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OB protein binds specifically to the choroid plexus of mice and rats

(body energy balance/food intake/neural networks/*ob* gene product/leptin)

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ABSTRACT **Binding studies were conducted to identify the anatomical location of brain target sites for OB protein, the *ob* gene product. ^{125}I-labeled recombinant mouse OB protein or alkaline phosphatase-OB fusion proteins were used for *in vitro* and *in vivo* binding studies. Coronal brain sections or fresh tissue from lean, obese *ob/ob*, and obese *db/db* mice as well as lean and obese Zucker rats were probed to identify potential central OB protein-binding sites. We report here that recombinant OB protein binds specifically to the choroid plexus. The binding of OB protein (either radiolabeled or the alkaline phosphatase-OB fusion protein) and its displacement by unlabeled OB protein was similar in lean, obese *ob/ob*, and obese *db/db* mice as well as lean and obese Zucker rats. These findings suggest that OB protein binds with high affinity to a specific receptor in the choroid plexus. After binding to the choroid plexus receptor, OB protein may then be transported across the blood–brain barrier into the cerebrospinal fluid. Alternatively, binding of OB protein to a specific receptor in the choroid plexus may activate afferent neural inputs to the neural network that regulates feeding behavior and energy balance or may result in the clearance or degradation of OB protein. The identification of the choroid plexus as a brain binding site for OB protein will provide the basis for the construction of expression libraries and facilitate the rapid cloning of the choroid plexus OB receptor.**

Obesity is a complex, increasingly prevalent, and important health problem throughout the world (1, 2). Human obesity is characterized by increased adipose tissue mass resulting from an interaction of genetic predisposition to metabolic efficiency (35–50%) and environmental/lifestyle factors (50–65%) (1–4). Obesity is also characterized by reduced insulin sensitivity of muscle and fat, exaggerated insulin secretion, and hyperinsulinemia as well as high rates of lipid deposition in adipose tissue (1–7). Since obesity is associated with non-insulin-dependent diabetes mellitus (NIDDM), hypertension, hyperlipidemia, coronary heart disease, stroke, and certain cancers, the increasing prevalence of; the lack of effective treatment for; and the psychological, social, and economic costs of obesity are matters of growing concern in the scientific, medical, and public health communities (1, 2). Recent studies have clearly demonstrated that obesity is, at its basis, a disease of biological dysregulation. That is, the steady-state body weight of an individual results from an integration of multiple biological factors, which are at least partially genetically determined. Perturbations in body weight, in both directions, from this steady-state weight are resisted and corrected by robust physiological mechanisms (8).

The complex molecular mechanisms by which discrete ingestive behavior, continuous energy expenditure, and dynamic energy storage in adipose tissue are integrated have remained largely unknown. However, several lines of evidence strongly suggested the existence of circulating signals that are proportional to adipose tissue mass, possibly coming from adipose tissue, and that regulate ingestive behavior and energy balance (5–15). According to this hypothesis, when adipose tissue mass expands, the circulating concentration of signal molecule(s) would increase and act on the neuronal networks in the brain controlling food intake and energy balance and lead to decreased food intake and/or increased energy expenditure (5–15).

The experimental study of obesity has been facilitated and enhanced by the identification and characterization of several single-gene mutations in rodents. One of the most extensively studied genetic models of obesity has been the obese *ob/ob* mouse that was first described 45 years ago (16). The affected C57BL/6J *ob/ob* mice inherit profound, early onset obesity as a recessive trait, whereas homozygous (+/+) and heterozygous (+/*ob*) littermates remain lean (5–7, 9–12, 17, 18). When the *ob* gene was placed on a diabetes-susceptible background, such as C57BL/KsJ, the affected obese mice exhibited a phenotype that resembles NIDDM (9–12). Yet, the molecular bases of the obesity in *ob/ob* mice eluded investigators despite numerous, elegant, and extensive behavioral, physiological, pharmacological, biochemical, and molecular studies (5–7, 9–12, 17, 18) until the recent cloning of the mouse *ob* gene and its human homologue (19).

Two other single-gene mutations, *db/db* in mice and *fa/fa* (also known as Zucker) in rats, have also been important animal models in obesity research. They exhibit similar phenotypes, characterized by both obesity and hyperglycemia, that resemble NIDDM (9–12). Molecular genetic studies have demonstrated that the *fa* gene in rats is the rat homologue of the mouse *db* gene (20). Experimental parabiosis of lean mice and *ob/ob* and *db/db* obese mice suggested that *ob/ob* mice were deficient in a circulating factor but retained the ability to respond to it by decreasing food intake and body weight when parabiosed to lean or *db/db* mice. In contrast, parabiosis studies with *db/db* mice indicated that although *db/db* mice have adequate circulating levels of this factor, they were unable to respond to it (9–12).

