Leptin Increases Axonal Growth Cone Size in Developing Mouse Cortical Neurons by Convergent Signals Inactivating Glycogen Synthase Kinase-3β

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We examined the effects of the adipocyte hormone leptin on the development of mouse cortical neurons. Treatment of neonatal and adult mice with intraperitoneal leptin (5 mg/kg) induced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in pyriform and entorhinal cortex neurons. Stimulation of cultured embryonic cortical neurons with leptin evoked Janus kinase 2 and ERK1/2 phosphorylation and activated the downstream effector 90-kDa ribosomal protein S6 kinase. Moreover, leptin elicited the phosphorylation of the phosphatidylinositol 3-kinase (PI3K) pathway (3, 4). Leptin-mediated GSK3β phosphorylation was prevented by the MEK/ERK inhibitor PD98059, the phosphatidylinositol 3-kinase inhibitor LY294002, or the protein kinase C inhibitor GF109203X. Exposure of cortical neurons to leptin also induced Ser-41 phosphorylation of the neuronal growth-associated protein GAP-43, an effect prevented by LY294002 and GF109203X but not by PD98059. Ser-41-GAP-43 phosphorylation is usually high in expanding axonal growth cones. Neurons exposed to 100 ng/ml leptin for 72 h displayed reduced rate of growth cone collapse, a shift of growth cone size distribution toward higher values, and a 4-fold increase in mean growth cone surface area compared with control cultures. The leptin-induced growth cone spreading was hampered in cortical neurons from Leprdb/db mice lacking functional leptin receptors; it was associated with localized Ser-9-GSK3β phosphorylation and mimicked by the GSK3β inhibitor SB216763. At concentrations preventing GSK3β phosphorylation, PD98059, LY294002, or GF109203X reversed the leptin-induced growth cone surface enlargement. We concluded that the leptin-mediated regulation of growth cone morphogenesis in cortical neurons relies on upstream regulators of GSK3β activity.

The adipocyte-derived hormone leptin acts as satiety signal in hypothalamic nuclei to regulate energy homeostasis (1, 2). Mice lacking leptin (Leprdb/db mice) display obesity and several associated abnormalities (1). Excluding very rare cases of humans with genetic obesity, obese human subjects have high circulating leptin levels and hypothalamic insensitivity to leptin (1).

Five leptin receptors (LEPRs)2 in the mouse have been identified, including long (LEPRb) and short isoforms (LEPRa and LEPRγc). LEPRb, which is ineffective in Leprdb/db mice, phosphorylates Janus kinase 2 (JAK2), which in turn phosphorylates LEPRb tyrosine residues to mediate downstream signaling (3, 4). Recruitment of the signal transducer and activator of transcription-3 (STAT3) is broadly considered a molecular signature of hypothalamic leptin signaling and drives subsequent induction of genes, including that of the feedback inhibitor, suppressor of cytokine signaling-3 (SOCS3). LEPRb stimulation can also activate the extracellular signal-regulated kinase (ERK) signaling in the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (3, 4).

The existence of LEPRs in extrahypothalamic brain areas, including neocortex, entorhinal, and pyriform cortex and hippocampus (5, 6), suggests that leptin, besides controlling food intake, might affect cognitive abilities. The hormone actually facilitates memory mechanisms in mice (7). The finding of circulating leptin levels in fetal life (8) and the 5–10-fold increase of serum leptin levels observed in neonatal mice when compared with the <10 ng/ml found in adult mice (9) first suggested that leptin might intervene as a modulatory factor during development. LEPRs are also expressed in diverse areas of the developing brain (10–12). Leprdb/db mice show decreased levels of synaptic proteins, which are partially restored by postnatal leptin treatment, indicating that leptin deficiency might interfere with neuronal maturation (13). Recent papers show that leptin modulates axonal growth and synaptic plasticity within the hypothalamus (14, 15). In particular, leptin increases neurite extension in the arcuate nucleus during mouse perinatal development, thus playing an early trophic role within those circuits that will be the target of leptin physiological actions in adult life (14). However, these studies did not clarify whether the trophic effects of leptin are directly exerted on arcuate nucleus neurons, nor did the studies investigate the intracellular signals activated by leptin in the developmental and plastic changes described so far. Additional ques-

2 The abbreviations used are: LEPR, leptin receptor; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription-3; SOCS3, suppressor of cytokine signaling-3; RSK, ribosomal protein S6 kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; GSK3β, glycogen synthase kinase-3β; PBS, phosphate-buffered saline; E15, embryonic day 15; DIV, days in vitro; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAP-43, growth-associated protein-43; LTP, long-term potentiation; Biotin, 2-biotin(2-hydroxyethyl)amine-2-hydroxymethylpropane-1,3-diol; TRITC, tetramethylrhodamine isothiocyanate; MEK, MAPK/ERK kinase; P, postnatal day; E, embryonic day.

