Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization

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Abstract Expression of the leptin receptor gene has been examined in mouse hypothalamus and other brain regions by in situ hybridization. With a probe recognizing all the known splice variants, receptor mRNA was evident in several brain regions (cortex, hippocampus, thalamus), with strong expression in the hypothalamus (arcuate, ventromedial, paraventricular and ventral premammillary nuclei), choroid plexus and leptomeninges. A probe specific to the long splice variant of the leptin receptor (Ob-Rb), containing the putative intracellular signaling domain, again revealed strong expression in the hypothalamus; there was, however, minimal hybridization to choroid plexus and leptomeninges. These results indicate that the hypothalamus is a key site of leptin action, although other brain regions are also targeted.

Key words: Leptin receptor; In situ hybridization; Hypothalamus; *Ob* gene; Splice variant; Arcuate nucleus

1. Introduction

Leptin, the protein product of the recently cloned *ob* (obese) gene [1], is secreted by adipocytes and appears to be important in the maintenance of energy balance. Peripheral injections of recombinant leptin have been shown to reduce the body weight, adiposity and food intake of obese *ob/ob* mice [2–4]. Leptin is also active when administered centrally [4], suggesting that certain areas of the brain may be targets for leptin action. This was confirmed when [^{125}I]leptin binding sites were identified in the choroid plexus of the mouse brain [5]. This tissue was then used in the expression cloning of a leptin receptor [5]. Leptin receptor gene expression was subsequently detected in the mouse hypothalamus by RT-PCR [5]. However, so far, the precise localization of leptin receptor mRNA in the brain is unknown.

Given the pivotal role of the hypothalamus in the regulation of appetite, body weight and energy balance, the resolution of leptin receptor gene expression to specific hypothalamic nuclei is of critical importance to the elucidation of the physiological function of leptin. Additionally, the leptin receptor gene has been shown to have at least 6 splice variants [6,7]. The receptor originally cloned from the choroid plexus [5] corresponds to the Ob-Ra splice variant [7], which, in common with the variants Ob-Rc, Ob-Rd and Ob-Re, has a short intracellular domain. Its function may be to transport leptin across the blood-brain barrier [7]. By contrast, the splice variant, Ob-Rb, encodes a receptor with a long intracellular domain. By analogy with the class I cytokine receptors to which the leptin receptor is most closely related, this domain is thought to be essential for intracellular signal transduction [5]. It is significant, therefore, that the *db/db* mouse, a mutant previously considered to have a leptin receptor defect, has an insertion leading to premature termination of the intracellular domain. RT-PCR studies indicate that the mRNA for Ob-Rb is expressed abundantly in the mouse hypothalamus [7], consistent with a role in mediating the effects of leptin on energy balance. Accordingly, using in situ hybridization we have examined receptor gene expression in mouse hypothalamus using two different probes. The first hybridizes to part of the common extracellular domain of the receptor gene, and thus recognizes each of the splice variants, Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re [7], while the second has specificity for the Ob-Rb (long intracellular domain) splice variant. We show here that the leptin receptor gene is expressed in several regions of the mouse brain, with strong expression in the choroid plexus and in parts of the hypothalamus. A similar pattern of expression was observed for the long splice variant, Ob-Rb.

2. Materials and methods

2.1. PCR and probe synthesis

Probes were generated from mouse brain cDNA using receptor-specific PCR primers. The probe to the common leptin receptor sequence (which recognizes the splice variants Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re), a 473 bp product, was generated using the primers 5′-CAGATTCGATATGGCTTAATGGG-3′ (+1704 to +1726) and 5′-GTTAAAATTCACAAGGGAAGCG-3′ (+2177 to +2156; Genebank U42467 [5]). The probe specific to the long form of the receptor, Ob-Rb, a 533 bp product, was generated using the primers 5′-GTGTGAGCATCTCTCCTGGAG-3′ (+2829 to +2849) and 5′-ACCACACCAGACCCTGAAAG-3′ (+3362 to +3343; Genebank U49107 [7]). Reverse transcription using the Superscript Preamplification system (Gibco BRL) was performed on 1 μg of mRNA from total mouse brain (extracted using the Microfast Track kit; Invitrogen). PCR was performed on a Hybaid Omnigene thermal cycler using the following conditions: [94°C (4 min) – 1 cycle]; [94°C (1 min), 55°C (1 min), 72°C (1 min) – 35 cycles]; [72°C (10 min) – 1 cycle]. The PCR reaction contained 2.5% of the cDNA product, 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.01% Triton X-100 (Promega), 5 μM of each primer, 200 μM dNTPs (Promega), 1.5 mM $MgCl_2$ and 1.5 U Taq polymerase (Promega) in a final volume of 25 μl. A single PCR product, of the predicted size, was observed in each case by gel electrophoresis, and was purified using Wizard PCR preps (Promega) and cloned directly into pGEM-T (Promega). The sequence and orientation of the inserts were confirmed by automated sequencing. Plasmids were linearized with *Sac*I or *Apa*I for transcription with T7 or SP6 RNA polymerase to generate antisense and sense riboprobes.

