TRAF1 may inhibit antigen-induced apoptosis in a transgenic animal model (*21*). Also, NF-κB can regulate c-IAP2, but this protein is unable, on its own, to inhibit TNF-mediated apoptosis under NF-κB–null conditions but rather provides survival function through activating NF-κB (*18*). IAPs can inhibit cytochrome c–induced caspase activity and the proteolytic processing of caspase-3 (*17*). Consistent with this, c-IAP1 and c-IAP2 expression alone blocked etoposide-induced processing of caspase-3 and apoptosis in HT1080I cells. However, although cytochrome c release and caspase-3 activation occurred in TNF-mediated apoptosis in NF-κB–inhibited cells, the overexpression of c-IAP1 and c-IAP2 was insufficient to inhibit TNF-induced processing of caspase-3 and to render cells resistant to apoptosis. Thus, TNF and etoposide may have different caspase requirements to efficiently kill cells, which supports our conclusion that the recruitment of c-IAP1 and c-IAP2 to the receptor complex in response to TNF, presumably through interactions with TRAF1 or TRAF2, is required to inhibit the apical caspase, caspase-8. However, it is also possible that TNF-induced killing is somehow stronger than that induced by etoposide and requires inhibition at the apex of the cell death pathway. These observations underscore the importance of the activation of TRAF1 and TRAF2 as well as c-IAP proteins in suppressing TNF-induced cell death. The fact that most cells survive a TNF-α challenge supports the hypothesis that a rapid defense mechanism induced by the activation of NF-κB is required to block death signaling at the initiating and not at the executing stage of apoptosis because inhibiting the latter response may only delay cell death (*8, 14*).

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Biological Action of Leptin as an Angiogenic Factor

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Leptin is a hormone that regulates food intake, and its receptor (OB-Rb) is expressed primarily in the hypothalamus. Here, it is shown that OB-Rb is also expressed in human vasculature and in primary cultures of human endothelial cells. In vitro and in vivo assays revealed that leptin has angiogenic activity. In vivo, leptin induced neovascularization in corneas from normal rats but not in corneas from *fa/fa* Zucker rats, which lack functional leptin receptors. These observations indicate that the vascular endothelium is a target for leptin and suggest a physiological mechanism whereby leptin-induced angiogenesis may facilitate increased energy expenditure.

Leptin, a circulating hormone secreted by adipocytes, influences body weight homeostasis through effects on food intake and energy expenditure (*1*). It also modulates other physiological actions, including lipid metabolism, hematopoiesis, pancreatic β cell function, ovarian function, and thermogenesis (*2*). Despite this multiplicity of biological effects in extraneural tissues, the leptin receptor is expressed predominantly in the hypothalamus (*3*). Alternative splicing of a single transcript encoded by the *db* gene produces several variants of the leptin receptor, including a transmembrane full-length, long form (OB-Rb) expressed at high levels in discrete hypothalamic regions (*4*). The OB-Rb form has a cytoplasmic domain that transduces the leptin signal through the Jak-STAT pathway (*5, 6*).

The discovery of leptin and its receptor strongly supports the hypothesis that adipose tissue mass is regulated by a hormone that is

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produced by adipocytes and released into the bloodstream (*7*). This endocrine concept of white adipose tissue implies a plastic microvascular bed that not only provides adequate blood supply for this endocrine function or for proper lipid utilization and storage but also undergoes the requisite adaptive changes that occur during physiological or pathological fluctuations in adiposity (*8*).

