Developmental changes in plasma leptin and hypothalamic leptin receptor expression in the rat: peripubertal changes and the emergence of sex differences

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Abstract

Leptin, the peptide hormone product of the *ob* gene, regulates food intake and energy expenditure at the hypothalamic level via the long-form of the leptin receptor (Ob-Rb). Leptin also plays a key role in determining the onset of puberty, but there is controversy as to whether leptin provides a trigger for puberty or is a permissive signal. Thus, although leptin administration can advance puberty onset in rodents, circulating leptin appears stable across puberty. While these data suggest a permissive role for leptin in rat puberty, it is possible that a change in hypothalamic response to leptin (e.g. via increased Ob-Rb expression) could enhance leptin action and thus trigger puberty without a rise in circulating leptin. In the present study we assessed developmental changes in hypothalamic Ob-Rb mRNA and protein expression in female and male rats from late fetal to postpubertal life. Quantitative RT-PCR showed that Ob-Rb mRNA increased ($P < 0.05$) by around fivefold from fetal to postpubertal life in both females and males. These increases in Ob-Rb mRNA expression were gradual, but did not increase significantly between postnatal day 30 (pre-puberty) and

day 51 (post-puberty). By day 51, hypothalamic Ob-Rb mRNA expression was higher ($P < 0.05$) in females relative to males. Hypothalamic Ob-Rb protein showed a comparable developmental pattern (approximate threefold increase from fetal to postpubertal life), although a significant increase (15%; $P < 0.05$) was observed between days 30 and 51 in females. Plasma leptin levels exhibited a dynamic pattern in both male and female rats during the prepubertal period, characterised by a precipitous fall after birth, relative stability to day 5, then a rapid increase to a transient peak on day 12. Plasma leptin then remained unchanged from day 15 in female rats but increased in males after puberty, thus confirming the well-recognised sex difference in adult rat leptin levels. In conclusion, this study shows that developmental increases occur not only in plasma leptin but also in hypothalamic Ob-Rb expression, suggesting that both are likely to influence the timing of puberty onset. Moreover, our data show that sex differences in both hypothalamic Ob-Rb and plasma leptin emerge only after puberty.

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Introduction

Leptin is a 16 kDa peptide hormone product of the *ob* gene expressed in a variety of tissues, predominantly adipocytes, and acts via its receptor to regulate food intake and energy expenditure at the hypothalamic level (Campfield *et al.* 1995, Pellymounter *et al.* 1995). Leptin is also known to act as a metabolic signal to the reproductive system (Cunningham *et al.* 1999) that, in turn, is regulated by body weight and nutritional status (Barash *et al.* 1996). As leptin is known to regulate gonadotrophin-releasing hormone secretion (Nagatani *et al.* 1998) and mutations in the *ob* gene in mice prevent the attainment of sexual maturity (Zhang *et al.* 1994), leptin has been proposed as a metabolic signal to the timing of puberty (Cheung *et al.* 1997). While a contribution of leptin to the timing of

normal sexual maturation is clear, there is controversy as to whether leptin *per se* is a trigger for puberty onset or serves only in a permissive role (Ahima *et al.* 1997, Chehab *et al.* 1997, Almog *et al.* 2001, Cheung *et al.* 2001). If leptin were a trigger for puberty, an increase in circulating levels would be expected yet this is not universally observed (Gruaz *et al.* 1998, Matsuda *et al.* 1999, Cheung *et al.* 2001, Watanobe & Schioth 2002). On the other hand, a change in the hypothalamic response to leptin (e.g. via increased expression of the leptin receptor) could potentially enhance leptin action and thus trigger puberty without any change in circulating leptin levels. The leptin receptor is a member of the class I cytokine receptor family, and six multiple splice variants have been identified (for review see Tartaglia 1997). These splice variants fall into three categories: a long form (Ob-Rb), located

primarily within the hypothalamus, capable of full signal transduction; a number of C-terminally truncated forms (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf), of which Ob-Ra is the most commonly studied and is thought to act as a transporter of leptin; and a soluble receptor (Ob-Re) that lacks both the intracellular and transmembranous domains and acts as a circulating leptin binding protein. Cheung *et al.* (2001) recently reported no change in the expression of Ob-Rb mRNA in several hypothalamic nuclei measured by *in situ* hybridisation; the authors suggested, however, that their methodology may lack the sensitivity to detect biologically significant change. In the present study, therefore, we used real-time RT-PCR to quantitate hypothalamic Ob-Ra, Ob-Rb, and Ob-Re mRNA expression from fetal to postpubertal life in male and female rats; expression patterns for hypothalamic Ob-Rb were also confirmed by Western analysis, and plasma leptin levels were measured over the same period. These analyses enabled determination of the extent of sex differences in plasma leptin and hypothalamic leptin receptor expression. Previous studies indicate that while plasma leptin is clearly higher in adult male compared with female rats (Landt *et al.* 1998, Pinilla *et al.* 1999), the developmental emergence of this sex difference remains uncertain, and the possibility that hypothalamic leptin receptor expression differs between males and females has not been explored.