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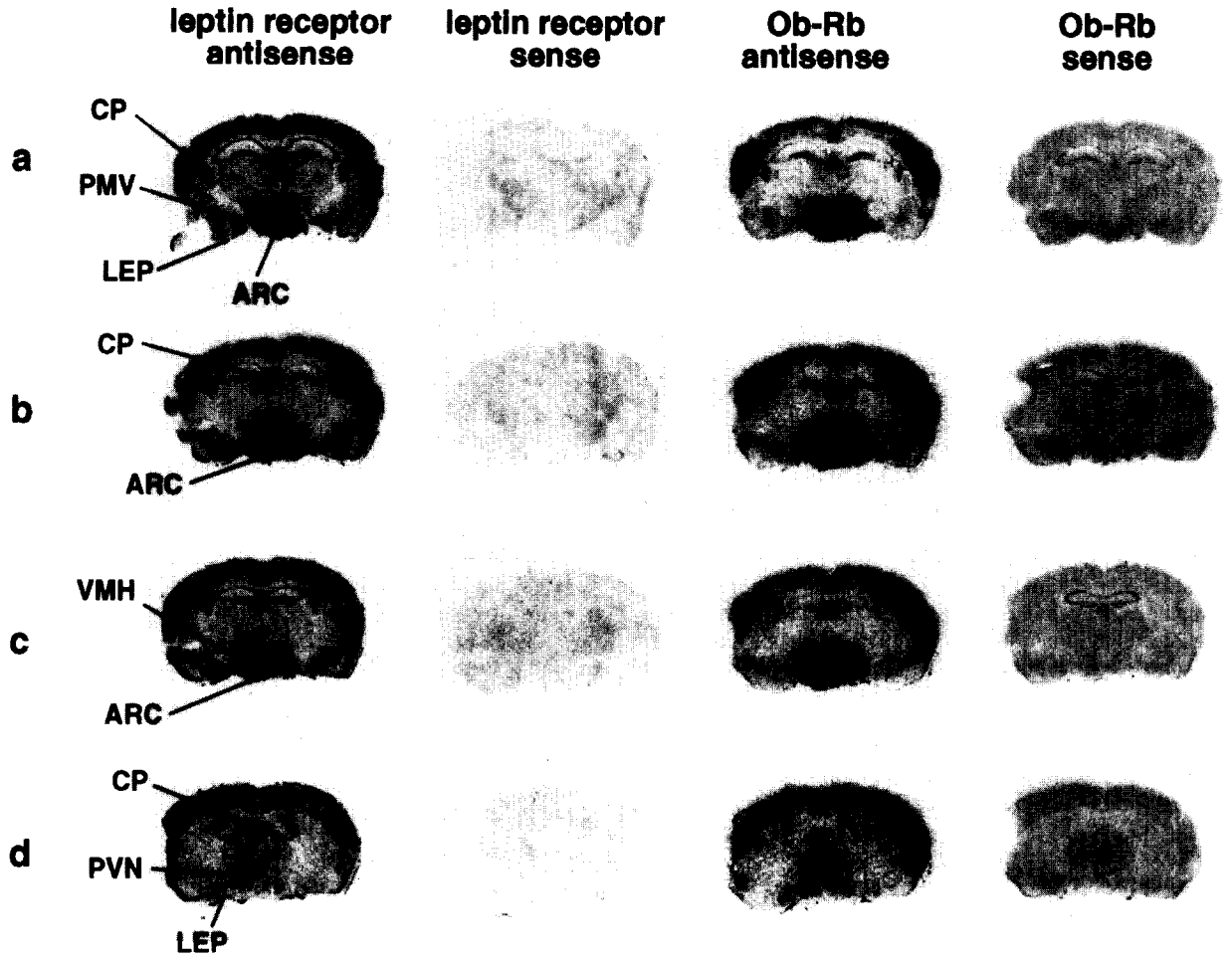