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§ The online version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

** The on-line version of this article contains supplemental Fig. 1.
tions consider the possible trophic actions of leptin on extrapyramidal circuits.

We investigated the role of leptin in the development of mouse cerebral cortex neurons, and we discovered that the hormone acts as a trophic factor in the elaboration of cortical growth cones by modulating MEK/ERK, PI3K/Akt, and PKC signaling pathways that converge in the inhibition of glycolgen synthase kinase-3β (GSK3β).

MATERIALS AND METHODS

In Vivo Leptin Administration and Immunohistochemistry—Lepr<sup>db/db</sup> male mice or wild type C57BL/Ks congeners (Harlan, S. Pietro al Natisone, Italy) were treated with a single intraperitoneal injection of recombinant mouse leptin (National Hormone and Peptide Program, Torrance, CA) or vehicle at a dose of 5 mg/kg body weight at postnatal days (P) 5, 10, 15, 20, and 25 or at adult age (3 months). Forty five min later, mice were anesthetized with 100 mg/kg ketamine (Ketavet; Farmacia Sponsa, Italy) and 10 mg/kg xylazine (Rompum; Bayer AG, Leverkusen, Germany) and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Brains were quickly removed, post-fixed in the same fixative for 36 h, then cut in 40-μm coronal sections on a vibratome, collected as a series, and kept in PB until further use. The first section of each series was Nissl-stained to recognize structures according to Ref. 16. Free floating sections were washed in phosphate-buffered saline (PBS, which was used for all subsequent washes), quenched in 1% H<sub>2</sub>O<sub>2</sub> for 30 min at room temperature, washed, and blocked in 3% normal horse serum (Vector Laboratories, Burlingame, CA) in PBS for 1 h at room temperature. Sections were incubated overnight at 4 °C with a mouse monoclonal antibody recognizing diphosphorylated ERK1/2 (1:1000 dilution, Sigma), washed, and then incubated in 1:200 biotinylated IgG anti-mouse horse serum (Vector Laboratories) in PBS for 30 min at room temperature. After washing and incubation in avidin-biotin-peroxidase reagent (Vector Laboratories) for 1 h at room temperature, signals were revealed with diaminobenzidine (Sigma). Finally, sections were mounted on gelatin-coated slices, air-dried, cleared with xylene, and coverslipped with Entellan®.

Phospho-STAT3 immunohistochemistry was performed as described (17) using the anti-phospho-STAT3 (Tyr-705) rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000. Negative controls were obtained by using preimmune instead of primary antiserum. Brightness and contrast of the final images (TIFF files) were adjusted using the Photoshop 6 software (Adobe Systems, Mountain View, CA).

Neuronal Cultures and Treatments—Primary cortical neurons were prepared as described by Brewer et al. (18) with minor modifications. Embryonic day 15 (E15) cortices were isolated from CD1 mouse brains (Charles River Breeding Laboratories, Calco, Italy), pooled, mechanically dissociated into a single-cell suspension in Neurobasal medium (Invitrogen) containing 2% B27 supplement (Invitrogen), 500 μg/ml glutamine (Euroclone, Pero, Italy), 100 units/ml penicillin, and 100 μg/ml streptomycin (Euroclone) (N-B27 medium), and centrifuged for 5 min at 200 × g. Cells were plated onto poly-D-lysine (Sigma)-coated glass coverslips or dishes (2.5–7.5 × 10<sup>4</sup> cells/cm<sup>2</sup>) and cultured in N-B27 medium for diverse days in vitro (DIV). Cultures contained >99% neurons, as assessed by MAP2 immunostaining (data not shown). Cells were treated with murine recombinant leptin (National Hormone and Peptide Program), PD98059 (Calbiochem), GF109203X (Calbiochem), LY294002 (Cell Signaling Technology), or SB216763 (Tocris, Avonmouth, UK), for times and concentrations indicated in the text or figure legends. Phosphorylation of signaling molecules was assayed on cells maintained in B27-free culture medium 5 h before exposure to test molecules; signaling experiments at DIV 4 (not shown) and DIV 8 gave superimposable results.