Materials and Methods

Animals

Albino Wistar rats aged between 9 and 12 weeks were obtained from the Animals Resources Centre (Murdoch, Australia) and maintained under controlled conditions as previously described (Burton & Waddell 1994). Rats were mated overnight and the day on which spermatozoa were present in the vaginal smear was designated gestational day 1 (g1). The day of birth, usually g23 in this colony of rats, was designated postnatal day 0 (p0). Pup sex was determined by examination of external genital morphology and litters were standardised to 10 pups before the third day postpartum and, where applicable, were weaned on p30. The onset of puberty in this colony occurs on day 41 in females (vaginal opening) and day 48 in males (preputial separation) (Smith & Waddell 2000). All procedures involving animals were conducted only after approval by the Animal Experimentation Ethics Committee of The University of Western Australia.

Blood sampling and tissue collection

For collection of fetal blood and hypothalamus, pregnant rats were anaesthetised at g22 with halothane/nitrous oxide and fetuses were removed. Blood samples from

female and male g22, p0, p1, p2, p3, and p5 rats were obtained by decapitation (at g22 and p0, blood samples from 4 fetuses/newborns from each litter were pooled to provide sufficient volume). Blood samples were collected from anaesthetised rats at p9, p12, p15, p20 by decapitation, and at p30 and p51 via the exposed dorsal aorta. Hypothalami were obtained from rats at selected stages (g22, p0, p5, p9, p12, p15, p30, and p51) as previously described (Zakrzewska *et al.* 1999); briefly, each hypothalamus was dissected from a frontal brain slice between the optic chiasm and the mammillary bodies. Slices extended laterally to the perihypothalamic sulcus and superiorly to the anterior commissure. Hypothalami were immediately frozen in liquid nitrogen for real-time quantitative RT-PCR or Western blot analysis.

Radioimmunoassays

Blood samples collected as described above were centrifuged at 11 000 r.p.m. for 5 min and plasma was stored at -20°C until assayed. Plasma leptin concentrations were measured using a radioimmunoassay kit supplied by Linco Research (St Charles, MO, USA). The intra- and inter-assay coefficients of variation were 4% and 8% respectively.

Real-time quantitative RT-PCR

Total RNA was isolated from g22, p12, p30, and p51 hypothalami, reverse transcribed and the resultant cDNA purified and quantified as previously described (Seeber *et al.* 2002). The cDNA primers to specific rat Ob-Ra, Ob-Rb and Ob-Re (Takaya *et al.* 1996) were positioned to span intron–exon junctions to distinguish cDNA from genomic DNA and were as described previously (Smith & Waddell 2002). External standards were generated from regular PCR products using the Ob-Ra, Ob-Rb, and Ob-Re primers as previously described (Smith & Waddell 2002).

Quantitative PCR and melting curve analyses were performed in 10 μl reaction volumes in capillary tubes using the LightCycler system (Roche Diagnostics, Indianapolis, IN, USA) as previously described (Smith & Waddell 2002). Melting curve analysis showed a single PCR product for each Ob-R isoform and this was confirmed by gel electrophoresis (data not shown). Fluorescence values were analysed and a standard curve constructed using the LightCycler software, and sample values were expressed as fg RNA/ μg cDNA. The intra- and interassay coefficients of variation were as follows: Ob-Ra, 0.9% and 1.2%; Ob-Rb, 0.6% and 2.4%; Ob-Re, 0.2% and 0.3% respectively.

