

# Developmental increases in plasma leptin binding activity and tissue Ob-Re mRNA expression in the rat

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## Abstract

Leptin's actions are mediated via the long form of its receptor, Ob-Rb, but access to this receptor on target cells is also influenced by truncated leptin receptor isoforms Ob-Ra and Ob-Re. Plasma leptin binding activity is primarily attributed to Ob-Re, which can restrict leptin passage to extravascular tissue. In this study we investigated whether plasma leptin binding activity changes from fetal to adult life in male and female rats, and whether tissue expression of Ob-Re mRNA changes during development. Plasma leptin binding activity was low in the fetus and prepubertal rats but then increased in male rats by more than three-fold from pre- to post-puberty and by a further two-fold by 7 months of age. A more modest increase in plasma leptin binding activity was observed in females such that a clear sex difference became evident after puberty. There was also a reduction in hypothalamic Ob-Rb protein content between puberty and adult life in female rats. Combined with the higher levels of plasma leptin binding activity, this change in hypothalamic

Ob-Rb expression is likely to lead to a more leptin-resistant state in aging females. To assess possible sources of circulating leptin binding activity, Ob-Re mRNA expression was measured by quantitative RT-PCR in several tissues from male rats soon after puberty and at 7 months of age. All tissues examined (testis, epididymis, adrenal, liver, adipose and spleen) expressed Ob-Re mRNA, and there was a dramatic, age-related increase in expression (>300-fold) in the spleen. These data show that, in addition to the developmental increase in hypothalamic Ob-Rb expression previously reported, plasma leptin binding activity increases several fold from fetal to adult life in the rat. This suggests that the actions of leptin depend not only on its synthesis in adipose tissue and Ob-Rb expression in target cells, but also on factors that regulate tissue expression of Ob-Re and thus leptin transport within plasma.

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## Introduction

Leptin, the 16 kDa peptide product of the *ob* gene, is synthesized primarily by adipocytes and acts via the hypothalamus to decrease food intake and increase metabolic rate (Campfield *et al.* 1995, Pelleymounter *et al.* 1995). Leptin also impacts on a range of other physiological processes including immune (Clarke & Henry 1999, Henson & Castracane 2000) and reproductive function (Loffreda *et al.* 1998, Fantuzzi & Faggioni 2000), most notably by providing a key signal for the timing of puberty (Cheung *et al.* 1997, Nagatani *et al.* 1998). Accordingly, puberty onset in *ob/ob* mice occurs only after administration of exogenous leptin (Zhang *et al.* 1994). The leptin receptor is a member of the class I cytokine receptor family and six multiple splice variants have been identified in rodents (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

including Ob-Ra which 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. In rodents, the Ob-Re isoform is encoded by a specific mRNA and is secreted (Gavrilova *et al.* 1997, Tartaglia 1997, Huang *et al.* 2001), whereas in the human it is derived from shedding of the extracellular portion of membrane-bound leptin receptors (Ge *et al.* 2002). Regardless of its origin, leptin binding to soluble leptin receptors is now recognised as an important regulator of leptin access to target and metabolic tissues (Huang *et al.* 2001, Eleftheriou *et al.* 2004). Indeed, a dramatic increase in plasma leptin binding activity in rodent pregnancy (Gavrilova *et al.* 1997, Seeber *et al.* 2002) reduces the metabolic clearance rate (MCR) of maternal leptin (Smith & Waddell 2003b) and thus contributes to the marked rise in maternal plasma leptin levels (Gavrilova *et al.* 1997, Seeber *et al.* 2002). While the placenta is the major source of this binding activity in pregnancy (Gavrilova *et al.* 1997,

Smith & Waddell 2002), its source and variability in non-pregnant animals is unknown. Recently, we demonstrated that hypothalamic Ob-Rb expression increases progressively from the late fetal to the peripubertal period in both sexes (Smith & Waddell 2003a). These data suggest that enhanced responsiveness of the hypothalamus to leptin may contribute to puberty onset, but whether plasma leptin binding activity also changes during development, particularly in the peripubertal period, is unknown. Studies across human development show that circulating leptin binding activity falls from pre- to postpuberty (Quinton *et al.* 1999, Kratzsch *et al.* 2002, Mann *et al.* 2003), suggesting that access of plasma leptin to its hypothalamic targets may be enhanced over this period. In the present study, therefore, we measured plasma leptin binding activity in fetal, prepubertal, postpubertal and adult male and female rats. This analysis showed a clear increase in plasma leptin binding activity over development, particularly in males, and so to assess possible sources of this binding activity Ob-Re mRNA expression was determined in a range of tissues from young and adult rats.

