Leptin-mediated Cell Survival Signaling in Hippocampal Neurons Mediated by JAK STAT3 and Mitochondrial Stabilization

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Leptin plays a pivotal role in the regulation of energy homeostasis and metabolism, primarily by acting on neurons in the hypothalamus that control food intake. However, leptin receptors are more widely expressed in the brain suggesting additional, as yet unknown, functions of leptin. Here we show that both embryonic and adult hippocampal neurons express leptin receptors coupled to activation of STAT3 and phosphatidylinositol 3-kinase-Akt signaling pathways. Leptin protects hippocampal neurons against cell death induced by neurotrophic factor withdrawal and excitotoxic and oxidative insults. The neuroprotective effect of leptin is antagonized by the JAK2-STAT3 inhibitor AG-490, STAT3 decoy DNA, and phosphatidylinositol 3-kinase/Akt inhibitors but not by an inhibitor of MAPK. Leptin induces the production of manganese superoxide dismutase and the anti-apoptotic protein Bcl-xL, and stabilizes mitochondrial membrane potential and lessens mitochondrial oxidative stress. Leptin receptor-deficient mice (db/db mice) are more vulnerable to seizure-induced hippocampal damage, and intraventricular administration of leptin protects neurons against seizures. By enhancing mitochondrial resistance to apoptosis and excitotoxicity, our findings suggest that leptin signaling serves a neurotrophic function in the developing and adult hippocampus.

Leptin is a 16-kDa protein produced primarily by adipose cells from which it is released into the circulation and transported across the blood-brain barrier. Leptin regulates food intake and energy homeostasis by activating receptors on neurons in the hypothalamus. Mice defective in leptin production are phenotypically hyperphagic and obese (1, 2). Leptin also serves functions apart from those related to food intake and energy expenditure in mammals, including regulation of fertility (3–5), immune responses (6, 7), and bone formation (8–10). Leptin signaling is mediated by the leptin receptor (ObRb),2 which is a member of the class I cytokine receptor superfamily. Leptin binding to Ob-Rb results in activation of the tyrosine kinase JAK-2, which in turn phosphorylates tyrosine residues on the intracellular domain of the receptor, providing a binding site for STAT3. The activated STAT3 proteins dimerize and translocate to the nucleus, where they regulate transcription of various genes (11).

Beyond regulating energy homeostasis and neuroendocrine functions by acting on neuronal targets in the hypothalamus, leptin may have more widespread actions in the brain. Leptin receptors are expressed in the hippocampus, cerebral cortex, and other regions of the adult rodent brain (12, 13), but the function of leptin in these brain regions is unknown. However, it was reported that leptin can modulate the excitability of hippocampal neurons by activating potassium channels (14) and that leptin receptor-deficient rodents have impaired spatial learning ability (15), suggesting that leptin signaling may influence neuronal excitability and synaptic plasticity. Here we show that hippocampal neurons express leptin receptors coupled to the JAK/STAT and PI 3-kinase Akt signaling pathways. Activation of these pathways by leptin promotes neuronal survival by a mechanism involving increased production of the antioxidant enzyme Mn-SOD and the anti-apoptotic protein Bcl-xL. Hippocampal neurons in leptin receptor-deficient mice are more vulnerable to seizure-induced death, and administration of leptin to normal mice protects hippocampal neurons against seizures.