Neuronal Cultures from Lepr<sup>db/db</sup> Mice and Genotyping for the Lepr<sup>db/db</sup> Mutation—Pregnant female mice heterozygous for the Lepr<sup>db/db</sup> mutation (Lepr<sup>db/−−</sup>), mated to Lepr<sup>db/−</sup> male breeder mice, were purchased from Harlan (Oxon, UK). Cortices were isolated at E15 from several embryos of two litters and individually dissociated. Cortical neurons from each embryo were plated separately and fed as described above with N-B27 medium. We applied a PCR restriction fragment length polymorphism method for identifying the embryonal Lepr<sup>db/db</sup> genotype as described (19). Briefly, cerebellar tissue from each embryo was digested in the presence of proteinase K (Sigma) overnight at 55 °C with agitation. DNA extracted by standard procedures served as a template in a PCR using primers and cycling conditions described previously (19). The PCR products were digested in the presence of AccI restriction enzyme (Promega, Madison, WI) at 37 °C for 4 h. Digests were analyzed in 3.5% agarose gel (Sigma) containing ethidium bromide (0.5 μg/ml). Digestion with AccI yielded 85- and 24-bp fragments in Lepr<sup>−/−</sup> mice, 58-, 27-, and 24-bp fragments in Lepr<sup>db/−</sup> mice, and 85-, 58-, 27-, and 24-bp fragments in the heterozygote Lepr<sup>db/−−</sup> mice. We failed to observe any gross difference between neuronal cultures obtained from Lepr<sup>−/−</sup> and Lepr<sup>db/db</sup> mice in terms of cell number or in vitro differentiation properties in NB-27 medium (data not shown).

Reverse Transcription–PCR—Total RNA (1.5 μg) was extracted with an RNeasy kit (Qiagen, Hilden, Germany) and reverse-transcribed in the presence of oligo(dT) (18) (Ambion, Austin, TX) and 200 units of Superscript II Reverse Transcriptase (Invitrogen) for 50 min at 42 °C. Transcripts were amplified with Taq DNA polymerase (Promega) in 50 μl of standard buffer containing 50 pmol of LEPRb primers or 12.5 pmol of primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For LEPRb PCR, 32 cycles were run at 94, 58, and 72 °C, 45 s each, with primers described by Hoggard et al. (10) (amplified fragment, 534 bp); for GAPDH, 26 cycles were run as above with primers described by Wurmback et al. (20) (amplified fragment, 199 bp). Primers were from MWG Biotech AG, Ebersberg, Germany. PCR fragments were separated by electrophoresis on 1.2% agarose gel and visualized by ethidium bromide staining. Quantitation was performed by densitometric scanning using a computerized image system (Gel-Pro Analyzer, Media Cybernetics, Silver Spring, MD). Omission of the reverse transcriptase step to provide negative PCR controls gave no amplified bands (data not shown).