We hypothesized that leptin might play an important physiological role in the microvasculature. To test this hypothesis, we first determined whether the leptin receptor is expressed in human umbilical vein endothelial cells (HUVECs) using confocal immunofluorescence microscopy and rabbit polyclonal antibodies to synthetic peptides based on the sequence of the human leptin receptor (*9*). With antibodies specific for the intracellular domain (or region) of the OB-Rb form of the receptor (anti–OB-R_{int}) (*9*), a strong signal was detected (Fig. 1A, panel 1). This signal is characterized by a scattered, punctuate, intracellular staining, suggesting that the bulk of OB-Rb resides in a vesicular compartment. In contrast, an intense perinuclear staining was seen (Fig. 1A, panels 2 and 3) when antibodies to extracellular epitopes of the receptor were used (anti–OB-R_{ext}) (*9*). The same pattern was found in endothelial cells (ECs) from other sources, including microvascular and aortic bovine ECs and human adipose or dermal microvascular ECs (*10*). In immunoblots prepared with total HUVEC extracts, a single >200-kD protein band was detected with anti–OB-R_{int} (Fig. 1B, lane 1). This species was also seen with anti–OB-R_{ext}, but in this case an additional 170-kD band was observed (Fig. 1B, lane 2). To demonstrate the immunological specificity of the antibodies used, we studied frozen sections of adipose tissue from *db/db* leptin receptor–deficient mice or *db/+* heterozygous normal littermates. A strong immunostaining was seen in the vascular endothelial lining of *db/+* mice but not in that of *db/db* mice (Fig. 1C). Finally, an intense immunostaining was observed in vascular structures of human dermis and adipose tissue when anti–OB-R_{int} was used (Fig. 1D) (*11*). Thus, the OB-Rb long form of the receptor is expressed in ECs. This expression was confirmed by reverse transcriptase polymerase chain reaction analysis of HUVEC RNA with OB-Rb–specific primers (*10*). The presence of the leptin receptor in ECs suggests that the endothelium may be a target for leptin action.

To determine whether the leptin receptor in ECs is functional, we evaluated its signaling properties. First, we found that treatment of HUVECs with leptin markedly stimulates tyrosine phosphorylation of OB-Rb (Fig. 2A) (*12*). Second, we examined the ability of leptin to induce tyrosine phosphorylation of the transcription factor Stat3 (*5*, *6*). Treatment of HUVECs with leptin rapidly stimulated Stat3 phosphorylation, as demonstrated by immunoblotting of cellular extracts with antibodies specific for the tyrosine-phosphorylated form of Stat3 (Fig. 2B) (*13*). Finally, we determined whether leptin-induced Stat3 tyrosine phosphorylation enhances its DNA-binding activity. Electrophoretic mobility gel shift assays performed with nuclear extracts from HUVECs treated with leptin [or interferon-γ (IFN-γ) as a positive control] revealed increased formation of a DNA-protein complex with the use of a Stat3-binding probe (Fig. 2C) (*14*, *15*). Thus, the endothelial leptin receptor is functionally competent with respect to ligand-induced tyrosine phosphorylation and activation of Stat3.

We next investigated whether leptin has angiogenic activity. First, in vitro experiments were performed. In a modified Boyden chamber assay (*16*), cultured HUVECs exhibited a robust directional migration in response to leptin treatment with an apparent half-maximal concentration of about 4 nM (Fig. 3A). Vascular endothelial growth factor (VEGF) (*17*) was used as a positive control in this assay. Leptin also promoted the formation of capillary-like tubes in three-dimensional (3D) collagen gels containing HUVECs (*18*, *19*) (Fig. 3B). In contrast to the control (Fig. 3B, panels 1 and 2), exposure of HUVECs to VEGF (Fig. 3B, panels 3 and 4) or to leptin (Fig. 3B, panels 5 and 6) induced formation of elongated, bifurcating tubules that pervaded the gel matrix. The tubes formed in the presence of leptin displayed a reticular array reminiscent of tissue microvasculature (Fig. 3B, panels 5 and 6). Finally,