Western blot analysis

Hypothalami (g22, p0, p5, p9, p12, p15, p30, and p51) were homogenised (250 μl , 10 mM Tris buffer containing

1.5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride and 100 µg/ml trypsin inhibitor), and then centrifuged at $105\,000 \times g$ for 30 min. Supernatant protein (30 µg) was resolved by SDS-PAGE (7% separating) and transferred to nitrocellulose membrane (Hybond C-Super, Amersham Pharmacia Biotech, Sydney, Australia). Consistency of protein loading and transfer among samples was confirmed by Ponceau S dye staining (ICN Biomedicals, Aurora, OH, USA). Membranes were incubated for 1 h in blocking solution containing 5% non-fat milk powder, then overnight at 4 °C with Ob-R antibody (diluted 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were identified by incubation with horseradish peroxidase conjugate donkey anti-goat secondary antibody (diluted 1:5000, Chemicom International, Temecula, CA, USA) and signals were visualised using a chemiluminescence detection kit (SuperSignal Substrate, Pierce Chemical, Rockford, IL, USA). The resultant autoradiographs were quantified by densitometry using Scion Image analysis software (Release beta 3b; www.scioncorp.com) as previously described (Burton *et al.* 1998). Because the primary antibody was generated against the common region of the Ob-R it theoretically cross-reacts with all three major isoforms; by Western analysis, however, only the 120 kDa Ob-Rb isoform (Wang *et al.* 1998) was detected (see Results).

Statistical analysis

All data are expressed as means $\pm$ S.E.M. where a minimum of three animals was used for each experimental variable and each litter represented an 'n' of one. Variation in body weight, plasma leptin concentration, Ob-R mRNA and protein related to age and sex was assessed by one-way or two-way ANOVAs as appropriate. Where the *F*-test for the ANOVA reached statistical significance ($P < 0.05$), differences among specific means were assessed by least significant difference (LSD) test (Snedecor & Cochran 1989). Comparison of hypothalamic Ob-Rb protein in males and females at day 52 of age was made by unpaired *t*-test.

Results

Body weight and plasma leptin concentrations

Body weight varied significantly with both age ($P < 0.001$) and sex ($P < 0.05$) and there was significant interaction between age and sex ($P < 0.001$, two-way ANOVA). Specifically, body weights were similar between males and females until post-puberty (p51), when males were significantly heavier (30%, $P < 0.001$, see Fig. 1). Plasma leptin concentrations varied significantly with age in both male and female rats ($P < 0.001$, see Fig. 1). The key

Figure 1 Developmental changes in the concentration of plasma leptin and body weight in female and male rats. Values (ng/ml) are the mean $\pm$ S.E.M. of $n=3-6$ per group. Body weight results: significant variation occurred with age ($P < 0.001$) and sex ($P < 0.05$) and there was significant interaction ($P < 0.001$, two-way ANOVA). Thus, males and females only differed ($P < 0.001$) at post-puberty (p51). Leptin results: variation occurred with age ($P < 0.001$) in both females (LSD_{0.05} = 2.03–2.66) and males (LSD_{0.05} = 1.59–2.27); see Results for specific comparisons.

features of this developmental change were that leptin concentrations fell precipitously at birth but then increased ($P < 0.05$) after postnatal day 5 to peak at day 12 in both sexes. Plasma leptin then declined markedly by day 15 in males ($P < 0.05$) and females ($P < 0.01$), and although there appeared to be a subsequent increase in females by day 20, this was not statistically significant. Thereafter, plasma leptin remained unchanged in females up to post-puberty (p51), whereas in males it increased ($P < 0.05$) from pre-puberty (p30) to post-puberty (p51). Thus, sex differences in plasma leptin became evident only after puberty onset, being more than 40% lower in females by day 51 ($P < 0.001$).

Figure 2 Quantification of hypothalamic Ob-Rb and Ob-Re mRNA at g22, p12, p30, and p51 by real-time RT-PCR. Values are expressed as fg RNA/ μ g total cDNA and are the mean $\pm$ S.E.M. of $n=3$ per group. Significant variation with age was detected in Ob-Rb (males, $P<0.01$; females, $P<0.05$). Ob-Re expression also varied ($P<0.05$) with age in females but not in males. In males and females values without common notations differ significantly ($P<0.05$, LSD test).

RT-PCR analysis of hypothalamic leptin receptor mRNA expression

Hypothalamic expression of Ob-Rb mRNA increased with age ($P<0.001$) and there was a significant interaction between age and sex ($P<0.05$, two-way ANOVA). Specifically, Ob-Rb expression in females increased from postnatal day 12 to peak at post-puberty (p51, $P<0.05$, one-way ANOVA, see Fig. 2); overall, there was an approximate fivefold increase in Ob-Rb mRNA from fetal to postpubertal life in females. A similar pattern of hypothalamic Ob-Rb mRNA was observed in males, but this expression appeared to fall after puberty and was significantly lower ($P<0.05$) than that in females at day 51 (see Fig. 2). The hypothalamus also expressed Ob-Re mRNA but at a lower level (40 to 80% less) than that of

Ob-Rb (see Fig. 2), and in females Ob-Re expression increased from late fetal life (g22) to post-puberty (p51; $P<0.05$, one-way ANOVA). In males, however, Ob-Re expression remained unchanged over this period. Hypothalamic expression of Ob-Ra mRNA was detected at much lower levels than either Ob-Rb (97–99% less) or Ob-Re and did not change over development (data not shown).