## 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 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 was deemed to occur at the time of vaginal opening in females and preputial separation in males (Smith & Waddell 2000). All procedures involving animals were conducted only after approval by the Animal Ethics Committee of The University of Western Australia.

### Blood sampling and tissue collection

At g22 pregnant rats were anesthetized with halothane/nitrous oxide and fetuses removed. Blood samples were obtained by decapitation from 4 female and 4 male g22 fetuses (from a single litter) and pooled to provide sufficient volume. Blood samples were collected from anesthetised rats at p12 by decapitation, and at p30 and p51 via the exposed dorsal aorta. Brains were removed from rats at p51 and 7 months of age and the hypothalamus dissected (Zakrzewska *et al.* 1999) and immediately frozen in liquid

nitrogen. Tissue samples for Ob-Re mRNA analysis (spleen, liver, adipose, adrenal, testis and epididymis) were obtained from separate groups of anaesthetised male rats at p51 and 7 months and immediately frozen in liquid nitrogen for subsequent real-time quantitative RT-PCR.

### Plasma leptin binding activity

Plasma leptin binding activity was assayed as described previously (Gavrilova *et al.* 1997, Seeber *et al.* 2002). Briefly, plasma samples (5 µl) were incubated with 5 µl of <sup>125</sup>I-leptin (approximately 5 ng/ml and 20 000 c.p.m.) overnight at 4 °C. Sample buffer (20 µl) containing bromophenol blue was added, samples were vortexed and electrophoresed in 12% Tris/glycine gels at 160 V. The gel was dried and placed against film for 48 h at -80 °C. The band corresponding to specifically bound <sup>125</sup>I-leptin was scanned and leptin binding activity quantified by analysis of pixel intensity using Scion Image analysis software (Release beta 3b; Scion Corporation, Frederick, MD, USA) as previously described (Burton *et al.* 1998).

### Radioimmunoassays

Blood samples collected as described above were centrifuged at 13 000 g for 5 min and plasma 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.

### Western blot analysis of hypothalamic Ob-Rb

Hypothalamic Ob-Rb protein expression was measured in postpubertal (p51) and adult rats (7 months of age) as previously described (Seeber *et al.* 2002). Briefly, hypothalami were homogenized (10 mM Tris buffer containing 1.5 mM EDTA, 1 mM DTT, 1 mM PMSF and 100 µg/ml trypsin inhibitor) then centrifuged at 105 000 g for 30 min. Supernatant protein (30 µg) was resolved by 7% SDS-PAGE electrophoresis and transferred to nitrocellulose membrane (Hybond C Super, Amersham Biosciences, Australia). Membranes were blocked in Tris-buffered saline (TBS)-Tween (0.1 M Tris, 0.15 M NaCl, 0.1% Tween-20; pH 7.5) containing 5% non-fat milk powder for 1 h then exposed for 2 h to leptin receptor antibody (K-20, Santa Cruz Biotechnology; 1:400 dilution in TBS-Tween buffer, containing 1% non-fat milk powder). Membranes were washed then incubated with a horseradish peroxidase-conjugated donkey anti-goat secondary antibody (1:5000; Santa Cruz Biotechnology) for 1 h. Immunoreactive bands were visualised using a chemiluminescence detection kit (SuperSignal Substrate, Pierce Chemical, Rockford, IL, USA), with membranes placed against film for 1 min, and resultant images quantified by

densitometry using Scion Image analysis software (Release beta 3b) as previously described (Burton *et al.* 1998).