EXPERIMENTAL PROCEDURES

Hippocampal Cultures, Experimental Treatments, and Quantification of Neuronal Survival—Hippocampi were removed from embryonic day 18 Sprague-Dawley rats, and cells were plated in 35- or 60-mm diameter plastic dishes or glass coverslips on a polyethyleneimine substrate in minimum essential medium with Earle’s salts supplemented 10% with heat-activated fetal bovine serum and containing 1 mM L-glutamine, 1 mM pyruvate, 20 mM KCl, and 26 mM sodium bicarbonate (pH 7.2). Following cell attachment, the culture medium was replaced with Neurobasal Medium containing B27 supplements (Invitrogen), 2 mM L-glutamine, 1 mM HEPES, and 0.001% gentamicin sulfate. All experiments were performed...
in 8–10-day-old-cultures. Leptin (PeproTech), glutamate (Sigma), and FeSO₄ (Sigma) were prepared as 200–500× stocks in sterile water. AG490 (ICN), wortmannin (Sigma), PD98059, and LY294002 (Cell Signaling Technology) were prepared as 500× stocks in dimethyl sulfoxide. STAT3 decoy DNA and control DNA were purchased from IDT; the following sequences were the same as described previously (16): STAT3 decoy DNA, 5’-CATTTCCGTAAATC-3’, and 3’-GTA-AAGGGCATTAG-5’; control DNA, 5’-CATATCCCTTA-AATC-3’, and 3’-GTATAGGGAATTAG-5’. Just before treatment, the medium was changed to Neurobasal Medium lacking B27 supplements. Withdrawal of trophic factor support was accomplished by replacing the culture maintenance medium with Locke’s buffer as described previously (17). Neuronal survival was quantified by counting viable neurons in pre-mixed microscope fields (10× objective) before experimental treatment and at different time points after treatment. Viability of neurons was established by morphological criteria. Neurons with intact neurites of uniform diameter and a soma with a smooth appearance were considered viable, whereas neurons with fragmented neurites and a vacuolated and/or swollen soma were considered nonviable. Counts were made in at least four separate cultures per treatment condition.

Assessment of Mitochondrial Membrane Potential and Reactive Oxygen Species—Cells were incubated for 30 min in the presence of 100 nM TMRE dye (Molecular Probes) at 37 °C and were then washed twice in Locke’s buffer. The dye dihydroorhodamine 123 (DHR) was used to quantify relative levels of mitochondrial reactive oxygen species (this dye is particularly sensitive to oxidation by peroxynitrite and hydroxyl radical) using methods described previously (18). Images of cellular TMRE or DHR fluorescence were acquired using a Zeiss LSM 510 confocal microscope with excitation at 488 nm and emission at 510 nm. The average pixel intensity in user-defined areas corresponding to neuronal cell bodies was determined using Zeiss software. All images were coded and analyzed without knowledge of experimental treatment history of the cultures.

Immunocytochemistry and Immunoblot Analyses—The methods were similar to those described previously (19). Briefly, cells were fixed in 4% paraformaldehyde, incubated for 5 min in a solution of 0.2% Triton X-100 in PBS, and placed in blocking solution (5% goat serum in PBS). Cells were then exposed to the primary ObRb antibody (1:3000 dilution; Chemicon) overnight at 4 °C, followed by incubation for 1 h with biotinylated goat anti-rabbit secondary antibody (1:200) and 30 min in the presence of ABC reagent (Vector Laboratories). The immunoreactive product was detected using peroxidase-labeled avidin and diaminobenzidine. For immunoblot analysis, equal amounts of solubilized proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated in a blocking solution containing 5% nonfat milk at 4 °C overnight. The membrane was then incubated for 2 h at room temperature with one of the following primary antibodies: anti-β-tubulin II (1:1000; Zymed Laboratories Inc.); anti-Bcl-xL (1:1000; Upstate Biotechnology); anti-Mn-SOD (1:2000; Upstate Biotechnology); anti-phosphorylated STAT3 (1:1000; Cell Signaling); anti-STAT3 (1:1000; Cell Signaling); phosphorylated Akt (1:1000; Cell Signaling); anti-β-actin (1:5000; Sigma); and anti-GLUT3 (1:2000; Chemicon). The blot was then exposed for 1 h to horseradish peroxidase-conjugated secondary antibody (1:3000; Jackson ImmunoResearch), and immunoreactive protein was visualized using a chemiluminescence-based detection kit according to the manufacturer’s protocol (Amersham Biosciences).

Mice, Kainate Treatment, and Quantification of Neuronal Survival—Male leptin receptor-deficient mice (db/db mice) and C57BL/6 control mice were purchased from The Jackson Laboratories. Three-month-old mice were anesthetized with isoflurane, and kainate (0.1 μg in a volume of 0.5 μl) was administered unilaterally via stereotaxic injection into the dorsal aspect of the right hippocampus (D/V, −1.4; M/L, +2.4; A/P, −2.0). Mice were euthanized 24 h after kainate injection and perfused transcardially with saline followed by cold buffered 4% paraformaldehyde. Coronal brain sections were cut at 30 μm on a freezing microtome and stained with cresyl violet. Nissl-positive surviving neurons were counted in three 40× fields in regions CA1 and CA3 of the kainate-treated hippocampus in six adjacent sections located between 0.2 and 0.6 mm rostral to the injection site. The mean number of neurons/field was determined for each mouse. Neurons were scored as undamaged if they were Nissl-positive with a round to oval shaped cell body that exhibited no evidence of cell shrinkage. All slides were coded and analyzed in a blinded manner.