Western blot analysis of Ob-R in the hypothalamus

Consistent with the changes in hypothalamic Ob-Rb mRNA expression, Western analysis showed a clear band of immunoreactivity at 120 kDa corresponding to the Ob-Rb protein (Wang *et al.* 1998) at all stages examined (see Fig. 3). This Ob-Rb expression increased ($P<0.001$) progressively from a minimum in the fetus (g22) to a maximum at post-puberty (p51) in both sexes. The increase above fetal hypothalamic Ob-Rb levels was first observed at postnatal day 12 (p12) in both sexes ($P<0.05$), and this rise continued between days 30 and 51 in females ($P<0.05$) but not in males. As a result, Ob-Rb protein levels in males were around 20% lower than those in females by day 51, although there was no significant overall sex difference evident by two-way ANOVA. However, when additional samples ($n=4$ each) from day 51 males and females were run on a single Western blot, hypothalamic Ob-Rb protein was similarly reduced in males (79.4 ± 7.5 arbitrary units) compared with females (100.0 ± 3.6) and in this case the difference did reach statistical significance ($P<0.05$, unpaired *t*-test).

Discussion

In this study we investigated the developmental patterns of hypothalamic Ob-Rb expression and plasma leptin levels from fetal to postpubertal life in male and female rats. The major findings were that hypothalamic Ob-Rb mRNA expression was relatively low and stable in the fetal and neonatal periods, increased gradually prior to puberty in both sexes, then reached maximal levels after puberty in females. Comparable patterns were observed for hypothalamic Ob-Rb protein. With respect to sex differences in the leptin system, our data show that higher levels of plasma leptin in males relative to females become evident only after puberty. At earlier stages of development the distinct neonatal pattern of plasma leptin, characterised by a marked fall at birth followed by a transient peak at day 12, is similar in males and females. Sex differences in hypothalamic Ob-Rb expression also became evident only after puberty, but in this case expression in females exceeded that in males.

The failure of *ob/ob* mice to undergo sexual maturation underscores the central role of leptin in promoting the onset of puberty (Zhang *et al.* 1994). Nevertheless, there

Figure 3 Western blot analysis and quantification of Ob-Rb protein in the rat hypothalamus during development. Values are expressed in arbitrary density units and are the mean $\pm$ S.E.M. of $n=3-6$ per group. Significant variation occurred with age in both males and females (males, $P<0.001$; females, $P<0.001$); values without common notations differ significantly ($P<0.05$ LSD test).

has been considerable controversy as to whether leptin serves a permissive or a triggering role in this regard. Thus, administration of exogenous leptin to prepubertal rodents was shown to advance the onset of puberty (Ahima *et al.* 1997, Chehab *et al.* 1997, Almog *et al.* 2001). On the other hand, if leptin were a trigger for normal sexual development, one would expect an increase in circulating leptin levels in the lead up to puberty. Two studies have reported a rise from pre- to post-puberty in rats (Gruaz *et al.* 1998, Watanobe & Schioth 2002) whereas others have observed that plasma leptin remains stable across the peripubertal period (Matsuda *et al.* 1999, Cheung *et al.* 2001). Our data show an increase in plasma leptin from

day 30 (prepubertal) to day 51 (postpubertal) in male but not female rats. In the latter, however, unchanging plasma leptin levels were accompanied by a slight increase in hypothalamic Ob-Rb expression from pre- to post-puberty suggesting that females may become more responsive to plasma leptin around the time of puberty. Thus, even though developmental changes in plasma leptin and hypothalamic Ob-Rb were relatively minor in the peripubertal period, it remains possible that a threshold level of both is required to induce puberty, and so a triggering role for leptin cannot be entirely ruled out.