Fig. 1. Autoradiographic localization of leptin receptor and Ob-Rb splice variant gene expression in coronal sections of mouse brain through the region containing the hypothalamus. Images were produced by 20 μm sections taken at 240 or 480 μm intervals through the hypothalamic region of a single representative animal (intervals: a–b, 240 μm; b–c, 240 μm; c–d, 480 μm). Adjacent sections were hybridized with antisense or sense probe to the common leptin receptor sequence, or with antisense or sense probe to the Ob-Rb splice variant. CP, choroid plexus; LEP, leptomeninges; ARC, arcuate hypothalamic nucleus; PMV, hypothalamic premammillary nucleus, ventral; VMH, ventromedial hypothalamic nucleus; PVN, paraventricular hypothalamic nucleus. Magnification approx. ×2.7.

2.2. In situ hybridization

Lean mice of the 'Aston' variety (approx. 8 weeks of age) were drawn from a colony maintained at the Rowett Research Institute. The study of leptin receptor gene expression in the hypothalamus and adjacent brain areas of the mouse used techniques described in detail elsewhere [8,9]. Briefly, 20 μm coronal sections were cut from the mammillary bodies through to the optic chiasm of 6 mouse brains. Sections were mounted on poly(L-lysine)-coated slides and stored at −70°C. After fixation in 4% paraformaldehyde in 0.1 M phosphate buffer, and acetylation in 0.1 M triethanolamine (pH 8)/0.25% acetic anhydride for 10 min, hybridization was performed using ^{35}S-labelled cRNA probes at concentrations of $1.5–2\times10^7$ cpm/ml. Probes were prepared in a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8), 1 mM EDTA, 0.05% tRNA, 10 mM dithiothreitol, 1×Denhardt's solution and 10% dextran sulphate, and were hybridized to sections overnight at 55–58°C. After hybridization, slides were treated with RNase A, desalted, with a final high stringency wash (30 min) in 0.1×SSC at 60°C, and dehydrated. Dried slides were apposed to Hyperfilm β-max (Amersham) for film autoradiography or coated with autoradiographic emulsion (LM-1; Amersham). Brain areas expressing the mRNAs of interest were identified by reference to adjacent stained sections and the atlas of the rat brain [10].

3. Results

The distribution of leptin receptor gene expression and the splice variant, Ob-Rb, was examined by in situ hybridization. Using the probe to the common extracellular domain of the leptin receptor gene, hybridization was widespread, but discretely localized, throughout the mouse brain (Fig. 1; leptin receptor antisense). Hybridization signals were not detected using the respective sense probe (Fig. 1; leptin receptor sense). The leptin receptor gene was strongly expressed in the choroid plexus (CP), leptomeninges (LEP) and hypothalamus, with less intense regions of hybridization in the cortex, hippocampus and thalamus (Fig. 1a–d). Within the hypothalamus, leptin receptor gene expression was present throughout the arcuate nucleus (ARC; Fig. 1a–c), in the ventral premammillary nucleus (PMV; Fig. 1a), the ventromedial nucleus (VMH; Fig. 1c) and the paraventricular nucleus (PVN; Fig. 1d). In addition to these areas, there were more diffuse regions of gene expression in the dorsomedial hypothalamus and lateral hypothalamus (Fig. 1b). Gene expression was not detected in the hypothalamus anterior to the PVN. Dark field photomicrographs showing probe hybridization to leptin receptor mRNA in the ARC, PMV and PVN of a second representative animal are presented in Fig. 2.