Osmotic Pump Implantation—Alzet osmotic minipumps model 1007D (Durect Corp.) were filled with either saline or leptin dissolved in saline (adjusted to deliver 3 μg/day for 6 days). Animal surgery was performed under 5% isoflurane anesthesia. Three-month-old C57BL/6 male mice were implanted with a stainless steel cannula into lateral ventricle via a hole drilled in the skull (Alzet brain infusion kit 3 catalog number 8851) and positioned under stereotaxic guidance (anterior/posterior −0.34 mm from bregma, medial/lateral −1.00 mm, dorsal/ventral −1.2 mm from the surface of the skull). The Alzet minipumps were implanted subcutaneously in the back of the animal and connected to the cannula. The cannula and minipumps were removed 6 days after either saline or leptin infusion, and kainate (0.1 μg in a volume of 0.5 μl) was infused into the right dorsal hippocampus. Twenty four hours later mice were killed; the brains were removed, and coronal sections were cut and stained with cresyl violet (Nissl staining).

Statistical Analysis—Statistical comparisons were made using analysis of variance and the Scheffe test for pairwise comparisons.

RESULTS

Leptin Receptor Activation Promotes Neuronal Survival under Conditions of Reduced Trophic Support, and Oxidative or Excitotoxic Stress—To determine whether hippocampal neurons are capable of responding to leptin, we first performed immunocytochemical analysis of cultured embryonic rat hippocampal neurons using an antibody against the long form of the leptin receptor Ob-Rb. Neurons exhibited specific leptin receptor immunoreactivity, with a subcellular distribution con-
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FIGURE 1. Leptin receptors are expressed in cultured hippocampal neurons, and leptin protects the neurons against death induced by glutamate, trophic factor withdrawal, and Fe\(^{2+}\). a and b, immunofluorescence staining for the neuronal marker β-tubulin (green) and the leptin receptor ObRb (red); the nucleus was stained with 4′,6-diamidino-2-phenylindole (blue). Neurons exhibited considerable ObRb immunoreactivity in the cell body and neurites. c, cultures were pretreated for 24 h with leptin at the indicated concentrations (1, 10, and 100 nM) and were then exposed to 50 μM glutamate for 24 h. Neuron survival was quantified, and values are the mean ± S.E. of determinations made in four different cultures. d, cultures were pretreated with 10 nM leptin for 24 h and were then either subjected to trophic factor deprivation or exposed to 0.5 μM Fe\(^{2+}\) for 24 or 48 h. Neuron survival was quantified, and values are the means ± S.E. of determinations made in 4–6 cultures.* \(p < 0.05\); ** \(p < 0.01\) compared with the corresponding values in vehicle-treated control cultures.

Excessive calcium influx through glutamate receptor channels and voltage-dependent calcium channels (23). Hippocampal cultures were pretreated for 24 h with saline or leptin at three different concentrations (1, 10, and 100 nM) and were then exposed to an excitotoxic concentration of glutamate (50 μM) for 24 h. In cultures not receiving leptin, glutamate killed ∼70% of the neurons (Fig. 1c). More neurons survived in cultures that had been pretreated with either 10 or 100 nM leptin, whereas 1 nM leptin was ineffective.

When deprived of trophic support, cultured hippocampal neurons undergo apoptosis (17, 24). In the absence of leptin, trophic factor withdrawal resulted in the death of more than 40% of the neurons during a 48-h period (Fig. 1d). In cultures pretreated with leptin (10 nM) prior to withdrawal of trophic support, cell survival was significantly increased with fewer than 20% of the neurons dying during the 48-h period (Fig. 1c). Oxidative stress plays an important role in neuronal death resulting from trophic factor deprivation (24, 25) and excessive glutamate receptor stimulation (23). We therefore tested the ability of leptin to prevent neuronal death induced by exposure to Fe\(^{2+}\), an oxidative insult that induces hydroxyl radical production and membrane lipid peroxidation (26, 27). In control cultures, ∼55% of the neurons survived a 24-h exposure to Fe\(^{2+}\) (5 μM), whereas in cultures pretreated with leptin (10 nM), neuronal survival was significantly improved to ∼75% (Fig. 1c). The neuroprotective effect of leptin was maintained through 48 h of exposure to Fe\(^{2+}\) (Fig. 1c).