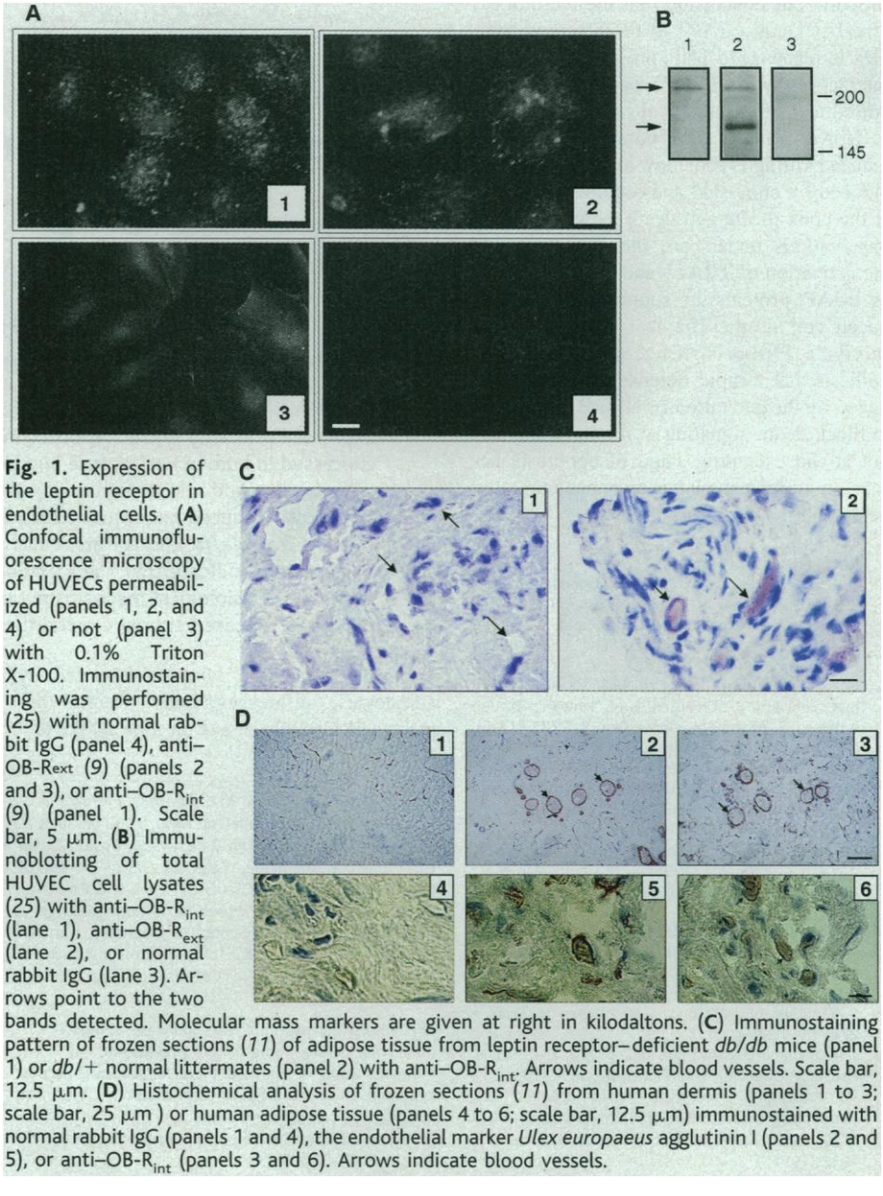

Fig. 1. Expression of the leptin receptor in endothelial cells. (**A**) Confocal immunofluorescence microscopy of HUVECs permeabilized (panels 1, 2, and 4) or not (panel 3) with 0.1% Triton X-100. Immunostaining was performed (*25*) with normal rabbit IgG (panel 4), anti–OB-Rext (*9*) (panels 2 and 3), or anti–OB-R_{int} (*9*) (panel 1). Scale bar, 5 μm. (**B**) Immunoblotting of total HUVEC cell lysates (*25*) with anti–OB-R_{int} (lane 1), anti–OB-R_{ext} (lane 2), or normal rabbit IgG (lane 3). Arrows point to the two bands detected. Molecular mass markers are given at right in kilodaltons. (**C**) Immunostaining pattern of frozen sections (*11*) of adipose tissue from leptin receptor–deficient *db/db* mice (panel 1) or *db/+* normal littermates (panel 2) with anti–OB-R_{int}. Arrows indicate blood vessels. Scale bar, 12.5 μm. (**D**) Histochemical analysis of frozen sections (*11*) from human dermis (panels 1 to 3; scale bar, 25 μm) or human adipose tissue (panels 4 to 6; scale bar, 12.5 μm) immunostained with normal rabbit IgG (panels 1 and 4), the endothelial marker *Ulex europaeus* agglutinin I (panels 2 and 5), or anti–OB-R_{int} (panels 3 and 6). Arrows indicate blood vessels.