The *ob* gene encodes a 4.5-kb adipose tissue mRNA with a highly conserved, 167-aa open reading frame. The predicted amino acid sequence of the gene product was 84% identical between mouse and human and was consistent with a secreted protein. The mutation identified in the *ob* gene in *ob/ob* mice indicates that these mice do not produce a mature OB protein (also known as leptin) (19). The positional cloning of the

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Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; AP, alkaline phosphatase.

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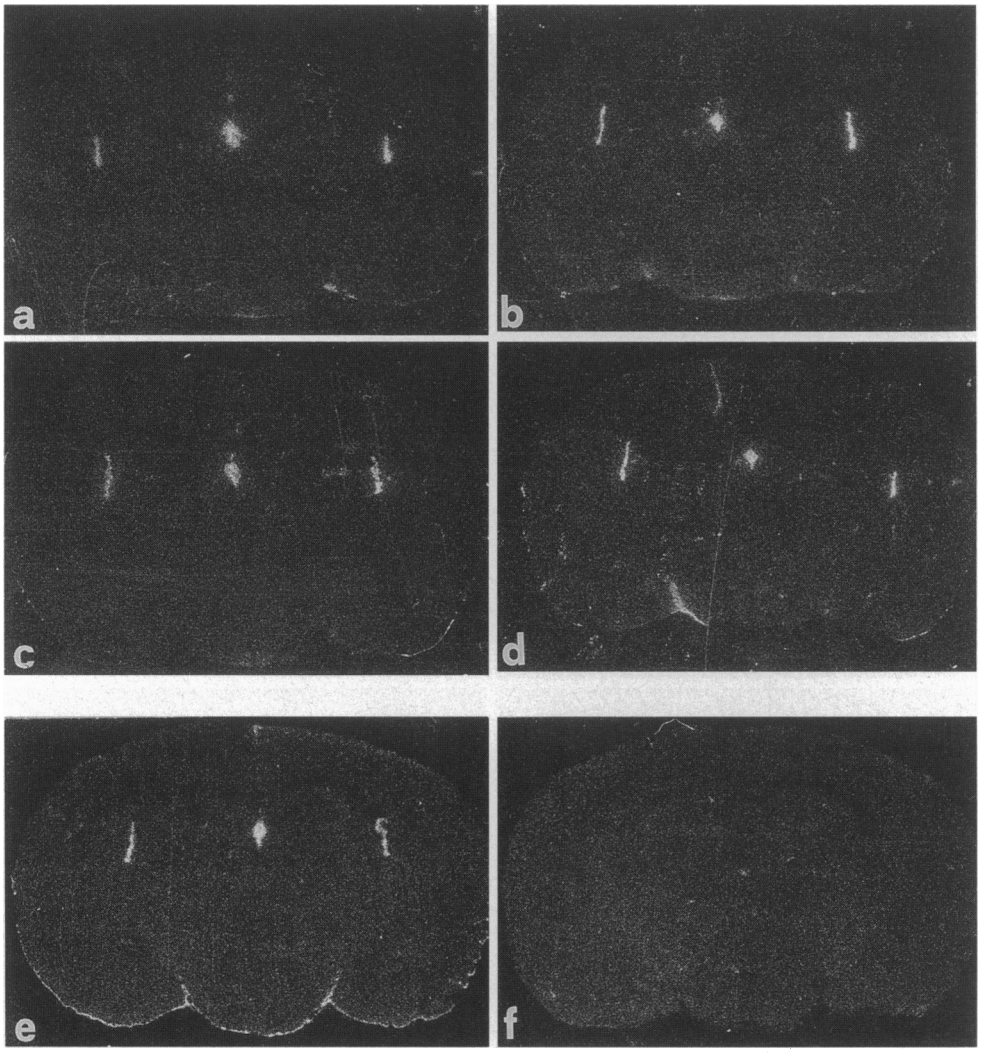