Western Immunoblots—Protein extracts were obtained from neuronal cultures, quantified, and processed for Western blot analysis as described by Valerio et al. (21). Denatured protein samples (25–50 μg each) were electrophoresed onto 4–12% NuPAGE BisTris Pre-Cast Gels (Invitrogen) and electroblotted to polyvinylidene difluoride membranes (Amersham Biosciences). Filters were blocked 1 h in 5% nonfat dry milk in TBS-T (0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween 20) and incubated overnight at 4 °C with primary antibodies (described below). After washing, membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Biosciences), and then immunodetection was performed by the enhanced chemiluminescence procedure (ECL plus kit; Amersham Biosciences). Primary antibodies were as follows: anti-leptin receptor, long form, rabbit polyclonal antibody (1:100 dilution; Linco Research, St. Charles, MO); anti-JAK2 rabbit polyclonal antibody (1:1000; Cell Signaling Technology); anti-phospho-JAK2 (Tyr-1007/Tyr-1008) rabbit polyclonal antibody (1:1000; BIOSOURCE); anti-STAT1 rabbit polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-STAT1 (Tyr-701) rabbit polyclonal antibody (1:1000; Cell Signaling Technology); anti-phospho-JAK2 (Tyr-1007/Tyr-1008) rabbit polyclonal antibody (1:1000; BIOSOURCE); anti-STAT1 rabbit polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-STAT1 (Tyr-701) rabbit polyclonal antibody (1:1000; Cell Signaling Technology); anti-phospho-JAK2 (Tyr-1007/Tyr-1008) rabbit polyclonal antibody (1:1000; BIOSOURCE); anti-STAT1 rabbit polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-STAT1 (Tyr-701) rabbit polyclonal antibody (1:1000; Cell Signaling Technology); anti-phospho-JAK2 (Tyr-1007/Tyr-1008) rabbit polyclonal antibody (1:1000; BIOSOURCE); anti-STAT1 rabbit polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-STAT1 (Tyr-701) rabbit polyclonal
antibody (1:1000; Upstate Biotechnology, Inc., Lake Placid, NY); anti-STAT3, anti-phospho-STAT3 (either Tyr-705 or Ser-727), anti-ERK44/42, anti-phospho-ERK44/42 (Thr-202/Tyr-204), anti-RSK, anti-phospho-p90RSK (Thr-359/Ser-363), anti-Akt, anti-phospho-Akt (Ser-473), anti-GSK3β, anti-phospho-GSK3β (Ser-9) (all rabbit polyclonal antibodies, used at 1:1000 dilution; Cell Signaling Technology); rabbit polyclonal anti-SOCS3 antibody (1:250; Abcam, Cambridge, UK); mouse monoclonal anti-α-tubulin antibody (1:20,000; Sigma); anti-GAP-43 antibody (clone 7D4/1, 1:3000) and anti-phospho-GAP-43 antibody (clone 2G12/C7, 1:3000) were the gifts of K. F. Meiri, Boston (22). After the visualization of phosphoproteins, filters were stripped with the ReBlot Western blot recycling kit (Chemicon International, Temecula, CA) and further used for the visualization of total proteins (phosphorylated and not phosphorylated). Quantification of at least three immunoblots with protein extracts from separate cell preparations was performed by densitometric scanning of exposed film using Gel-Pro Analyzer software.

Immunofluorescence and Image Analysis—Neurons grown on coverslips were fixed for 20 min at room temperature in 4% paraformaldehyde, 4% sucrose in 0.1 M PB, and permeabilized with 0.1% Triton X-100. The following primary antibodies were applied 1 h at room temperature: mouse monoclonal anti-GAP-43 (1:100; Chemicon International); mouse monoclonal anti-α-tubulin (1:1000; Sigma); rabbit monoclonal anti-GSK3β (1:50; Cell Signaling Technology); goat polyclonal anti-phospho-GSK3β (Ser-9, 1:200; Santa Cruz Biotechnology). Cells were then incubated in Cy3-, TRITC-, and/or Cy2-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) at room temperature for 1 h. When applied, staining with Oregon Green 488 phalloidin (1:15; Molecular Probes, Eugene, OR) was performed for 1 h at the end of immunofluorescence procedure. Coverslips were mounted in FluorSave (Calbiochem). Fluorescence was acquired using an Olympus IX51 microscope equipped with a digital camera (Olympus, Hamburg, Germany). Leptin-mediated morphological changes were quantified on axonal growth cones, defined as GAP-43-positive fan-shaped structures at the neuritic tip, with a width at least three times greater than their neurite of origin. Growth cone collapse was scored in club-shaped growth cones displaying complete loss of lamellipodia and filopodia. In each coverslip 50 cortical neurons were analyzed at ×100 magnification. At least three separate experiments conducted in triplicate were performed. Growth cone area and phospho-GSK3β/GSK3β immunostaining intensity ratio were calculated using the Image ProPlus software (Media Cybernetics).

Statistical Analysis—Statistical analyses were performed by one-way analysis of variance followed by t test as post-hoc analysis. Data are presented as means ± S.E.