Leptin Stabilizes Mitochondrial Membrane Potential and Suppresses Oxidative Stress—Mitochondrial alterations, including oxyradical production and membrane permeability changes, play a pivotal role in the death of neurons induced by trophic factor deprivation and excitotoxic and oxidative insults (28–30). We therefore determined whether leptin modifies mitochondrial changes involved in the death of neurons subjected to excitotoxic and oxidative insults. The fluorescent probe TMRE was used to assess mitochondrial membrane potential (31), and dihydrorhodamine 123 was used to measure mitochondrial reactive oxygen species levels (32). Exposure of cultures to Fe\(^{2+}\) or glutamate resulted in a significant decrease in the amount of TMRE fluorescence in neurons within 16 h (Fig. 2a). Quantification of the fluorescence revealed that the magnitude of the decrease was ∼50% in glutamate-treated neurons and 60% in Fe\(^{2+}\)-treated neurons (Fig. 2b). Treatment with leptin significantly attenuated the decreased levels of TMRE fluorescence induced by glutamate and Fe\(^{2+}\) (Fig. 2b). These findings suggest that the neuroprotective effect of leptin is associated with preservation of mitochondrial membrane potential.

Exposure of cultures to Fe\(^{2+}\) and glutamate resulted in significant increases in the level of DHR fluorescence in neurons within 6 h, indicating an increase in the levels of mitochondrial reactive oxygen species (Fig. 2c); Quantification of the fluorescence revealed that the magnitude of the increase was more than 3-fold in cultures treated with Fe\(^{2+}\) and more than 2-fold in cultures treated with glutamate (Fig. 2d). In contrast, cultures pretreated with leptin exhibited a significant attenuation of glutamate- and Fe\(^{2+}\)-induced DHR fluorescence. Mitochondrial potential was also preserved, and reactive oxyradical species production was suppressed in neurons treated with leptin and subjected to trophic factor deprivation. When deprivation of trophic support continued for 48 h, DHR fluorescence continued to increase (Fig. 2d).

Previous studies have shown that binding of leptin to its receptor results in activation of the JAK/STAT and PI 3-kinase/Akt signaling pathways (11, 20). Because neurotrophic factors that activate either the JAK/STAT (21) or PI 3-kinase/Akt (22) pathways can promote neuronal survival, we determined that activate either the JAK/STAT (21) or PI 3-kinase/Akt (22) signaling pathways (11, 20). Because neurotrophic factors including oxyradical production and membrane permeability changes, play a pivotal role in the death of neurons induced by trophic factor deprivation and excitotoxic and oxidative insults (28–30). We therefore determined whether leptin modifies mitochondrial changes involved in the death of neurons subjected to excitotoxic and oxidative insults. The fluorescent probe TMRE was used to assess mitochondrial membrane potential (31), and dihydrorhodamine 123 was used to measure mitochondrial reactive oxygen species levels (32). Exposure of cultures to Fe\(^{2+}\) or glutamate resulted in a significant decrease in the amount of TMRE fluorescence in neurons within 16 h (Fig. 2a). Quantification of the fluorescence revealed that the magnitude of the decrease was ∼50% in glutamate-treated neurons and 60% in Fe\(^{2+}\)-treated neurons (Fig. 2b). Treatment with leptin significantly attenuated the decreased levels of TMRE fluorescence induced by glutamate and Fe\(^{2+}\) (Fig. 2b). These findings suggest that the neuroprotective effect of leptin is associated with preservation of mitochondrial membrane potential.

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suggest that leptin signaling can stabilize mitochondrial function and thereby protect the cells against death induced by several different insults.