FIG. 1. (*a–d*) Autoradiographic reverse images of brain sections from C57BL/6J lean (*a* and *c*) and *ob/ob* (*b* and *d*) mice after i.v. administration of ^{125}I-labeled recombinant mouse OB protein. Fresh-frozen coronal plane sections (20 μm), including the hypothalamus, were thaw-mounted on glass slides 15 min (*a* and *b*) and 30 min (*c* and *d*) after i.v. injection of ^{125}I-labeled mouse OB protein and exposed to tritium-sensitive film (Amersham) for 2 weeks. (*e* and *f*) Autoradiographic reverse images of ^{125}I-labeled mouse OB protein binding *in vitro* to brain sections of a C57BL/6J mouse (*e*, total binding; *f*, nonspecific binding). Sections were incubated for 90 min at 4°C with 0.5 nM ^{125}I-labeled mouse OB protein in incubation medium (50 mM Tris·HCl/0.2% BSA/0.1 mM phenylmethylsulfonyl fluoride/0.1 phenantroline, pH 7.5). Nonspecific binding was determined by incubation of adjacent sections in the presence of 0.5 μM unlabeled mouse OB protein added to the labeled mouse OB protein in the incubation medium. After two washes with ice-cold incubation medium, the sections were exposed to tritium-sensitive film (Amersham) for 2 weeks. (×6.) Note the dense and displaceable binding of radiolabeled OB protein to the choroid plexus.

mouse *ob* gene and its human homologue has provided the basis to investigate the potential role of OB protein in body weight regulation.

Several groups have recently demonstrated that the administration of recombinant mouse OB protein leads to reduced food intake and body weight of obese *ob/ob* and lean, but not *db/db*, mice (21–26). Our demonstration that mouse OB protein reduced food intake and body-weight gain when administered directly into the lateral ventricles of the brains of obese *ob/ob* and lean mice indicated that OB protein can act directly on one or more brain areas (21). To identify the anatomical locations of these potential central target sites for OB protein, we have conducted binding studies using brain tissue from mice and rats. ^{125}I-labeled recombinant mouse OB protein was used in binding studies on coronal brain sections prepared for autoradiography from lean, obese *ob/ob*, and obese *db/db* mice as well as lean and obese Zucker rats. In companion binding studies, an alkaline phosphatase (AP)-OB fusion protein was used with freshly dissected brain tissues from the same strains of mice and rats. We report here that recombinant OB protein binds specifically with high affinity to the choroid plexus of mice and rats. The binding of OB protein and its displacement by unlabeled OB protein was similar in lean, obese *ob/ob*, and obese *db/db* mice as well as lean and obese Zucker rats. The implications of these results for the identification of OB protein receptors are discussed.

METHODS

Recombinant OB Protein. A biologically active form of authentic recombinant mouse OB protein that does not require denaturation and renaturation was overexpressed in *Escherichia coli* and purified to near homogeneity (21). Recombinant human OB protein was purified from baculovirus supernatants with a monoclonal antibody (2A5) column against human OB protein and was judged by Coomassie blue staining to be >95% pure. The ^{125}I-labeled recombinant mouse and human OB protein [specific activity = 90 μCi/μg (1 Ci = 37 GBq)] was prepared by the Iodogen method as described by the manufacturer (Pierce).