RESULTS

In Vivo Studies of Leptin Signaling—We acutely treated mice with leptin at various postnatal ages and evaluated the levels of phosphorylated STAT3 in diverse brain areas. Because we did not found any appreciable difference in leptin-driven signals at the examined ages, only the data at P20 will be showed. In untreated mice, grouped cells showed a weak nuclear phospho-STAT3 immunoreactivity in the hypothalamic arcuate nucleus (Fig. 1A). As expected, intraperitoneal leptin injection (5 mg/kg) increased phospho-STAT3 immunoreactivity in the hypothalamus of neonatal and adult mice. In particular, the arcuate, ventromedial, and dorsomedial nuclei contained numerous strongly stained cells (Fig. 1C). The increase in phospho-STAT3 immunostaining was not appreciable in Leprdb/db mice (data not shown). In contrast to mouse hypothalamus, intraperitoneal leptin injection did not affect STAT3 phosphorylation in mouse cerebral cortex at any serial level. As an example, the absence of phospho-STAT3 immunoreactivity is showed in pyriform cortex of untreated (Fig. 1B) and leptin-treated mice (Fig. 1D).
The activation of ERK1/2 in the pyriform cortex was evaluated in untreated mice at various postnatal ages. In basal conditions, phospho-ERK1/2 immunostaining was better appreciated in the nuclei of the layer II pyramidal cells at P10, when the peak of endogenous leptin occurs (9) (supplemental Fig. 1). The basal phospho-ERK1/2 signal lowered during postnatal development and was faintly detectable from P25 thereafter, consistently with the low serum leptin levels (9) (supplemental Fig. 1). At P20, pale phospho-ERK1/2 immunostaining was detectable in the mouse pyriform cortex of untreated mice (Fig. 2A), mostly in the nuclei of the layer II pyramidal cells (Fig. 2B). After leptin administration, the pyriform cortex showed an increase in the number of phospho-ERK1/2-positive cells (Fig. 2C). Layer II pyramidal cell nuclei were more intensely stained and their somata and projections strongly labeled (Fig. 2D). The entorhinal cortex, usually found negative in the controls, showed signs of phospho-ERK1/2 activation after leptin treatment (data not shown). Leptin administration in Leprdb/db mice did not induce phospho-ERK1/2 staining either in the pyriform (Fig. 2, A and B) or in the entorhinal cortex (data not shown). Moreover, intraperitoneal leptin injection failed to activate ERK1/2 in the neocortex at any examined developmental age (data not shown).

Leptin Increases MKK/ERK and PI3K/AKT/GSK3β Cascades in Mouse Cortical Neurons—We found that primary neurons isolated from E15 mouse embryonic cortices express the LEPRb isoform in a developmentally regulated manner (Fig. 3, A and B). LEPRb mRNA was clearly detectable at DIV 1 and increased during in vitro neuronal maturation from DIV 1 up to DIV 12 (Fig. 3A). LEPRb protein was found at DIV 1 and gradually increased during in vitro differentiation of cortical neurons, peaking around DIV 8 (Fig. 3B). Neuronal cultures were acutely stimulated with leptin to verify the possible activation of JAK2. A time-dependent increase in JAK2 phosphorylation was rapidly observed. Phospho-JAK2 levels were significantly induced at 2 min and peaked 5 min after the addition of 100 ng/ml leptin to cortical neurons (Fig. 3C).

To examine possible leptin-mediated effects on the STAT pathway, neuronal cultures were treated with increasing doses of leptin for 5 or 30 min. Up to a 500 ng/ml concentration of leptin did not induce STAT3 phosphorylation either at Tyr-705 or Ser-727 (Fig. 4A). Although the role of other STAT proteins in physiological leptin signaling is not recognized, the hormone activates STAT1 in adipose tissue (3). Despite this, leptin at different concentrations and incubation times failed to phosphorylate STAT1 in cortical neurons (Fig. 4A). Accordingly, leptin (100 ng/ml) treatment of cortical neurons up to 24 h was unable to induce the expression of the SOCS3 protein (Fig. 4B), which is induced by leptin in a STAT3-dependent manner in the hypothalamus (4).