Neuroprotective Effect of Leptin Involves Activation of JAK-STAT3 Signaling and Up-regulation of the Expression of Mn-SOD and Bcl-xL.—Binding of leptin to ObRb results in the activation of JAK2, a member of the Janus kinase family of tyrosine kinases (33, 34). Phosphorylation of JAK2 on tyrosine 1138 recruits STAT3 to the ObRb-JAK2 complex, which results in tyrosine phosphorylation and subsequent translocation of STAT3 to the nucleus where it regulates gene transcription. Immunoblot analysis revealed a rapid phosphorylation of STAT3 in hippocampal neurons in response to leptin, which occurred within 5 min of exposure to leptin and reached a peak at 30 min (Fig. 3a). When neurons were treated with AG490, a specific inhibitor of JAK2 phosphorylation (35, 36), STAT3 phosphorylation was attenuated. AG490 treatment also resulted in increased levels of two lower molecular weight phospho-STAT3 immunoreactive bands (Fig. 3a), which may be enzymatic cleavage products of STAT3 (37). Examination of phospho-STAT3 immunoreactivity in leptin-treated and control neurons revealed increased levels of nuclear phospho-STAT3 in leptin-treated neurons compared with untreated control neurons (not shown) and AG490-treated neurons (Fig. 3b). Phospho-STAT3 immunoreactivity was also present in glial fibrillary acidic protein-positive astrocytes treated with leptin although, compared with neurons, relatively little of the immunoreactivity was present in the nucleus of astrocytes (Fig. 3c).

We next evaluated the effects of AG490 on neuronal survival in leptin-treated and control hippocampal cultures. Neurons were treated for 24 h with leptin alone or in combination with AG490 and were then exposed to Fe2+ for 24 h and neuronal survival was quantified. AG490 completely blocked the neuroprotective effect of leptin (Fig. 3d). AG490 also blocked the neuroprotective effects of leptin in cultures exposed to glutamate and trophic factor withdrawal (Fig. 3e). These results suggest that JAK2 and STAT3 activation are required for leptin-mediated cell survival signaling in hippocampal neurons.

We next determined whether STAT3 nuclear translocation and DNA binding are necessary for leptin-mediated neuron survival signaling. We employed a decoy DNA approach in which neurons were treated with double-stranded DNA oligonucleotide with a sequence corresponding to the STAT3 consensus sequence (see “Experimental Procedures”). Previous studies have demonstrated the effectiveness and specificity of such decoy DNAs in blocking the function of transcription factors in neurons (32). In this study, gel shift analysis confirmed the specific binding of the decoy DNA to STAT3 (data not shown). Hippocampal neurons were treated for 24 h with leptin, in the presence of either STAT3 decoy DNA or a control DNA oligonucleotide, and then exposed to glutamate for 24 h. The ability of leptin to protect neurons against glutamate-induced death was significantly attenuated in cultures treated with STAT3 decoy DNA at a concentration of 1 μM, and a higher concentration of STAT3 decoy DNA (10 μM) exacerbated glutamate toxicity (Fig. 4a).

Previous studies of non-neural cells have shown that STAT3 induces the expression of the anti-apoptotic protein Bcl-xL (38) and the mitochondrial antioxidant enzyme Mn-SOD (39). Immunoblot analysis of cell lysates from control and leptin-treated cultures showed that leptin signaling increases the levels of both Bcl-xL and Mn-SOD in hippocampal neurons (Fig. 4b). Treatment of cultures with AG490 or STAT3 decoy DNA blocked the ability of leptin to increase the level Mn-SOD and Bcl-xL (Fig. 4, c and d), indicating a requirement for JAK2 and STAT3 in leptin-induced Mn-SOD expression.
STAT3 and the PI3K-Akt Pathways Mediate Mitochondrial Stabilization by Leptin—Because leptin induced the expression of Bcl-xL and Mn-SOD, we determined whether STAT3 and PI3K-Akt pathways mediated mitochondrial stabilization by leptin. Treatment of neurons with STAT3 decoy DNA abolished the ability of leptin to prevent glutamate-induced mitochondrial membrane depolarization (Fig. 5a) and ROS production (Fig. 5b). Treatment of neurons with the PI 3-kinase inhibitor LY294002 also blocked the ability of leptin to prevent glutamate-induced mitochondrial membrane depolarization (Fig. 5c) and ROS production (Fig. 5d). These findings suggest that both STAT3 and PI3-kinase-Akt pathways are involved in mitochondrial stabilization by leptin. The PI 3-Kinase-Akt Pathway but Not the MAPK Pathway Is Involved in the Neuroprotective Action of Leptin—In addition to the JAK2-STAT3 pathway, leptin can also activate PI3-kinase-Akt and MAPK pathways (40–42). Because activation of the latter pathways can promote neuronal survival, we evaluated their involvement in the neuroprotective effect of leptin. We detected an increase in the amount of phosphorylated Akt in