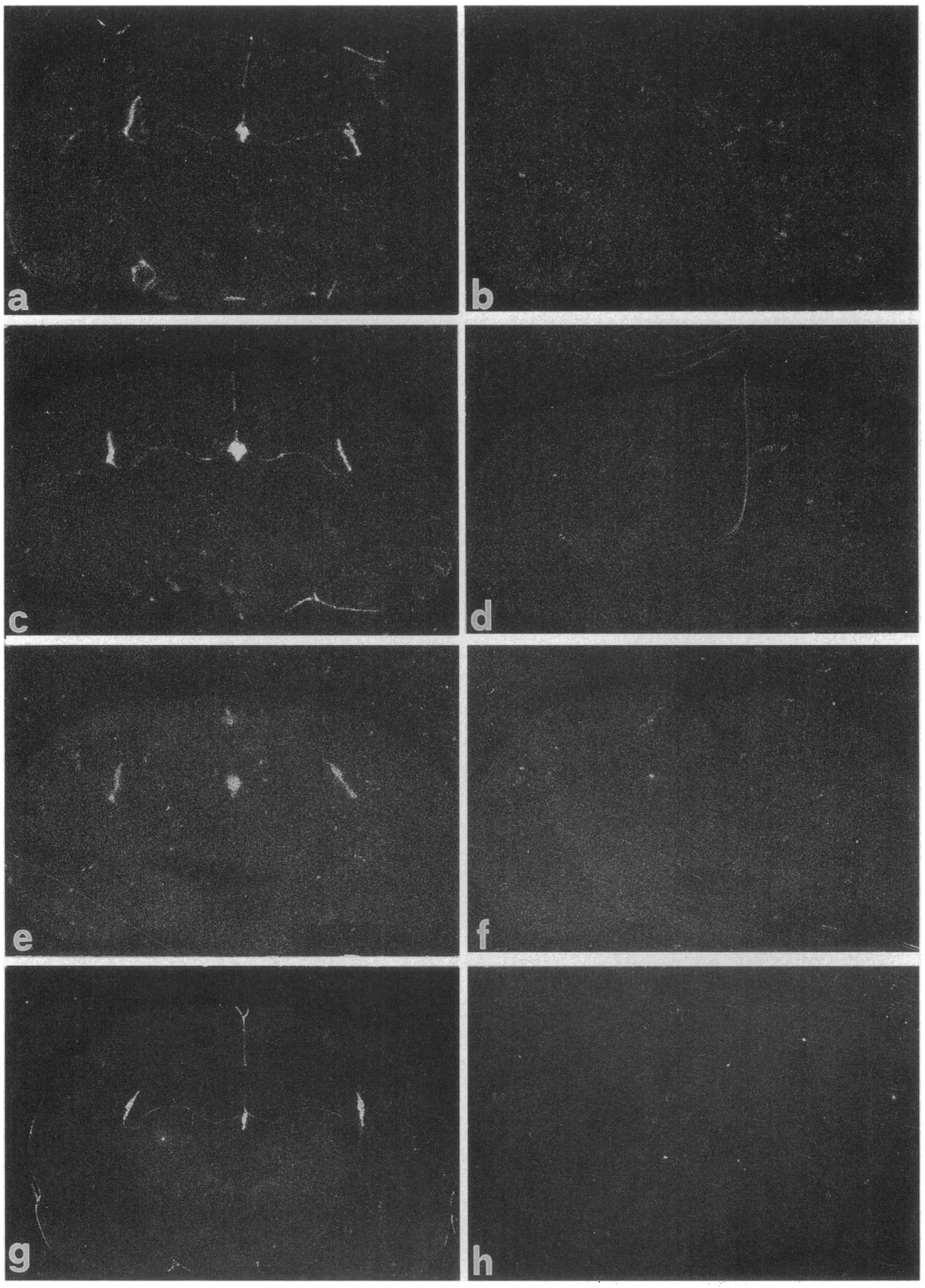

FIG. 2. Autoradiographic reverse images of *in vitro* binding of ^{125}I-labeled mouse OB protein to choroid plexus in coronal brain sections from C57BL/6J lean (*a* and *b*) and *ob/ob* (*c* and *d*) mice, and C57BL/KsJ *db/db* (*e* and *f*) mice, and albino RORO (*g* and *h*) rats. Sections were incubated with ^{125}I-labeled mouse OB protein as described in Fig. 1 *e* and *f*. Total binding, *a*, *c*, *e*, and *g*; nonspecific binding, *b*, *d*, *f*, and *h*. (mouse brains, ×6; rat brains, ×4.) Note the similar dense and displaceable binding of radiolabeled OB protein to the choroid plexus in the three strains of mice and in lean rats.

AP-OB Fusion Protein. To produce the mouse AP-OB fusion construct, an AP fusion vector encoding an AP molecule with its own signal peptide (APtag-3) was first generated by replacing sequences between the *Hin*dIII and *Xho*I sites of APtag-2 (27) with PCR-amplified sequences of secreted placental AP. A *Bgl*II site was placed so that fusions introduced into this site would be in-frame with the AP protein. The sequence encoding the predicted mature form of mouse OB protein was then PCR-amplified from the corresponding cDNA. Restriction sites at the end of the amplification primers were cut with *Bam*HI and *Bgl*II and inserted into the *Bgl*II site of APtag-3.

The plasmid was transiently transfected into COS-7 cells (11.25 μg/150-mm plate). Cells were grown to confluence, and then, after 3 additional days, the media were centrifuged, filtered (0.45-μm pore size), and stored at 4°C with 20 mM Hepes (pH = 7.0) and 0.05% sodium azide. Conditioned media were quantified in a 96-well plate reader as described (28) except that homoarginine was omitted from all assays.

Animals. Lean (+/+) and obese C57BL/6J *ob/ob* mice and obese C57BL/KsJ *db/db* mice were obtained from the Jackson Laboratory. Lean and obese (*fa/fa*) rats were obtained from Charles River Breeding Laboratories, and lean albino RORO rats were obtained from the Roche–Basel colony. Animals were housed with free access to food and water in a 12/12 light/dark cycle.

