exposure levels designated 100% in all groups. In fact, vehicle treatment of levels of CYP2C11 in the combined MSG and GH group were considerably lower (~35%) than that in the control group resulting in a calculated higher GH induction level in the combined treatment group).

Neonatal GH irreversibly imprints the STAT5b signal transduction pathway mediating episodic GH induction of CYP2C11

The terminal transducer in the signaling transduction pathway mediating episodic GH induction of CYP2C11 is STAT5b. This transcription factor must be activated (i.e., phosphorylated), homodimerized and translocated to the nucleus, where it binds to a promoter site initiating the transcription of the *Cyp2c11* gene (Waxman & Frank 2000). Primary hepatocytes were cultured in the presence

Figure 5

Neonatal growth hormone (GH) imprints adult growth hormone regulation of STAT5b activation, nuclear translocation and binding to the CYP2C11 promoter. (Left panel) Nuclear levels of activated STAT5b (pSTAT5b) in cultured primary hepatocytes exposed to either the episodic male-like GH profile or the episodic profile containing GH vehicle alone for 6 days. Results are presented as a percentage of nuclear pSTAT5b in hepatocytes exposed to vehicle arbitrarily designated as 100%. Values are means $\pm$ s.d. of at least 6 rats/data point. * $P < 0.01$ vs hepatocytes exposed to the episodic vehicle GH profile from rats of the same neonatal treatment, i.e. CONTROL, MSG, GH or MSG+GH as described in Fig. 1. (Right panel) Representative ChIP blots (2 of 5/group) demonstrating episodic GH regulation (δ GH) described above, of pSTAT5b binding to the *Cyp2c11* promoter in hepatocytes derived from adult male rats neonatally treated with either diluents (CONTROL), MSG, GH or MSG+GH as described in Fig. 1.

of the masculine episodic GH profile or episodic GH vehicle alone for 6 days. The cells were harvested 30 min after the last GH pulse; an optimal time to measure the STAT5b response (Verma *et al.* 2005). In agreement with the transduction requirements of GH-induced transcription of CYP2C11, the concentration of activated nuclear phospho-STAT5b (pSTAT5b) increased 2-fold in episodic GH-exposed hepatocytes from control rats, whereas the same hormone treatment produced a 20–25% decline in nuclear levels of pSTAT5b in adult hepatocytes from neonatal MSG-alone-treated rats (Fig. 5). In contrast, exposure of adult hepatocytes from the combined neonatal MSG and GH treatment to episodic GH resulted in a 2-fold increase in nuclear pSTAT5b concentrations, similar to control hepatocytes. Neonatal GH treatment alone, however, resulted in only ~50% increase in the nuclear translocation of pSTAT5b reflecting the lower induced levels of CYP2C11 (Fig. 4B2).

The binding of pSTAT5b to the *Cyp2c11* promoter was evaluated in adult hepatocytes from the various neonatal treatments by ChIP assays (Fig. 5, right). In agreement with the expected sequence of signaling events, robust binding of pSTAT5b to the promoter occurred in control hepatocytes exposed to the masculine-like GH profile. In contrast, none of the episodic GH-treated hepatocytes from the MSG-treated rats exhibited detectable binding of the activated transcription factor to the *Cyp2c11* promoter. Hepatocytes from the combined neonatal treatment (MSG+GH) were found to display pSTAT5b promoter-binding levels similar to those of hepatocytes from control male rats. Lastly, the density of the pSTAT5b bands in the ChIP assays in hepatocytes from the GH-alone-treated rats was somewhat lower than that of

bands from control and combined MSG- and GH-treated rats, indicating reduced binding, in agreement with the hepatocytes' lower levels of both nuclear pSTAT5b and CYP2C11 expression.

Developmental GH irreversibly imprints expression of the SOCS family of inhibitory proteins mediating GH action

The SOCS family of inhibitory proteins are negative regulators of GH signaling. Their actions are particularly important in regulating the effects of episodic GH on such male-dependent parameters as growth, musculoskeletal metabolism and CYP expression (Karlsson *et al.* 1999, Waxman & Frank 2000). Consequently, we decided to examine the long-term effects of neonatal GH on both the *in vivo*-like levels and *in vitro* responsiveness of four of the most commonly referenced SOCS, i.e. CIS, SOCS1, SOCS2 and SOCS3 (Fig. 6). Neonatal GH ablation (i.e. MSG treatment) had little effect on both the *in vivo* and *in vitro* adult expression of hepatic SOCS3 (Fig. 6D1 and D2), which may explain our earlier report demonstrating the ineffectiveness of the masculine episodic GH profile to regulate SOCS3 expression (Thangavel & Shapiro 2007). In contrast, the absence of circulating GH in adulthood of both the MSG and MSG+GH neonatal treatment groups resulted in lower CIS, SOCS2 and SOCS1 *in vivo* expression levels, most dramatically at the transcription level (Fig. 6A1, B1 and C1), a possible result of altered ubiquitination levels shown to be regulated by GH (Banerjee *et al.* 2013). These SOCS proteins are known to be induced by the episodic GH profile, but only in males and not in females even when females are renaturalized