proliferation assays with several types of human and bovine ECs (from microvascular and large vessel origin) exhibited variable mitogenic activity in response to leptin (*10*).

To test leptin's angiogenic activity in vivo, we surgically implanted Hydron polymer pellets containing phosphate-buffered saline (PBS), VEGF, or leptin into the corneas of normal or leptin receptor–deficient *fa/fa* Zucker fatty rats and monitored neovascularization (*20*). In normal rats, leptin caused a vigorous angiogenic response (Fig. 4A), whereas in *fa/fa* rats angiogenesis was seen only with VEGF; leptin had no effect in these animals (Fig. 4B).

The direct angiogenic action of leptin suggests a peripheral mechanism whereby the increase in energy expenditure produced by leptin (which together with the hypothalamus-mediated satiety effect contributes to body weight loss) may be facilitated. By providing a local angiogenic signal, leptin might improve the efficiency of lipid release from fat stores to maintain energy homeostasis. An artificially induced hyperleptinemic state in normal rats causes increased lipolysis and lipid oxidation (*21*), accompanied by augmented expression of genes encoding enzymes that regulate fatty acid metabolism and thermogenesis, including UCP2 (*22*). Similar observations have been made in lean C57BL/6J mice after systemic administration of leptin (*23*). In this case, increased lipid oxidation seems to coincide with an increase in adipose tissue vascularity (*23*). Thus, leptin produced in adipocytes is not only secreted into the bloodstream, but it may also act locally upon ECs in a paracrine fashion, causing increased fatty acid oxidation and an angiogenic response that maintains an appropriate balance between blood supply and fat depot size. In addition, leptin-induced angiogenesis may assist in heat dissipation at sites of active thermogenesis in the body, including adipose tissue. Our observations suggest that leptin, acting as a functional link between adipocytes and the vasculature, might also play an important extrahypothalamic role in the modulation of adipose tissue mass.