Autoradiography–*in Vitro*. Coronal brain sections were prepared from free-feeding mice and rats for autoradiography.

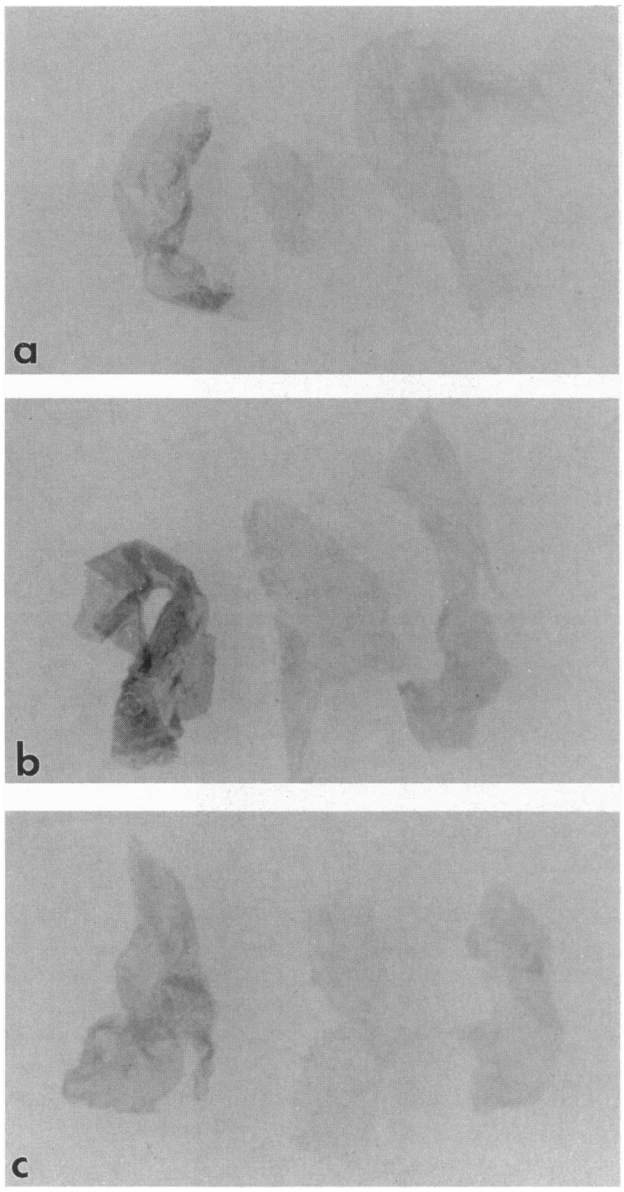

FIG. 3. AP-OB fusion binding to choroid plexus pieces from *ob/ob*, lean, and *db/db* mice. Freshly dissected pieces of choroid plexus from *ob/ob* (*a*), lean (*b*), or *db/db* (*c*) mice were exposed to 2 nM AP-OB fusion protein (*Left*), 2 nM unfused AP (*Center*), or 2 nM AP-OB fusion + 160 nM mouse OB protein (*Right*). Intensity of labeling is proportional to AP-OB fusion protein binding. Note the similar displacement of AP-OB fusion protein labeling by unlabeled OB protein in the choroid plexus in the three strains of mice.

Frozen coronal plane sections of mouse and rat brains, at the level of the hypothalamus, were thaw-mounted on glass slides. Sections were incubated for 90 min at 4°C with 0.5 nM ^{125}I-labeled mouse OB protein in incubation medium containing 50 mM Tris·HCl, 0.2% BSA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM phenantroline at pH 7.5. Nonspecific binding was determined by incubation of adjacent sections in the presence of 0.5 μM unlabeled mouse OB protein added to the labeled OB protein in the incubation medium. After two 5-min washes with cold incubation medium and a dip in distilled water, the sections were exposed together with ^{125}I-labeled plastic standards (Amersham) to Hyperfilm-^{3}H (Amersham) for 2 weeks.

Autoradiography–*in Vivo*. Lean (+/+) and obese *ob/ob* C57BL/6J mice were injected i.v. with a single dose of 1.125 mCi/kg (90 μCi/μg) ^{125}I-labeled recombinant mouse OB protein. After 15 min and 30 min, mice were killed under halothane anesthesia, the brains were removed and frozen, and 12-μm cryostat sections were thaw-mounted on glass slides. Binding was determined as described above.