Figure 6

Neonatal growth hormone (GH) imprints adult growth hormone regulation of CIS/SOCS expression. (A1, B1, C1, D1) Levels of 4 members of the CIS/SOCS family (i.e. CIS, SOCS2, SOCS1 and SOCS3) were measured in freshly isolated preplated hepatocytes from adult male rats neonatally administered diluents (CONTROL), MSG, GH or MSG+GH as described and statistically analyzed in Fig. 1. (A2, B2, C2, D2) Concentrations of the same CIS/SOCS family members in hepatocytes from the same rats (A1, B1, C1, D1) were exposed to either the episodic male-like GH profile or the hormone vehicle for 6 days in culture. Results are presented and statistically analyzed as described in Fig. 4B2.

with the male-dependent profile suggesting irreversible sex-dependent imprinting (Thangavel & Shapiro 2007).

Hepatocytes from the control treatment group exposed to the male-like GH profile responded with a 2- to 3-fold increase in CIS, SOCS2 and SOCS1 expression levels (Fig. 6A2, B2 and C2). In contrast, the SOCS expression levels in adult hepatocytes from the MSG-alone

treatment group were actually suppressed by the episodic GH profile, whereas cells from the combined MSG and GH neonatal treatment responded the same as controls to the masculine-like GH profile. As with other measured parameters, hepatocytes derived from GH alone-treated rats exhibited a muted response of CIS, SOCS2 and SOCS1 to the episodic GH profile.

GH imprinting is associated with permanent alterations in miRNA expression

miRNAs are naturally occurring small, single-stranded non-coding RNA molecules involved in post-transcriptional control of gene expression. In animals, miRNAs imperfectly hybridize, through partial sequence homology, to the 3'-UTR of target mRNAs causing a block in translation and/or increased mRNA degradation (Bartel 2009). Prediction of miRNA target mRNA was carried out by our use of the algorithms miRanda (John *et al.* 2004) and TargetScan (Lewis *et al.* 2003). Four miRNAs were identified by *in silico* analyses with high predictability to interfere with the expression and/or action of CYP2C11 and/or GH. Three of the miRNAs (miR-29b-3p, miR-130a-3p and miR-381-3p) exhibited a dramatic ≥ 3 -fold overexpression in freshly isolated hepatocytes from GH-devoid MSG and MSG plus GH-treated rats

(Fig. 7A1, B1 and C1). As these 3 miRNAs have high predictability pairing scores for such relevant mRNAs as *Cyp2c11*, *Socs2* and *Igf1* (Fig. 7D) known to be regulated by the masculine GH profile, their overexpression is in agreement with the subnormal *in vivo* expression levels of CYP2C11 (Fig. 4B1), SOCS2 (Fig. 6B1) and IGF1 (Pampori & Shapiro 1996) in episodic GH-ablated male rats. Furthermore, the 3 miRNAs were found to be completely unresponsive to the *in vitro* administration of the episodic GH profile in hepatocytes from all treatment groups neonatally exposed to GH, i.e. controls, GH alone and MSG+GH (Fig. 7A2, B2 and C2). Only hepatocytes derived from the MSG-alone treatment responded to the episodic GH profile with a significant reduction in miR-29b-3p and miR130a-3p concentrations, now similar to all the other treatment groups indicating that neonatal GH imprints the 3 miRNAs to be unresponsive to episodic

Figure 7

Adult expression of 3 rat-specific hepatic microRNAs (miR) neonatally imprinted by growth hormone (GH). (A1, B1, C1) Levels of 3 microRNAs (rno-miR-29b-3p, rno-miR-381a-3p, rno-miR-130a-3p) were measured in freshly isolated preplated hepatocytes from adult male rats neonatally administered diluents (CONTROL), MSG, GH or MSG + GH as described and statistically analyzed in Fig. 1. (A2, B2, C2) Levels of the same hepatic microRNAs from the same rats (A1, B1, C1) were exposed to either the episodic male-like GH profile or the hormone vehicle for 6 days in culture. Results are presented and statistically analyzed as described in Fig. 4B2. (D) Sequence of the 3 microRNAs and their complementary sites in the 3'-UTR of CYP2C11, SOCS2 and IGF1.

GH regulation. (miRNA-141-3p, an *in silico* predicted blocker of STAT5b expression, was unaffected, statistically, by all the neonatal treatments (data not presented)).