**FIGURE 3.** Leptin induces phosphorylation and nuclear translocation of STAT3, a pathway critical for the neuroprotective actions of leptin in hippocampal neurons. a, cultures were exposed to 10 nM leptin for the indicated time periods or were pretreated with 100 nM of the JAK-STAT inhibitor AG490 for 2 h and then exposed to 10 nM leptin for 30 min. Cell lysates were subjected to immunoblot analysis using an antibody against phosphorylated STAT3 (p-STAT3). Note that leptin increased the amount of phosphorylated STAT3 (p-STAT3), an effect blocked by AG490. b, neurons were pretreated with AG490 or vehicle (0.2% dimethyl sulfoxide) and were then exposed to 10 nM leptin for 2 h. Neurons were immunostained with p-STAT3 antibody (red), β-tubulin II (green), and the nuclear marker Topro3 (blue). Note that neurons pretreated with AG490 showed less STAT3 translocation into nucleus. c, left, confocal images of cells that were triple-labeled with antibodies against the neuron marker β-tubulin II (green) and phospho-STAT3 (red), in combination with the nuclear marker TOPRO3 (blue). Right, confocal images of cells that were triple-labeled with antibodies against the astrocyte marker glial fibrillary acidic protein (green) and phospho-STAT3 (red), in combination with the nuclear marker TOPRO3 (blue). Phospho-STAT3 immunoreactivity is evident in the nucleus of neurons but not in the nucleus of astrocytes. d, hippocampal neurons were pretreated with AG490 (10 or 100 nM) or vehicle (0.2% dimethyl sulfoxide) for 2 h and were then treated with leptin for 24 h. Neurons were then exposed (or not) to 5 μM FeSO₄ for 24 h, and neuronal survival was quantified. Values are the means ± S.E. of determinations made in four cultures. *, p < 0.05 compared with the value for cultures pretreated with leptin and then exposed to FeSO₄. e, hippocampal neurons were pretreated with 10 nM AG490 or vehicle for 2 h and were then treated with leptin for 24 h, followed by exposure to 50 μM glutamate or trophic factor withdrawal (TFW) for 24 h. Neuron survival was quantified, and values are the means ± S.E. of determinations made in four cultures. **, p < 0.01 compared with the value for untreated control cultures. #, p < 0.05; ##, p < 0.01 compared with the corresponding value for cultures subjected to trophic factor withdrawal or glutamate without leptin pretreatment.
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**Figures**

**Figure 4.** Levels of Mn-SOD and Bcl-xL are increased in response to leptin receptor activation. a, hippocampal neurons were pretreated with 1–10 μM of STAT3 decoy DNA or nonsense control DNA for 6 h and were then treated with 10 nM leptin for 24 h followed by exposure to 50 μM glutamate. Neuron survival was quantified, and values are means ± S.E. of determinations made in four cultures. ***, p < 0.01 compared with the value for cultures pretreated with leptin and then exposed to glutamate, #, p < 0.05 compared with the value for glutamate-treated cultures treated with 1 or 10 μM STAT3 decoy DNA and leptin. b, hippocampal neurons were exposed to 10 nM leptin or vehicle (control) for 24 h. Cell lysates were subjected to immunoblot analysis using antibodies against Bcl-xL, MnSOD, or actin. c, hippocampal neurons were pretreated for 2 h with 10 μM STAT3 decoy DNA, 100 nM AG490, or vehicle, and were then exposed to 10 nM leptin or vehicle (control) for 24 h. Cell lysates were subjected to immunoblot analysis using antibodies against Bcl-xL, MnSOD, or actin. d, hippocampal neurons were pretreated with 1–10 μM of either STAT3 decoy DNA or control DNA, followed by exposure for 24 h to 10 nM leptin. Cell lysates were subjected to immunoblot analysis using antibodies against MnSOD, Bcl-xL, and actin. Upper panel, representative blots. Lower panels, results of densitometric analysis of immunoblots. Values were normalized to the actin level. ***, p < 0.01 compared with control/vehicle value. #, p < 0.05 compared with value for cells treated with STAT3 control DNA.