Cortical neurons were exposed to leptin to assess the activation of ERKs. A dose-dependent increase in both ERK1 and ERK2 phosphorylation was observed with maximal effect at 100 ng/ml leptin (Fig. 5A).
Leptin-mediated ERK1/2 phosphorylation was rapid and transient, with the strongest increase observed 2 min after the stimulus (Fig. 5B). Lower levels of phosphorylated ERKs were detectable up to 30 min after the leptin stimulus (2.12 ± 0.32- and 2.41 ± 0.26-fold increase over control conditions for ERK1 and ERK2, respectively). Exposure of cultures to 10 μM PD98059, a pharmacological inhibitor of MEK in the ERK/MAPK pathway (23), totally prevented leptin-induced ERK1/2 phosphorylation (Fig. 5C). Treatment with the PI3K inhibitor LY294002 (10 μM) (24) did not modify leptin-induced ERK1/2 phosphorylation (Fig. 5C). Leptin has been reported to trigger PKC activation and elicit PKC-dependent ERK1/2 phosphorylation in peripheral cells (25). Application of the PKC inhibitor GF109203X (1 μM) (26) did not significantly affect leptin-mediated rise in phospho-ERK1/2 in cortical neurons (Fig. 5C). Phosphorylation of ERKs involves regulation of downstream targets, including ribosomal protein S6 kinases (RSKs) (27). Incubation of cortical neurons with leptin resulted in a rapid and significant increase of p90RSK phosphorylation, with maximal induction at 2 min and smaller inductions detectable up to 15 min of leptin exposure (Fig. 5D).

A number of molecules, including protein kinase B/Akt, have been established as downstream mediators of PI3K activity. Leptin (100 ng/ml) induced a rapid increase of Akt phosphorylation in cortical neurons, which peaked at 5 min and remained elevated for at least 15 min (Fig. 6A). Ten μM LY294002, a PI3K inhibitor acting upstream of Akt (24), completely blocked leptin-induced Akt phosphorylation (data not shown). GSK3β is known to be a target of PI3K/Akt-mediated phosphorylation at Ser-9 (28). Leptin (100 ng/ml) increased Ser-9 GSK3β phosphorylation, with significant effects observed at 5 min and maximal stimulation occurring at 15 min of treatment (Fig. 6B). Concomitant exposure to LY294002 (10 μM) totally prevented leptin-mediated GSK3β phosphorylation (Fig. 6C). GSK3β phosphorylation at Ser-9 can also occur via PKC (29) or p90RSK (30). Accordingly, leptin-induced GSK3β phosphorylation was prevented by the PKC inhibitor GF109203X (1 μM) and reduced by the MEK inhibitor PD98059 (10 μM) (Fig. 6C).

Leptin Stimulates GAP-43 Expression and Phosphorylation in Mouse Cortical Neurons—In cultured neurons, LEPRb is concentrated at axonal growth cones where it colocalizes with GAP-43 (31), a presynaptic protein maximally expressed during neural development, that modulates growth cone formation (32–34). Western immunoblotting using anti-GAP-43 antibody showed that prolonged treatment with leptin increased GAP-43 protein levels in mouse cortical neurons (Fig. 7A). Growth cone behavior is affected by the phosphorylation status of GAP-43 on Ser-41, a well characterized target of PKC-mediated phosphorylation (35, 36). Exposure of cortical neurons to 100 ng/ml leptin for 2 h promoted a significant increase of Ser-41-GAP-43 phosphorylation (Fig. 7B), the effect being prevented by application of 1 μM GF109203X to block PKC activity (Fig. 7B). The leptin-mediated increase in phospho-Ser-41-GAP-43 levels was unaffected by 10 μM PD98059, but it was reduced in the presence of 10 μM LY294002 (Fig. 7B), possibly due to the capability of leptin to activate atypical PKC isoforms via PI3K inhibition (37). GSK3β activity is inhibited via phosphorylation on a Ser-9 residue (38). Given the observed leptin-mediated increase in phospho-Ser-9-GSK3β levels, we investigated whether GAP-43 phosphorylation is modulated by GSK3β inactivation. Application of the selective pharmacological GSK3β inhibitor SB216763 (10 μM) (39) did not modify GAP-43 phosphorylation on Ser-41 (Fig. 7C).
Leptin Locally Inactivates GSK3β and Induces Growth Cone Expansion in Mouse Cortical Neurons—We then exposed DIV 1 in vitro-differentiating cortical neurons to 100 ng/ml leptin for 72 h and examined its effects on axonal growth cones by immunofluorescence with anti-GAP-43 antibody (Fig. 8A). Double labeling with anti-α-tubulin antibody and phalloidin was also used to define microtubules at the growth cone central domain and actin cytoskeleton (lamellipodia and filopodia) at the growth cone peripheral domain, respectively (40) (Fig. 8A). The addition of leptin to cortical neurons induced an increase in growth cone surface area, and a large proportion of growth cones exhibited the morphology of large paused growth cones, with loops of unbundled microtubules and extended lamellipodial veils and filopodial protrusions. The typical appearances of growth cones in control and leptin-treated cultures are shown in Fig. 8A. The leptin-mediated effect was quantified by analyzing the frequency distribution of growth cone areas (Fig. 8B). In untreated cultures, 24.8 ± 8.1% of growth cones underwent collapse and neuritic retraction, whereas leptin reduced this proportion to 11.3 ± 3.9%. A 4-fold raise in average growth cone extension was observed in leptin-treated cultures compared with that of untreated cultures from wild type Lepr+/mice (Fig. 8C). On the contrary, exposure to 100 ng/ml leptin did not evoke any change in the average growth cone size of cortical neurons obtained from Leprdb/db mice (Fig. 8C).
investigated the contribution of different signaling pathways to the leptin-mediated growth cone spreading. Coexposure of cortical neurons to either 10 \( \mu \text{M} \) PD98059, 1 \( \mu \text{M} \) GF109203X, or 10 \( \mu \text{M} \) LY294002 reduced the leptin-mediated enlargement of growth cone size (Fig. 8D), suggesting that both MEK and PI3K-related pathways as well as PKC-dependent signaling processes participate in the leptin-mediated axon morphogenetic effects. Similar to what was observed after leptin exposure, treatment with the selective GSK3\( \beta \) inhibitor SB216763 (10 \( \mu \text{M} \)) increased mean growth cone size (Fig. 8E). To determine whether GSK3\( \beta \) phosphorylation is locally affected at the growth cone by leptin treatment, we double-labeled neurons with an antibody against phospho-Ser-9-GSK3\( \beta \) and another against total GSK3\( \beta \). The ratio of phospho-GSK3\( \beta \)/GSK3\( \beta \) in the leptin-treated axonal growth cones was significantly higher than in control cultures (Fig. 9).