Discussion

As an endocrine-responsive tissue appears to be imprinted/programmed by the same hormone that regulates its adult function (Shapiro & Goldman 1979, Csaba 2008), it seemed reasonable to assume that the expression of adult hepatic CYPs by GH is accordingly imprinted by GH. To investigate the specific requirement for perinatal GH in the imprinting process, we blocked postpartum GH secretion in newborn male rats using MSG, a selective disrupter of developing hypothalamic GH-releasing hormone nuclei (Bakke *et al.* 1978, Dada *et al.* 1984) while having no direct effect on liver differentiation (Kaufhold *et al.* 2002). In addition, some of the MSG-treated neonates simultaneously received replacement rat GH during the 'critical developmental period'. We also included two control groups: vehicle alone or GH alone. Plasma GH levels were normal in both control groups during the first two weeks of life, whereas neonatal MSG virtually eliminated circulating GH after the first injection, and as designed, concurrent GH replacement maintained generally normal postnatal levels of the hormone. However, as soon as exogenous GH was withdrawn, plasma concentrations of the hormone declined to undetectable as observed in the MSG alone group. As adults, males from both control groups secreted the typical masculine GH profile (Pampori *et al.* 1991b, Agrawal & Shapiro 2000) characterized by episodic pulses of ~1 h duration separated by interpulses of 2–3 h containing undetectable concentrations of the hormone. The one exception being that the pulse amplitudes were somewhat lower in the GH alone group. Since the GH-releasing hormone's hypothalamic nuclei were permanently destroyed in the neonates by MSG, both MSG groups, with and without concurrent neonatal GH replacement, secreted no measurable levels of GH as adults. Nevertheless, and in spite of the same complete lack of circulating GH, the two MSG treatment groups expressed very different peripubertal and adult functions of what would normally be GH-dependent activities. That is, adults from the MSG-alone treatment group were profoundly obese, exhibited a 3-fold longer hexobarbital-induced sleep time than controls and a concomitant near-complete deficiency in hexobarbital hydroxylase-dependent CYP2C11. In contrast and irrespective of the same absence of plasma

GH, adult males neonatally treated with both MSG and GH exhibited only a late-onset mild obesity, a prolonged hexobarbital-induced sleep time that was still ~30% shorter than that resulting from the MSG-alone treatment and CYP2C11 expression levels (mRNA and protein) representing ~40% of controls. Evidently, even though there was no circulating GH after 12 days of age in the MSG+GH treatment group, the neonatal replacement GH alone appears to have programmed a permanent imprint that attenuated the abnormalities associated with congenital-like GH deficiency as characterized by the MSG alone treatment. The present findings identifying the effects of GH imprinting are analogous to those of the model developmental organizer/imprinter androgen. During the perinatal period when the brain is nonsexual and still differentiating, testicular androgens or one of their metabolites programme/imprint neural pathways required to fully express numerous masculine functions, such as gonadotropin secretory profiles, taste preferences and sexual behavior, when subsequently exposed to adult androgens (Feder 1981, Shapiro 1985a). Without hormonal imprinting, however, these masculine neural pathways are never established and/or are permanently refractory to androgens and their dependent masculine functions can never be expressed, regardless of adult hormone treatment. In this regard, it seemed more than reasonable to assume that androgens also masculinize (i.e., imprint) hepatic sex-dependent CYP isoforms, as neonatal orchidectomy had been reported to demasculinize drug metabolism (i.e., CYP isoforms). However, the permanence or irreversibility of the imprinting was either never tested by administering testosterone to neonatal castrates as adults (Chung & Chao 1980, Waxman *et al.* 1985) or, in fact, the defects produced by neonatal castration were found to be completely corrected when treated with the androgen as adults (Gustafsson & Stenberg 1974, Jansson *et al.* 1985b, Dannan *et al.* 1986, Bandiera & Dworschak 1992). (Androgens, including testosterone, have no direct effect on hepatic CYP expression, but rather can act at the hypothalamic–pituitary axis to regulate the sexually dimorphic secretion of GH, which, in turn, regulates sex-dependent CYPs (Kramer *et al.* 1979, Jansson *et al.* 1985a, Shapiro 1985b)). If the effects of neonatal orchidectomy on CYP expression can be completely corrected (i.e., reversible), by definition, and despite the researchers' conclusions, androgens do not imprint the sexually dimorphic expression of CYP isoforms. Moreover, our observation that neonatal MSG has no inhibitory effects on circulating testosterone levels during the first 15 days of life, inclusive of the

critical period of hypothalamic–pituitary–hepatic axis differentiation (Das *et al.* 2014), is further support that perinatal androgens are not imprinters of CYP sexual dimorphisms. Rather, our results indicate that just as the brain of the adult not imprinted by androgens is permanently incapable of responding to androgens with a display of masculine behaviors, the liver of the adult male rat not imprinted by GH is permanently incapable of responding to episodic GH induction of the predominant CYP2C11 isoform. Consequently, hepatocytes derived from adult males not imprinted by neonatal GH (MSG only treatment) were completely unresponsive to episodic GH induction of CYP2C11 expression. In contrast, when the same neonatal treatment was supplemented with rat GH, the adult hepatocytes were fully responsive to the episodic GH profile exhibiting a ~2.5-fold induction of CYP2C11 establishing perinatal GH as a requisite developmental organizer or imprinter.