#### Real-time quantitative RT-PCR

Total RNA was isolated from various tissues (adipose, liver, spleen, adrenal, testis and epididymis) obtained from postpubertal (p51) and adult (7 months of age) rats using RNAzol (Iso-TEX Diagnostics, Friendswood, TX, USA), except for adipose tissue which used RNeasy Lipid Tissue Mini Kit (Qiagen, Clifton Hill, Australia). The purified RNA (5 µg) was reverse transcribed and the resultant cDNA purified and quantified as previously described (Seeber *et al.* 2002). Primers specific to rat Ob-Re (Takaya *et al.* 1996) and L19 as a housekeeping internal control (Orly *et al.* 1994) were used for amplification and spanned introns to distinguish cDNA from genomic DNA, and external standards were generated from regular PCR products. 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). Fluorescence values were analysed and a standard curve constructed using the LightCycler software, and sample values were expressed relative to L19 expression.

#### Statistical analysis

All data are expressed as the mean ± S.E.M. where a minimum of three animals were used for each experimental variable and each litter represented an 'n' of one. Variation in plasma leptin binding activity related to age and sex was assessed by one- or two-way ANOVA, and differences among specific means were determined by LSD test (Snedecor & Cochran 1989). Unpaired *t*-tests were used to compare plasma leptin, leptin binding activity and hypothalamic Ob-Rb protein expression between male and female rats at any given age. For tissue expression of Ob-Re mRNA, the data were first log transformed to adjust for unequal variance, then analysed by two-way ANOVA.

## Results

#### Plasma leptin binding activity

Plasma leptin binding activity varied significantly with both age ( $P < 0.001$ ) and sex ( $P < 0.05$ ) and there was significant interaction between these sources of variation ( $P < 0.05$ ; Fig. 1). Therefore, age-related changes were assessed separately in males and females by one way ANOVAS. In females, plasma leptin binding activity increased gradually from very low levels at g22 and p12 to a maximum at 7 months ( $P < 0.05$ ). A more dramatic

increase ( $P < 0.001$ ) was observed in male rats, rising more than three-fold from pre- to post-puberty ( $P < 0.05$ ) and then by a further two-fold from post-puberty to seven months ( $P < 0.05$ ). Therefore, while plasma leptin binding activity was similar in male and female rats at prepubertal stages, it was higher ( $P < 0.05$ ) in males after puberty (p51 and 7 months).

#### Hypothalamic Ob-Rb protein expression

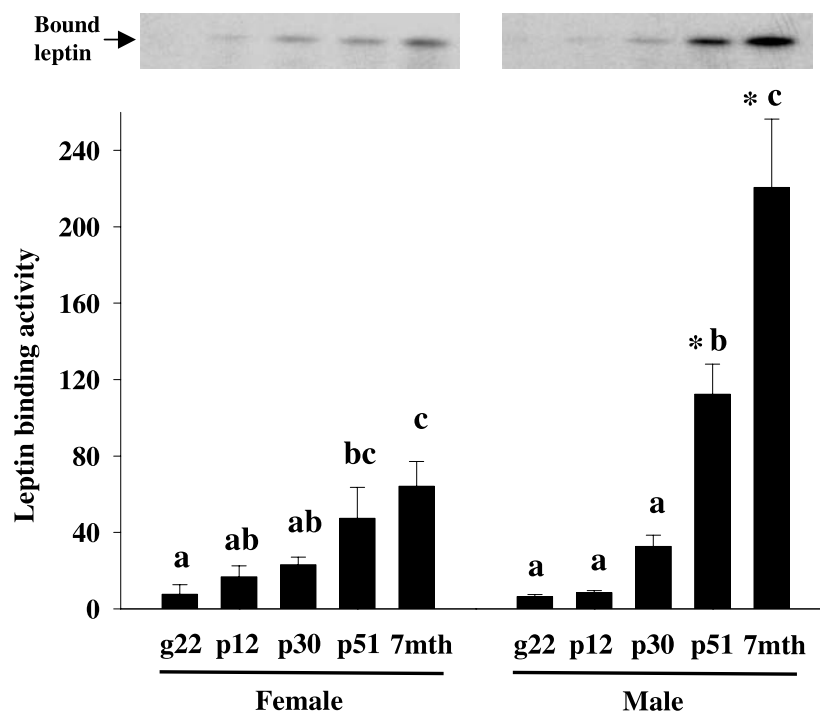
We previously demonstrated that hypothalamic Ob-Rb protein expression was higher in females compared with males in the immediate post-pubertal period (Smith & Waddell 2003a). A similar difference in Ob-Rb expression was observed at day 51 in the present work (females 21% greater,  $P < 0.05$ ), but by seven months of age this sex difference was no longer apparent (Fig. 2). Plasma leptin concentrations at seven months of age were quite variable and did not differ significantly between males ( $9.0 \pm 1.0$  ng/ml) and females ( $6.1 \pm 1.0$  ng/ml;  $P = 0.10$ ), even though males were 37% heavier than females (males,  $533 \pm 5$  g; females,  $334 \pm 9$  g;  $P < 0.001$ ).