**DISCUSSION**

It has been proposed that the leptin surge occurring in the perinatal period (9) promotes the maturation of hypothalamic circuits, which will be later functionally responsive to the hormone itself (14). Given the observation that leptin facilitates LTP, a form of synaptic plasticity at the basis of learning and memory (7), we hypothesized that the hormone could regulate axon morphogenesis also in brain areas involved in cognition. We found that developing mouse cortical neurons express LEPR\( \beta \), which gradually increases during the process of in vitro neuronal maturation. Exposure of cortical neurons to leptin activated JAK2, which is essential for the phosphorylation of LEPR\( \beta \) itself required for leptin regulation of energy balance in the hypothalamus (4). Treatment of mice with intraperitoneal leptin was able to activate the STAT3 pathway, which is regarded as a master leptin-mediated intracellular signal, being required for leptin regulation of energy balance in the hypothalamus (4). Treatment of mice with intraperitoneal leptin was able to activate the STAT3 pathway, which is regarded as a master leptin-mediated intracellular signal, being required for leptin regulation of energy balance in the hypothalamus (4). Treatment of mice with intraperitoneal leptin was able to activate the STAT3 pathway, which is regarded as a master leptin-mediated intracellular signal, being required for leptin regulation of energy balance in the hypothalamus (4). Treatment of mice with intraperitoneal leptin was able to activate the STAT3 pathway, which is regarded as a master leptin-mediated intracellular signal, being required for leptin regulation of energy balance in the hypothalamus (4). Treatment of mice with intraperitoneal leptin was able to activate the STAT3 pathway, which is regarded as a master leptin-mediated intracellular signal, being required for leptin regulation of energy balance in the hypothalamus (4). Treatment of mice with intraperitoneal leptin was able to activate the STAT3 pathway, which is regarded as a master leptin-mediated intracellular signal, being required for leptin regulation of energy balance in the hypothalamus (4).
the PI3K cascade in cultured cortical neurons as revealed by Akt phosphorylation. Signaling through ERKs and PI3K is typical of neurotrophins and leads to various and partially overlapping trophic effects on neurons, ranging from neuronal differentiation and plasticity to survival (41). ERK and PI3K pathways have been implicated in the leptin-mediated enhancement of LTP (7). Both signaling systems participate in the control of axon outgrowth (42). Finally, we found that leptin induces the Ser-9 phosphorylation of GSK3β, and in particular, it enhanced the phospho-Ser-9-GSK3β signal in the axonal growth cone. This serine/threonine kinase is normally active in cells and is primarily regulated through inhibition of its activity (38). Thus, leptin produces localized inactivation of the GSK3β kinase. Inactivation of GSK3β through Ser-9 phosphorylation is regarded as a point of convergence of diverse trophic signals, possibly being mediated by p90RSK in the MEK/ERK pathway (30), PI3K/Akt (28), or PKC (29). Accordingly, we observed that the MEK, PI3K, and PKC inhibitors PD98059, GF109203X, or LY294002, respectively, reduced the leptin-mediated GSK3β phosphorylation.