The present work also shows for the first time that the well established sex difference in plasma leptin is not present up to at least day 30, but is clearly evident after puberty (females around 40% lower than males by day 51). Moreover, the higher plasma leptin levels in males are accompanied by slightly lower hypothalamic Ob-Rb expression. The latter observation suggests that after puberty male rats may be less responsive to leptin than females, consistent with the clearly greater body weight and fat mass of adult males (Reed *et al.* 1930). Interestingly, the postpubertal sex difference of plasma leptin in the rat (male>female) is opposite to that in humans where levels in females clearly exceed those in males (Landt *et al.* 1998, Roemmich *et al.* 1998, Pinilla *et al.* 1999). Again this presumably reflects the greater percentage body fat in human females relative to males (Roemmich *et al.* 1998), again possibly related to differences in responsiveness to leptin. Sex differences in both leptin and leptin receptor are likely to reflect the actions and interactions of oestrogen and testosterone, but how such effects are mediated is unclear. It is known that leptin receptor expression in the rat choroid plexus, hypothalamus (Bennett *et al.* 1999) and ovary (Duggal *et al.* 2002) are minimal at cycle stages when oestrogen is high, suggestive of oestrogen inhibition of Ob-R expression. On the other hand, hypothalamic Ob-Rb expression in the present work was higher in females relative to males, suggesting that the suppressive effect of oestrogen is not central to the observed sex difference in Ob-Rb. To our knowledge the effects of testosterone on Ob-Rb expression have not been investigated. Leptin production also appears to be influenced by sex steroids, since plasma levels are maximal at pro-oestrus when oestrogen is high (Duggal *et al.* 2002), and testosterone stimulates leptin secretion by rat epididymal adipose tissue *in vitro* (Nazian 2001). Interestingly, testosterone does not affect leptin secretion by human omental adipose tissue from either males or females, although leptin secretion was inhibited by dihydrotestosterone and other androgens in females (Pineiro *et al.* 1999). Therefore, oestrogen and testosterone may interact to regulate leptin production, and their effects may vary among different species and/or fat depots. Further studies are required to determine such interactions and thus the nature of sex differences in plasma leptin and hypothalamic leptin receptor expression.

A dramatic fall in plasma leptin levels on postnatal day 1 suggests that high leptin levels in the fetus are likely to originate from the mother, since the rat fetus and placenta do not synthesise significant amounts of leptin *de novo* (Dessolin *et al.* 1997, Kawai *et al.* 1997, Amico *et al.* 1998). A similar postnatal decline in plasma leptin has been observed in newborn humans (Harigaya *et al.* 1999), consistent with leptin synthesis by the human placenta (Masuzaki *et al.* 1997). Plasma leptin levels then remained relatively low in the neonate until day 5 before increasing to a transient peak at day 12, and this pattern was comparable in males and females. A similar neonatal pattern of plasma leptin has been reported in both the rat (Rayner *et al.* 1997, Matsuda *et al.* 1999, Morash *et al.* 2001) and the mouse (Devaskar *et al.* 1997, Ahima *et al.* 1998), and a transient peak has also been observed in humans at 3 weeks of age (Harigaya *et al.* 1999). The present work further shows that neonatal leptin fluctuations occur on a background of stable but relatively low hypothalamic Ob-Rb mRNA and protein expression, consistent with a previous report showing low hypothalamic Ob-Rb immunolocalisation in the fetal and neonatal rat (Matsuda *et al.* 1999). Therefore, the hypothalamus is potentially a leptin target at these early stages of development, and leptin may regulate metabolic rate and food intake as occurs in the adult. If so, the initial fall in plasma leptin at birth would be expected to stimulate food intake during the first days of life, an effect that would appear to be highly adaptive. On the other hand, the subsequent peak at day 12 appears paradoxical given the high energy demands during this period, and so the fetal and neonatal hypothalamus may be relatively insensitive to leptin due to the low Ob-Rb expression. Accordingly, leptin administration to neonatal mice and rats fails to influence food intake but does increase metabolic rate (Stehling *et al.* 1996, Mistry *et al.* 1999), and neonatal mice subjected to food deprivation show no change in plasma leptin (Ahima *et al.* 1998). Further studies are required to identify the precise roles of leptin in the neonate and how they might change over the course of development.

Previous studies have reported expression of shorter isoforms of the leptin receptor, Ob-Ra and Ob-Re, in the adult rat hypothalamus (Lollmann *et al.* 1997), but the present work is the first to quantitate relative expression levels and developmental changes. Interestingly, expression of the soluble isoform, Ob-Re, increased in females during development, but its physiological role is unknown. Given that Ob-Re is thought to act as a leptin binding protein in plasma (Gavrilova *et al.* 1997, Seeber *et al.* 2002), its increased expression in the hypothalamus during development could influence the dynamics of leptin access to Ob-Rb.

In conclusion, this study shows a marked developmental increase in hypothalamic leptin receptor expression from late gestation to the prepubertal period. Peripubertal increases were also evident in hypothalamic Ob-Rb

expression (in females) and plasma leptin (in males), and although these changes were small it remains possible that both contribute to the timing of puberty onset. Our data further show that sex differences in both hypothalamic Ob-Rb expression (female>male) and plasma leptin levels (male>female) become evident after puberty.

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