Binding of AP-OB Fusion Protein to Mouse and Rat Choroid Plexus. Freshly isolated choroid plexi were incubated in tissue culture supernatants containing AP-OB fusion or unfused AP control supernatant for 75 min with gentle rotation at room temperature. Tissues were then treated to detect binding of the AP-OB fusion protein as described (29), except that development with the 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium chloride substrates was discontinued after 20 min.

RESULTS

To identify the anatomical location of the brain target sites for OB protein, *in vivo* and *in vitro* binding studies were conducted using radiolabeled OB protein. In the first series of experiments, ^{125}I-labeled recombinant mouse OB protein was injected i.v. into lean and obese *ob/ob* mice 15 or 30 min before they were killed. In both lean and *ob/ob* mice, radiolabeled OB protein accumulated in the choroid plexus and in the pia mater associated with vascular elements at both time points (Fig. 1 *a–d*). With the experimental design selected, no labeled OB protein was detected in other brain areas, including the hypothalamus, hind brain, and brainstem.

In the second series of experiments, slide-mounted coronal brain sections from lean, obese *ob/ob*, and *db/db* mice were incubated with radiolabeled mouse OB protein in the presence and absence of unlabeled OB protein. Specific, 90% displaceable binding [total binding, at 0.5 nM labeled OB protein, was 56.6 ± 0.01 Bq/mg (mean ± SEM)] was detected in the choroid plexus and the pia mater (associated with vascular elements) of lean mice (Fig. 1 *e* and *f*). Similar labeling of the choroid plexus was observed in brain sections prepared from mice that were exsanguinated before removal of the brain (data not shown). Furthermore, *in vitro* binding studies using brain sections from lean, obese *ob/ob*, and obese *db/db* mice also indicated specific, displaceable binding to choroid plexus and pia mater (Fig. 2 *a–f*). The intensity of labeling by OB protein and the degree of displacement by unlabeled OB protein was similar in lean, obese *ob/ob*, and obese *db/db* mice.

In an independent series of companion experiments, the binding studies using ^{125}I-labeled OB protein were confirmed using an AP-OB fusion protein. In these studies, freshly isolated choroid plexi from adult mouse brains were tested for bound AP activity of the AP-OB fusion protein. Staining of the choroid plexus from lean mice was observed using the mouse AP-OB fusion protein (Fig. 3*b*, *Left*), whereas no staining was observed in surrounding brain tissues (data not shown). In control experiments, using recombinant AP alone, no staining was detected (Fig. 3*b*, *Center*). When recombinant OB protein was included to compete for the binding of the AP-OB fusion protein, complete displacement of the staining was observed (Fig. 3*b*, *Right*). Additional *in vitro* studies using pieces of choroid plexus from obese *ob/ob* and obese *db/db* mice also indicated specific, displaceable binding of AP-OB fusion protein (Fig. 3 *a* and *c*, respectively). The intensity of labeling by OB protein and the degree of displacement by unlabeled OB protein were similar in lean, obese *ob/ob*, and obese *db/db* mice.

In vitro binding experiments were also conducted with radiolabeled OB protein on slide-mounted brain sections from lean and obese Zucker rats. Brain sections from rats were incubated with labeled OB protein in the presence or absence of unlabeled OB protein. Total binding, at 0.5 nM labeled OB protein, was 51.0 ± 0.2 Bq/mg and 57.3 ± 0.2 Bq/mg in the choroid plexus of lean and obese Zucker rats, respectively. Specific, displaceable, high-density binding to the choroid plexus and pia mater associated with vascular elements of lean