#### Developmental changes in Ob-Re mRNA expression

Expression of mRNA encoding the Ob-Re isoform of the leptin receptor was detected in all tissues examined at day 51 and at 7 months (Fig. 3). Expression levels varied with both age ( $P < 0.01$ ) and tissue ( $P < 0.01$ ) and there was significant interaction between these sources of variation ( $P < 0.01$ ). Therefore, specific age comparisons were made by unpaired *t*-tests and these showed age-related increases in Ob-Re mRNA expression in the spleen (300-fold increase,  $P < 0.01$ ), testis (2.4-fold,  $P < 0.05$ ) and adipose tissue (2-fold,  $P < 0.05$ ).

## Discussion

In the present study we show that a marked increase in plasma leptin binding activity occurs across development in the rat, and this is associated with upregulation of Ob-Re mRNA expression in a number of tissues, most notably the spleen. Leptin actions are mediated via Ob-Rb on target cells but are also influenced by at least two truncated receptor isoforms, Ob-Ra and Ob-Re. Thus, Ob-Ra is thought to facilitate leptin transport across anatomical barriers such as the blood-brain barrier (Kastin *et al.* 1999) and the placenta (Smith & Waddell 2003b), whereas the soluble form of the leptin receptor, Ob-Re, serves as a plasma binding protein that limits access of leptin to extravascular tissues and thus target cells. Such a role for circulating Ob-Re is supported by reports showing that adenovirus-mediated overexpression of Ob-Re increases plasma leptin binding activity and limits the effect of leptin on target tissues in mice (Huang *et al.* 2001,



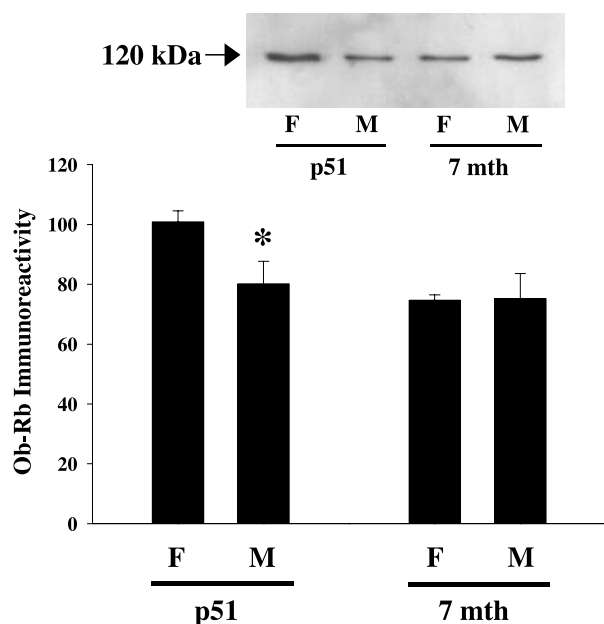
**Figure 1** Changes in plasma leptin binding activity at gestational day 22 (g22), postnatal (p) days 12, 30 and 51 and at seven months (7 mth) of age in female and male rats. Values are expressed in arbitrary density units and are the mean  $\pm$  S.E.M. of  $n=4$  per group. Significant variation occurred with age ( $P<0.001$ ) and sex ( $P<0.01$ ) and there was significant interaction ( $P<0.01$ ) between these sources of variation. Within each group (male or female), values without common notations (a, b and c) differ significantly ( $P<0.05$ ; LSD-test). \*  $P<0.05$  compared with corresponding age in females (unpaired t-test).