Strikingly, we found that by 72 h after leptin exposure the number of collapsing growth cones was decreased. Axonal growth cones became on average about four times larger than those of untreated controls and showed extended filopodia and lamellipodia and increased microtubule looping. As our cultures are highly purified, it is most likely that leptin exerts morphoregulatory effects by acting directly on developing cortical neurons. The leptin-mediated effect on growth cones was mediated through LEPRβ and was observed at concentrations compatible with those measured in mice plasma during the leptin surge occurring in early postnatal life (9). Through the use of specific inhibitors, we demonstrated that leptin effects on growth cone morphogenesis are mediated by the MEK/ERK1/2 and the PI3K pathways. Furthermore, we observed the involvement of PKC in the growth cone enlargement evoked by leptin. Axonal growth cone spreading has been related to GSK3β inactivation (43, 44), which occurs through Ser-9 phosphorylation (38). Lithium chloride, which inhibits GSK3β activity, mimics the effects of Wnt factors that induce the unbundling and looping of microtubules and augment growth cone size (43, 45). We observed that the selective GSK3β inhibitor SB216763 reproduces the leptin-mediated growth cone spreading. Thus, our data suggest that multiple leptin-driven signaling cascades converge at GSK3β inactivation to induce growth cone enlargement.

Axonal morphogenesis implies cytoskeletal rearrangements leading to growth cone extension, which involve growth-associated proteins interacting with actin microfilaments or microtubules (40). The actin-binding protein GAP-43 is necessary for growth cone spreading (32), the phenomenon being associated with increased GAP-43 phosphorylation at Ser-41, which depends on PKC activation (32, 35, 36). We found that leptin induced a significant increase of Ser-41 GAP-43 phosphorylation in a PKC- and PI3K-dependent manner and that Ser-41-GAP-43 phosphorylation was not affected by GSK3β inhibition. Clarification of the possible mechanism(s) by which GSK3β inactivation and GAP-43 phosphorylation cooperate to modulate growth cone maturation requires further investigation. It is worth noting that phosphorylated GAP-43 is a key protein in the genesis of LTP (34, 46). Intriguingly, in the neocortex of Lepdb/db and Lepd/db mice, elevated GAP-43 protein levels are found (13). By describing reduced LTP in Leprdb/db mice, Li et al. (47) speculated that the defective leptin signaling could damage learning mechanisms by causing an abnormal GAP-43 storage, possibly attributable to a disruption of GAP-43 phosphorylation. Our finding that leptin induces GAP-43 phosphorylation corroborates this hypothesis.

Aged obese subjects have impaired cognition (48) and are predisposed to temporal lobe cerebral atrophy (49) and dementia (50, 51). Because the obese, leptin-resistant Leprdb/db mice show degenerating cortical cells (13) and display poor performances in spatial memory tasks (47), it is intriguing to speculate that cognitive deficits in obese individuals are a consequence of leptin resistance in extrahypothalamic brain areas. It has been established that spatial memory consolidation requires ERK activity in entorhinal cortex (52), a cerebral area that is affected by neurodegeneration in preclinical Alzheimer disease (53). We found that the leptin-mediated increase in ERK1/2 phosphorylation is lost in Leprdb/db mice pyriform and entorhinal cortex. On the other side, olfactory memory, which involves pyriform cortex and is impaired in dementia patients (54), has not been investigated in Leprdb/db mice yet. Noticeably, GSK3 is emerging as a prominent drug target, and GSK3 inhibitors are being considered as therapeutics for various medical conditions, including neurodegenerative diseases (55, 56). Further studies are needed to elucidate the role of leptin in healthy and diseased aged brain and to assess the possible use of this hormone as a drug in the obesity-related neurodegenerative disorders.

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