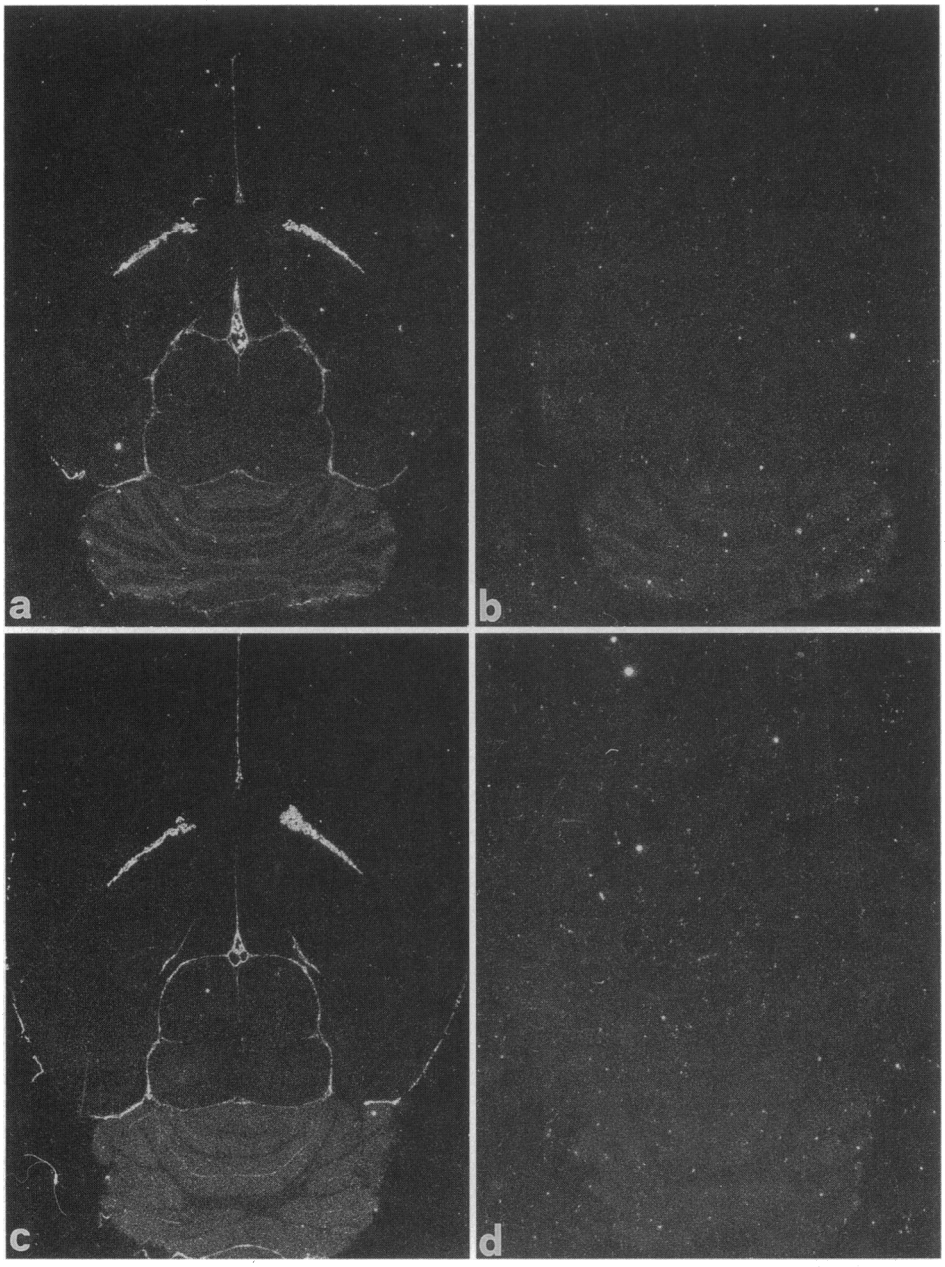

FIG. 4. Autoradiographic reverse images of ^{125}I-labeled human OB protein binding to horizontal sections of lean (*a* and *b*) and obese (*c* and *d*) Zucker rat brains *in vitro*. Sections were incubated with 0.5 nM ^{125}I-labeled human OB protein as described in Fig. 1 *e* and *f*. Total binding, *a* and *c*; nonspecific binding, *b* and *d*. Note the similar dense and displaceable binding of radiolabeled OB protein to the choroid plexus of lean and obese Zucker rats. (×4.)

(Fig. 4 *a* and *b*) and obese (Fig. 4 *c* and *d*) Zucker rats were observed. The presence of low-density, displaceable, but diffuse, binding in the thalamus and cerebellum was suggested in these brain sections from rats (Fig. 4). Total binding, at 0.5 nM labeled OB protein, was 12.3 ± 0.1 Bq/mg and 8.4 ± 0.1 Bq/mg in the cerebellum of lean and obese Zucker rats, respectively. The intensity of labeling by OB protein and the degree of displacement by unlabeled OB protein was similar in lean and obese Zucker rats. When tissues were incubated with the AP-OB fusion protein, similar specific, displaceable binding to the choroid plexus of lean and obese Zucker rats was observed (data not shown).