Eleftheriou *et al.* 2004). Because the developmental rise in plasma leptin binding occurred on a background of increasing hypothalamic expression of Ob-Rb (Smith & Waddell 2003a), it is apparent that leptin actions are determined by the complex interplay of leptin secretion by adipose tissue, leptin transport within and from the vascular compartment, and target tissue expression of Ob-Rb.

The developmental increase in plasma leptin binding activity was clearly more pronounced in males than females, rising several-fold between late fetal life and adulthood. Because this sex difference emerged only after puberty it may reflect the action of sex steroids, with testosterone stimulating and/or ovarian steroids inhibiting tissue expression of Ob-R<sub>e</sub>. Estrogen appears to suppress Ob-R<sub>b</sub> expression in the rat choroid plexus, hypothalamus (Bennett *et al.* 1999) and ovary (Duggal *et al.* 2002), although in the baboon placenta estrogen suppression induced by fetectomy was recently shown to reduce placental expression of the long form of Ob-R (Edwards *et al.* 2004). Interestingly, a putative estrogen response element has been identified on the Ob-R promoter in the rat (Lindell *et al.* 2001), but it remains unclear how this

might influence tissue expression of the specific Ob-R isoforms, including Ob-R<sub>e</sub>. In humans, plasma leptin binding activity is also higher in males but, surprisingly, plasma leptin binding activity is associated negatively with plasma testosterone in males and positively with estrogen in females (Mann *et al.* 2003). Clearly, further studies are required to determine the mechanisms underlying sex differences in plasma leptin binding activity in both rodents and humans.

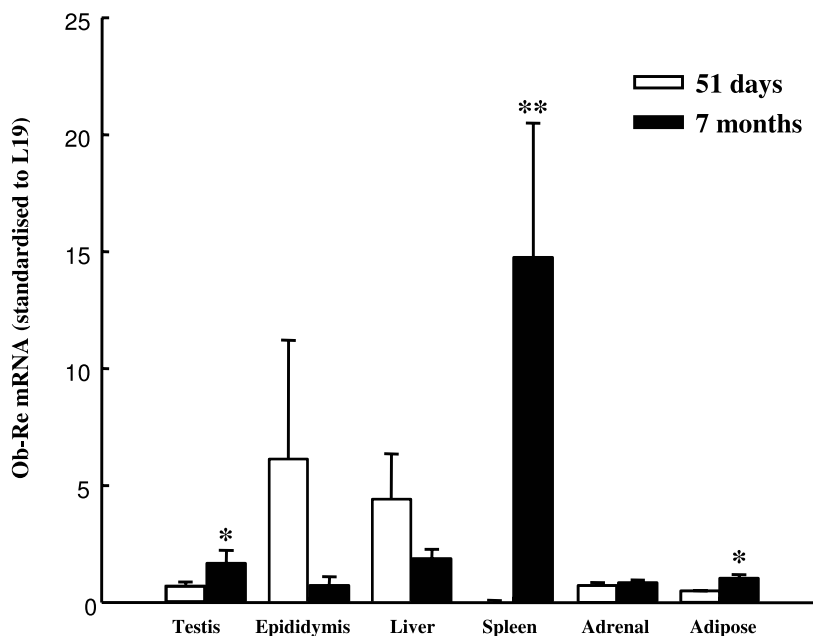
The increase in plasma leptin binding is likely to reduce the MCR of leptin relative to body weight, an effect recently observed in rat pregnancy (Smith & Waddell 2003b). This reduction in leptin MCR occurred just prior to term, coincident with maximal levels of leptin binding activity (Smith & Waddell 2003b) and likely reflects retention of leptin within the vascular compartment. During early postnatal development plasma leptin levels exhibit a dynamic pattern in both males and females, but then after puberty leptin rises more quickly in male rats (Smith & Waddell 2003a). The present study suggests that this divergence partly reflects the more rapid increase in plasma leptin binding activity and the associated fall in relative MCR of leptin in males.



**Figure 2** Western blot analysis and quantification of hypothalamic Ob-Rb protein in male (M) and female (F) rats at postnatal day 51 (p51) and at 7 months of age (7 mth). Values are expressed in arbitrary density units and are the mean ± S.E.M. of  $n=4$  per group. \* $P<0.05$  compared with female p51 value (unpaired  $t$ -test).