Adjacent hypothalamic sections were then probed for the

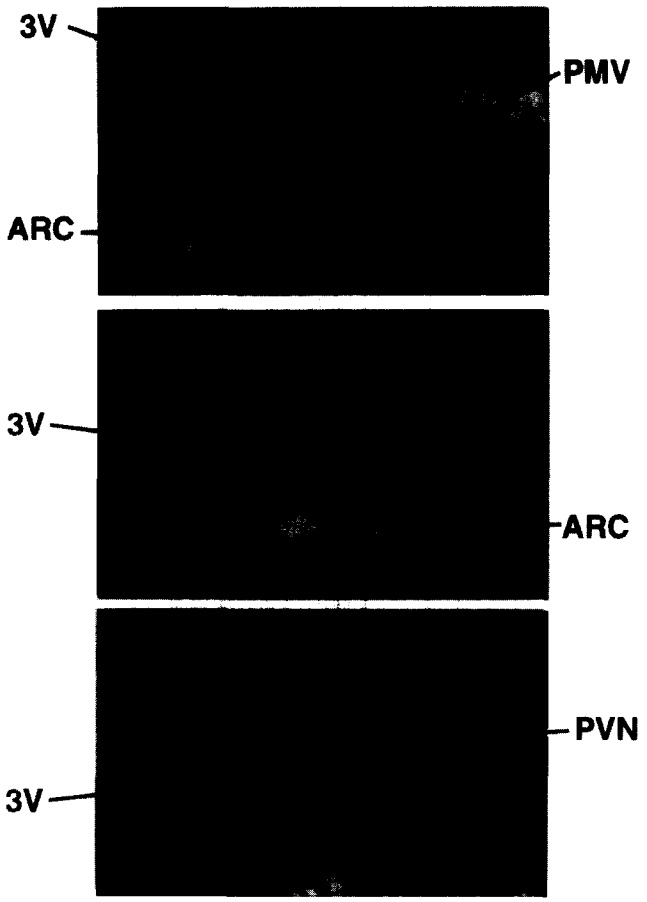

Fig. 2. Each panel is a dark field photomicrograph of a hypothalamic section on which in situ hybridization was performed using the probe to the common extracellular domain of the leptin receptor gene. Images were produced by 20 μm sections taken from a second representative animal. (Upper panel) Gene expression in the ARC and PMV (equivalent to Fig. 1a); (middle panel) gene expression in the ARC (equivalent to Fig. 1b); (lower panel) gene expression in the PVN (equivalent to Fig. 1d). ARC, arcuate hypothalamic nucleus; PMV, hypothalamic premammillary nucleus, ventral; PVN, paraventricular hypothalamic nucleus; 3V, third ventricle. Magnification approx. ×60.

long form splice variant, Ob-Rb (Fig. 1; Ob-Rb antisense). The background signal generated by the Ob-Rb sense probe was relatively high, with some non-specific association with the hippocampus (Fig. 1; Ob-Rb sense). The localization of Ob-Rb gene expression matched with the neuronal areas which hybridized with the common probe, but there was minimal hybridization to choroid plexus and leptomeninges (Fig. 1; Ob-Rb antisense). In the hypothalamus and adjacent brain areas, hybridization signals from the Ob-Rb probe were of comparable intensity to those observed with the common probe. Using the same primers we have demonstrated leptin receptor mRNA and Ob-Rb specific mRNA in both cortex and hypothalamus by RT-PCR (results not shown).

4 Discussion

We have localized leptin receptor gene expression to several hypothalamic nuclei which have been implicated in the regulation of energy balance; the ARC, VMH, PVN. In addition to the relatively discrete hybridization seen over these areas, a more diffuse signal was observed over dorsomedial and lateral hypothalamic areas. The significance of the high level of gene expression seen in the PMV of the mouse remains to be established. Furthermore, based on the relative densities of the hybridization signals produced by probes of similar concentration and specific activity, the leptin receptor gene expressed in the hypothalamus appeared to consist mainly of the Ob-Rb splice variant, encoding a leptin receptor with a long intracellular domain. The identification of Ob-Rb mRNA in the hypothalamus is consistent with RT-PCR data indicating that this variant is highly expressed in the hypothalamus [7]. The dense hybridization seen with the common leptin receptor probe to choroid plexus and leptomeninges, both of which form part of the blood-brain barrier, was not observed with the Ob-Rb probe; only minimal hybridization was observed with the latter probe. This finding is consistent with the original designation of the receptor gene in the choroid plexus as the Ob-Ra splice variant, which is speculated to be involved in the transport of leptin across the blood-brain barrier [5,7]. Similarly, the leptomeninges may also be involved in leptin transport across the blood-brain barrier.

While the findings of this in situ hybridization study are consistent with the effect of leptin on energy balance being mediated by interaction with an Ob-Rb receptor in the hypothalamus, other neuronal sites of receptor expression also expressed the Ob-Rb splice variant. Expression of Ob-Rb mRNA in other brain regions, including the thalamus, cortex, and possibly the hippocampus is indicative of a more complex and widespread regulatory role for the product of the *ob* gene. Although expression of leptin receptor mRNA was widespread in the brain and associated blood-brain barrier tissues, [^{125}I]leptin binding sites have only been detected in the choroid plexus to date [5,11], and the affinity of [^{125}I]leptin binding to the proteins encoded by the different receptor splice variants is unknown.

It also remains to be established whether the various hypothalamic and non-hypothalamic brain areas that express the common leptin receptor sequence or specific Ob-Rb mRNA all contain functional receptor proteins. The signal transduction pathways which are involved in the integration of leptin with other peripheral and central neuroendocrine systems involved in energy balance also require further investigation.

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