DISCUSSION

The results of our studies demonstrate that recombinant mouse OB protein binds specifically, with high affinity and high density, to the choroid plexus in lean, obese *ob/ob*, and obese *db/db* mice as well as lean and obese Zucker rats. Displaceable binding was also observed in the pia mater associated with vascular elements. Since choroid plexus binding of OB protein was observed when radiolabeled mouse OB protein was either injected i.v. or incubated with brain sections *in vitro*, these results indicate that circulating OB protein has access to, and can bind specifically to, the choroid plexus in mice and rats. This demonstrates that the choroid plexus is one of the brain target sites for specific OB protein binding and suggests the existence of a specific OB receptor in the choroid plexus. This demonstration does not exclude the existence of other binding sites for OB protein within the brain and peripheral tissues.

The specific, displaceable binding of either radiolabeled OB protein or AP-OB fusion protein to the choroid plexus was similar in lean, obese *ob/ob*, and obese *db/db* mice as well as

lean and obese Zucker (*fa/fa*) rats. Assuming that the *db* gene or its rat homologue, the *fa* gene, encodes the OB receptor, as is widely expected based on parabiosis studies (9–12), no mutations would be expected in the OB protein-binding regions, or regions that modulate binding, in the extracellular domain of the choroid plexus OB receptor in *db/db* mice or obese Zucker (*fa/fa*) rats. These observations do not exclude the possibility that mutations in other regions of the OB receptor (e.g., intracellular domains) may be identified in *db/db* mice or obese Zucker rats.

The identification of the choroid plexus as a binding site for OB protein will enable studies in which choroid plexus will provide a source of tissue from which to construct expression libraries for expression cloning of the choroid plexus OB receptor. Screening of these libraries using displaceable binding of either ^{125}I-labeled OB protein or the AP-OB fusion protein will lead to the cloning of the choroid plexus OB protein receptor.

These findings suggest the hypothesis that OB protein binds to the choroid plexus and is then transported [perhaps by a specific or nonspecific transporter protein (30–33)] across the blood–brain barrier into the cerebrospinal fluid (30). However, since mouse OB protein reduced food intake in lean and *ob/ob*, but not *db/db*, mice when administered directly into the cerebrospinal fluid in the lateral ventricle (21), which is functionally and anatomically behind the choroid plexus (30, 34), this indicates that the defect in *db/db* mice may reside distal to the choroid plexus between the cerebrospinal fluid and neural tissues. This interpretation is supported by our binding results, which suggest that the lack of response to administered recombinant OB protein in *db/db* mice cannot be explained by the lack of specific, displaceable binding to the choroid plexus. Thus, a second form of OB receptor might be found distal to the choroid plexus. This hypothesis may also be consistent with the recent demonstration of competitive binding of radiolabeled recombinant mouse OB protein to hypothalamic membranes prepared from lean rats (26). Although in our studies specific binding in the hypothalamus was not detected in the mouse, our autoradiographic studies in rats suggest the presence of low-density, displaceable, but diffuse, binding in the thalamus and cerebellum. Further studies using higher concentrations of OB protein [based on the reported K_d of 46 nM (26)] may be required to document the presence of a corresponding specific binding site in brain sections.

An alternate hypothesis that is also consistent with our results is that binding of recombinant OB protein to the choroid plexus could lead to the activation of afferent neural inputs to the neural network that regulates feeding behavior and energy balance. This hypothesis would require that OB protein placed in the lateral ventricle, as well as the circulation, could interact with the choroid plexus and, thus, activate the same neural network in lean and *ob/ob* mice but fail to activate it in *db/db* mice. A third possibility is that the choroid plexus binding site for OB protein is involved in the clearance or degradation of OB protein as appears to be the case for the abundant choroid plexus binding sites for insulin (30).

The experimental testing of these hypotheses and the localization of the downstream defect in *db/db* mice will require further research. A necessary first step will be to clone the OB receptor from the choroid plexus. The characterization of the choroid plexus OB receptor will then facilitate studies aimed at identification and localization of other OB protein-binding proteins and OB receptors and the signaling pathways associated with them. These studies will also aid in the elucidation of the neural pathways and networks and the underlying molecular mechanisms by which OB protein exerts its potent influence on feeding behavior and energy balance.

Note Added in Proof. As suggested in the *Discussion* section, the identification of the choroid plexus as a specific binding site for OB protein provided the basis for the recent expression cloning of the OB receptor (35). The binding results reported above are also consistent with the recent identification of a mutation of the intracellular domain of the OB receptor in obese *db/db* mice that are unresponsive to OB protein (36).

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