In addition to limiting leptin metabolism, increased plasma leptin binding activity is likely to restrict leptin access to Ob-Rb on target cells and thus result in a state of relative leptin resistance, comparable to that apparent in pregnancy (Gavrilova *et al.* 1997, Seeber *et al.* 2002). This effect of leptin binding may contribute to the greater body weight and associated increased body fat percentage of male relative to female rats (Reed *et al.* 1930). Indeed, age-related hyperleptinemia and increased food intake occur in male rats in association with reduced hypothalamic uptake of leptin (Fernandez-Galaz *et al.* 2001), and similar effects have been observed in diet-induced rodent models of obesity (Halaas *et al.* 1997, Van Heek *et al.* 1997, El-Haschimi *et al.* 2000). Interestingly, the present work confirms the clear sex difference (males > females) in hypothalamic Ob-Rb protein expression previously reported for rats aged 51 days (Smith & Waddell 2003a), but shows that this sex difference is lost by 7 months, coincident with a reduction in the sex difference in plasma leptin. Thus, the rise in plasma leptin in females between post-puberty and seven months of age may reflect a more leptin resistant state due to lower hypothalamic Ob-Rb.

While the placenta is clearly recognised as a major source of Ob-Re and thus plasma leptin binding activity in pregnant rodents (Gavrilova *et al.* 1997, Lammert *et al.* 2002, Smith & Waddell 2002), the source of circulating Ob-Re in non-pregnant rodents has remained uncertain.



**Figure 3** Expression of Ob-Re mRNA measured by real-time RT-PCR in various tissues from male rats immediately after puberty (p51) and at 7 months of age (adult=7mth). Values are expressed relative to L19 levels and are the mean ± S.E.M. of  $n=3$  per group. There was significant variation among tissues ( $P<0.01$ ) and with age ( $P<0.01$ ) and significant interaction ( $P<0.01$ ). Therefore, age comparisons were made for each tissue by unpaired  $t$ -test. \* $P<0.05$ , \*\* $P<0.01$  compared with corresponding value at p51.

Thus, one previous report provided no clear indication of Ob-Re mRNA expression by Northern analysis (Fei *et al.* 1997) whereas another detected Ob-Re mRNA by RT-PCR in a range of tissues but without accurate quantitation (Lollmann *et al.* 1997). We subsequently used quantitative RT-PCR to demonstrate Ob-Re mRNA expression in the hypothalamus of both male and female rats, and to show a clear developmental increase in this expression in females (Smith & Waddell 2003a). The present work extends these observations by showing that Ob-Re mRNA is expressed by several other tissues, most notably in the spleen where it increased more than 300-fold from the immediate post-pubertal period to the adult. A role for leptin in the immune system is well recognised (Fantuzzi & Faggioni 2000, Loffreda *et al.* 1998) and the spleen is known to express both Ob-Rb (Gainsford *et al.* 1996, Lollmann *et al.* 1997) and Ob-Re (Lollmann *et al.* 1997). Potentially, Ob-Re could play a local regulatory role within the spleen by mediating leptin access to Ob-Rb, and splenic secretion of Ob-Re into the general circulation is likely to contribute significantly to plasma leptin binding activity. Among the other tissues examined, Ob-Re mRNA expression appeared relatively low, particularly in the adult, and as such these are likely to make only a relatively minor contribution to circulating Ob-Re.

In contrast to Ob-Re mRNA expression by several tissues in rodents, circulating leptin binding activity in humans is generated entirely by ectodomain shedding of membrane receptors (Maamra *et al.* 2001, Ge *et al.* 2002). It is possible that this shedding mechanism also operates in rodents, thus providing an additional source of circulating Ob-Re, but to our knowledge this possibility has not been investigated. In any event, regulation of leptin binding activity is likely to differ substantially between rodents and humans, and in this context it is noteworthy that the increase in plasma leptin binding activity from pre- to post-puberty in rats is opposite to the decrease in humans (Quinton *et al.* 1999, Kratzsch *et al.* 2002, Mann *et al.* 2003). The latter may facilitate puberty onset in humans by increasing leptin access to its hypothalamic targets, whereas in the rat, increased hypothalamic expression of Ob-Rb (Smith & Waddell 2003a) would appear to be of greater importance to puberty onset.

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