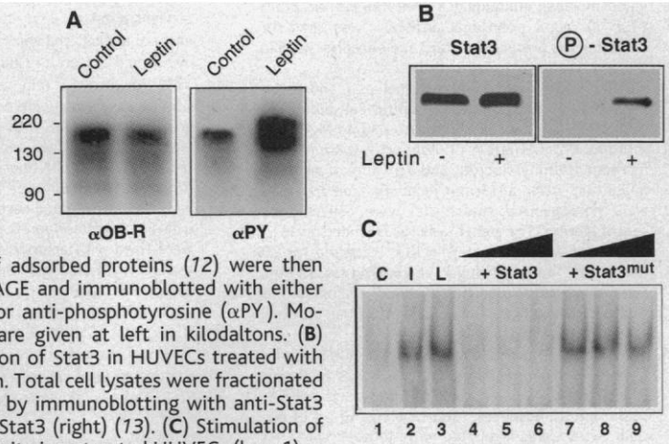

Fig. 2. Leptin signaling in endothelial cells. (**A**) Tyrosine phosphorylation of leptin receptor in HUVECs treated with 120 nM leptin for 5 min. Total soluble extracts were prepared and adsorbed to a leptin-affinity matrix (*12*). Equivalent amounts of adsorbed proteins (*12*) were then fractionated by SDS-PAGE and immunoblotted with either anti–OB-R_{int} (αOB-R) or anti-phosphotyrosine (αPY). Molecular mass markers are given at left in kilodaltons. (**B**) Tyrosine phosphorylation of Stat3 in HUVECs treated with 50 nM leptin for 15 min. Total cell lysates were fractionated by SDS-PAGE followed by immunoblotting with anti-Stat3 (left) or anti–phospho-Stat3 (right) (*13*). (**C**) Stimulation of Stat3 DNA-binding activity in untreated HUVECs (lane 1) or HUVECs treated with interferon-γ (IFN-γ) (30 U/ml) (lane 2) or 5 nM leptin (lane 3) for 10 min. Nuclear extracts (*14*) were incubated with a ^{32}P-labeled Stat3-binding oligonucleotide probe (Santa Cruz Biotechnology) (*15*). The resulting DNA-protein complexes were then separated by nondenaturing gel electrophoresis and detected by autoradiography (*15*). In competition reactions, leptin-treated cell extracts were incubated with unlabeled Stat3 probe (lanes 4 to 6) or a Stat3^{mut} probe (lanes 7 to 9) that cannot bind DNA (*15*).

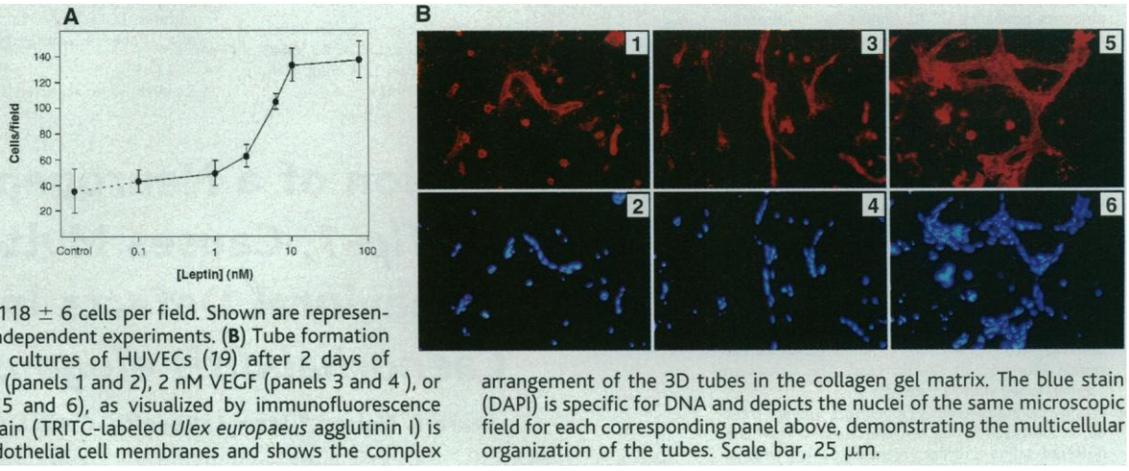

Fig. 3. In vitro angiogenic activity of leptin. (**A**) Directional migration of HUVECs as determined by the number of cells moving across a porous membrane in a Boyden chamber in response to leptin (*16*). Under these conditions, addition of 1 nM VEGF caused a migration response of 118 ± 6 cells per field. Shown are representative data from four independent experiments. (**B**) Tube formation in Type I collagen gel cultures of HUVECs (*19*) after 2 days of treatment with vehicle (panels 1 and 2), 2 nM VEGF (panels 3 and 4), or 10 nM leptin (panels 5 and 6), as visualized by immunofluorescence microscopy. The red stain (TRITC-labeled *Ulex europaeus* agglutinin I) is specific for human endothelial cell membranes and shows the complex arrangement of the 3D tubes in the collagen gel matrix. The blue stain (DAPI) is specific for DNA and depicts the nuclei of the same microscopic field for each corresponding panel above, demonstrating the multicellular organization of the tubes. Scale bar, 25 μm.