What, then, are the defects in the GH-inductive response of CYP2C11 in adult rats neonatally treated with MSG? That is, why cannot the hepatocytes respond to GH? In this regard, GH signaling in liver by the episodic profile (in contrast to the continuous profile) is initiated by hormone binding to and the resulting activation of GH receptor (GHR) on the surface of the target cells. This allows for the recruitment and/or activation of two molecules of Janus kinase 2 (JAK2), which then cross-phosphorylate each other, as well as phosphorylating the receptor on key tyrosine residues. STAT5b, a latent transcription factor, binds to these phosphorylated receptor docking sites and is, in turn, phosphorylated and homodimerizes and translocates to the nucleus, where it binds to promoter sites initiating transcription of GH-regulated genes. An important negative regulatory mechanism of GH signaling, the SOCS family of inhibitory proteins, which, depending on the protein, inhibit GH signaling by inactivating JAK2, by competing with STAT5b for common tyrosine-binding sites on the intracellular tail of the GHR and/or by a proteasome-dependent mechanism that results in the degradation of the (GH-GHR-JAK2)•SOCS complex (Waxman & Frank 2000, Landsman & Waxman 2005, LeRoith & Nissley 2005). At least 4 possible components in this signal transduction pathway were permanently altered by neonatal MSG. The response to episodic GH of hepatocytes from control rats (vehicle-treated more so than GH alone; the latter suggesting that too much of a hormone, just like too little, can have deleterious effects) increased cellular CIS, SOCS1 and SOCS2, as well as nuclear pSTAT5b, by 2- to 3-fold, whereas these transducers in hepatocytes from the MSG-treated rats were completely unresponsive

to the masculine GH profile. Not only did developmental exposure to MSG permanently suppress STAT5b activation (i.e. phosphorylation) and nuclear translocation, but it also completely blocked the binding of the transcription factor to the *Cyp2c11* promoter, explaining the inability of the masculine GH profile to induce CYP2C11 expression. In contrast, when MSG-treated pups were briefly injected with rat GH, induction levels of cellular CIS, SOCS1 and SOCS2 as well as nuclear translocation levels of pSTAT5b and its subsequent binding to the *Cyp2c11* promoter were statistically the same as vehicle controls demonstrating the successful imprinting of the developing liver by neonatal GH alone. The possible involvement of miRNAs in the suppression of adult CYP2C11 by neonatal MSG was also examined. We used multiple *in silico* prediction tools and identified complimentary sequence homology sites in downstream GH-activated genes, including *Cyp2c11* and *Socs2*. Three of the miRNAs, miR-29b, miR-130a and miR-381 were several fold overexpressed only in the adult GH-devoid, MSG-treated males in agreement with a report that miR-29b is suppressed by GH treatment (Cheung *et al.* 2009). Although it is not clear just what role, if any, the miRNAs play in GH imprinting, it is apparent from our findings that all three miRNAs are permanently refractory to GH regulation in only those animals neonatally imprinted by GH (i.e., vehicle alone, GH alone and MSG+GH). As miRNA studies are a nascent area of biological investigation, any conclusion that one of the purposes of GH imprinting is to permanently limit the inhibitory effects of miRNA on adult GH induction of CYP2C11 and other GH-dependent genes may seem reasonable (Banerjee *et al.* 2015), but remains speculative.

Clearly, then, neonatal exposure to MSG irreversibly suppresses GH activation of CYP2C11-dependent signal transduction at multiple steps. Whether the multiple defects in the signaling pathway are all independently induced or rather the result of an initial upstream defect (i.e. GHR) cascading dysfunctions down the signaling pathway is under investigation. In summary, our findings demonstrate for the first time that perinatal GH imprints a latently expressed programme, which is then subsequently responsive to GH during adulthood. The imprinting is obligatory and occurs during a critical developmental period, whereas the imprinted programme is only expressed in adulthood. As there is a myriad of metabolic functions in humans, including growth rates and lean body mass (e.g., note our Lee index findings); cardiovascular, bone, adipose and muscle physiology; protein, carbohydrate, lipid and electrolyte metabolism all regulated, at least in part, by GH, and like CYP expression, exhibit permanent

sexual dimorphisms (Thangavel & Shapiro 2007), it may be that these adult functions also require perinatal GH imprinting and could be vulnerable to pediatric drug therapy known to inadvertently disrupt GH secretion.

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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