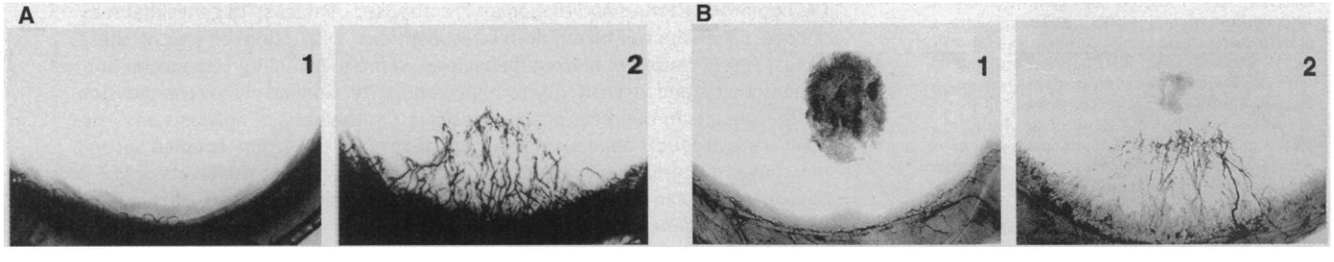

Fig. 4. In vivo angiogenic activity of leptin. (**A**) Corneal response (*20*) 7 days after implantation of a Hydron pellet containing PBS (panel 1) or 50 ng of leptin (panel 2). (**B**) Lack of corneal response in leptin receptor–deficient *fa/fa* Zucker fatty rats 7 days after implantation of a Hydron pellet containing 50 ng of leptin (panel 1). A strong positive response with 25 ng of VEGF in *fa/fa* rats is shown for comparison (panel 2).

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9. Peptides based on the sequence of the human leptin receptor (*4*) corresponding to regions within the intracellular or the extracellular domain were synthesized and coupled to keyhole limpet hemocyanin (KLH). The intracellular region peptides were (i) IC-1, for residues 1148 to 1165 at the COOH-terminal end of the receptor (CSTQTHKIMENKMCDLTV), and (ii) IC-2, for residues 1062 to 1078 (KLEGNFPEENNDKKSIY) (*24*). The extracellular region peptides were (i) EC-1, for residues 247 to 263 (ITDDGNLKISWSSPPLV), (ii) EC-2, for residues 473 to 487 (CSDIPSIHPISEPKD), and (iii) EC-3, for residues 753 to 767 (CVIVSWILSPSDYKL) (*24*). The KLH-peptide conjugates were used to generate polyclonal antibodies in rabbits and immunoglobulin (IgG) fractions prepared from bleeds with the highest enzyme-linked immunosorbent assay titers. Unless otherwise indicated, antibodies to IC-1 and IC-2 were combined in equal amounts, producing OB-R_{int} antibodies to intracellular epitopes of the leptin receptor's OB-Rb form. Likewise, equal amounts of antibodies to EC-1, EC-2, and EC-3 were mixed, producing OB-R_{ext} antibodies to extracellular epitopes of the leptin receptor.
10. M. R. Sierra-Honigmann *et al.*, data not shown.
11. Tissue specimens of human abdominal skin and subcutaneous fat were collected during cosmetic surgery. Subcutaneous adipose tissue specimens were also obtained from adult, female, leptin receptor–deficient *db/db* mice or normal *db/+* littermates. After delipidation, the tissue was flash-frozen and cut into 5-μm-thick sections. Sections were stained with anti–OB-R_{int}, *Ulex europaeus* agglutinin I, or normal rabbit serum. After incubation with primary antibodies, tissue sections were developed with secondary horseradish peroxidase–conjugated goat antibody to rabbit IgG with a Vectastain Elite ABC kit (Vector Labs). The slides were counterstained with hematoxylin.
12. Freshly isolated HUVECs (subculture 1) were treated as indicated in Fig. 2A and washed with tris-buffered saline and homogenized on ice with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors, and insoluble material was collected by centrifugation at 12,000*g* for 10 min at 4°C. Equal amounts of protein were precleared by incubation with Affi-gel-15 (Bio-Rad) beads for 2 hours at 4°C, and the resin was removed by centrifugation. Lysates were incubated for 6 hours at 4°C with 40 μl of leptin-Affigel beads [containing 30 μg of covalently coupled human recombinant leptin per milliliter of packed Affigel-15 resin; coupling was conducted following the manufacturer's instructions (Bio-Rad)]. Adsorbed complexes were washed with RIPA buffer and boiled with SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 10 min. Proteins were separated by SDS-PAGE with 4 to 20% gradient gels, followed by overnight transfer to nitrocellulose membranes. Immunoblots were developed with anti–OB-R_{int} (*9*) or with RC20 recombinant anti-phosphotyrosine (Transduction Laboratories, Lexington, KY).
13. Confluent monolayers of HUVECs grown in T75 flasks were washed with PBS twice, and total cell extracts were prepared by adding 1 ml of SDS-PAGE sample buffer supplemented with 100 μM dithiothreitol (DTT). Lysed cells were briefly sonicated, and insoluble material was removed by centrifugation. Supernates were collected, boiled for 3 min, and applied to a 4 to 20% gradient gel. Gels were processed for immunoblotting as described (*25*). The PhosphoPlus Stat3 (Tyr^{705}) Antibody kit (New England Biolabs), containing a phospho-specific Stat3 (Tyr^{705}) rabbit polyclonal antibody, was used for detecting the presence of Stat3 and phospho-Stat3 in the cell lysates.
14. HUVECs were washed with PBS, pelleted, and resuspended into a minimal volume of low-salt buffer [20 mM Hepes (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors]. After a 15-min incubation on ice, cells were homogenized, and nuclei were collected by centrifugation. The pellet was resuspended in low-salt buffer adjusted to 0.5 M KCl, extracted for 30 min at 4°C, and centrifuged at 25,000*g* for 30 min. Supernates were collected, dialyzed against buffer made 0.1 M with KCl, and divided into small portions kept at −80°C until used.
15. The double-stranded oligonucleotide Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA), was first end-labeled with T4 polynucleotide kinase and [γ-^{32}P]-adenosine triphosphate. After labeling, 10 μg of nuclear extract was incubated with 100,000 cpm (<1 pmol) of ^{32}P-labeled probe for 20 min at room temperature in binding buffer [20 mM tris-HCl (pH 7.9), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, bovine serum albumin (50 μg/ml), and polydeoxyinosine/polydeoxycytosine (2 μg/ml)]. Electrophoresis sample buffer was added to each sample before separation in a nondenaturing gel (89 mM tris, 89 mM boric acid, 10 mM EDTA, and 4.95% acrylamide). Gels were dried and exposed to autoradiography.
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Disruption of a Neuropeptide Gene, *flp-1*, Causes Multiple Behavioral Defects in *Caenorhabditis elegans*

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Neuropeptides serve as important signaling molecules in the nervous system. The FMRFamide (Phe-Met-Arg-Phe-amide)–related neuropeptide gene family in the nematode *Caenorhabditis elegans* is composed of at least 18 genes that may encode 53 distinct FMRFamide-related peptides. Disruption of one of these genes, *flp-1*, causes numerous behavioral defects, including uncoordination, hyperactivity, and insensitivity to high osmolarity. Conversely, overexpression of *flp-1* results in the reciprocal phenotypes. On the basis of epistasis analysis, *flp-1* gene products appear to signal upstream of a G protein–coupled second messenger system. These results demonstrate that varying the levels of FLP-1 neuropeptides can profoundly affect behavior and that members of this large neuropeptide gene family are not functionally redundant in *C. elegans*.

FMRFamide-related neuropeptides (FaRPs) represent a large family of peptides that have been implicated as neurotransmitters or neuromodulators in many invertebrate and vertebrate behaviors, including muscular control (*1*), cardioregulation (*2*), pain modulation (*3*),