Leptin Signaling Protects Hippocampal Neurons in Adult Mice against Seizure-induced Degeneration—To determine whether leptin signaling influences neuronal survival in vivo, we employed leptin receptor-deficient mice (db/db). As expected, the body weights of db/db mice were significantly greater than wild-type mice, whereas brain weights of db/db mice and wild-type mice were not different (Fig. 7a). As hippocampal neurons are vulnerable to excitotoxicity in temporal lobe epilepsy (43), we induced seizures in wild-type and db/db mice by focal injection of kainate into the dorsal hippocampus. Kainate caused the degeneration of neurons in regions CA3 and CA1 of the hippocampus of both wild-type and db/db mice (Fig. 7, b–d). However, the numbers of CA3 and CA1 neurons damaged by kainate were significantly greater in db/db mice compared with wild-type mice.

We next performed an experiment to determine the potential of leptin treatment as a therapeutic intervention. Leptin was infused into the brain through an intraventricular cannula connected to an osmotic mini-pump at a rate of 3 μg per day for 6 days. To determine whether the infused leptin activated ObRb in hippocampal neurons, we compared the level and subcellular localization of STAT3 immunoreactivity in brain sections from control and leptin-treated mice. In control mice treated with PBS, STAT3 immunoreactivity was present in the molecular layers throughout the hippocampus, with little or no immunoreactivity in the cell bodies and nucleus of pyramidal neurons (Fig. 8a). In contrast, high levels of STAT3 immunoreactivity were present in the cell bodies and nucleus of CA3 and CA1 neurons in mice treated with leptin (Fig. 8a). We also performed immunoblot analysis of hippocampal tissue samples from PBS- and leptin-treated mice, and we found that levels of phospho-STAT3 were clearly increased in response to leptin, whereas levels of phospho-Akt were not significantly increased by leptin (supplemental Fig. 2). We next determined whether leptin treatment can protect hippocampal neurons against seizure-induced damage. Kainate caused the degeneration of neurons in regions CA3 and CA1 of the hippocampus of both control and leptin-treated mice (Fig. 8, b–d). However, the numbers of CA3 and CA1 neurons damaged by kainate were significantly lower in leptin-treated mice compared with PBS-treated control mice.

DISCUSSION

Actions of leptin on the physiology of hypothalamic neurons involved in the regulation of appetite are well estab-
lished, and it was recently reported that leptin has trophic action on hypothalamic neurons (44). Leptin receptors are also expressed in hippocampal cells (45, 46), but the function of leptin in the hippocampus is unknown. Activation of ObRb by leptin results in tyrosine phosphorylation of JAK2, leading to STAT3 phosphorylation and nuclear translocation (33). We found that an inhibitor of STAT3 (AG490) and STAT3 decoy DNA abolished the cell survival-promoting effect of leptin. Treatment of hippocampal neurons with AG490 also blocked the ability of leptin to activate Akt, indicating a role for STAT3 upstream of Akt in the PI 3-kinase pathway. PI 3-kinase inhibitors attenuated the neuroprotective actions of leptin. Our findings therefore suggest that leptin promotes the survival of hippocampal neurons by activating JAK2-STAT3 and PI 3-kinase-Akt pathways. Leptin induced the expression of Mn-SOD and Bcl-xL, two mitochondria-associated proteins known to protect neurons against excitotoxicity and apoptosis (18, 47). We found that leptin can prevent glutamate-induced cleavage of PARP1 (supplemental Fig. 3). Because cleavage of PARP1 occurs in neurons undergoing apoptosis, the effect of leptin on PARP1 is consistent with an anti-apoptotic action of leptin signaling in neurons. Previous studies have provided evidence that STAT3 regulates the transcription of the Mn-SOD and Bcl-xL genes (48–50), suggesting that the latter genes may be direct targets of STAT3 in hippocampal neurons.

FIGURE 5. Leptin stabilizes mitochondrial membrane potential and suppresses ROS production by a STAT3-mediated mechanism in neurons exposed to glutamate. a, hippocampal neurons were pretreated for 2 h with 10 μM of either STAT3 decoy DNA or control DNA and were then treated for 24 h with 10 nM leptin. Cultures were then exposed to 50 μM glutamate for 12 h, and TMRE fluorescence intensity was quantified. **, p < 0.01 compared with values in vehicle-treated control cultures; #, p < 0.05 compared with corresponding value for STAT3 decoy plus leptin-treated cultures. b, hippocampal neurons were pretreated for 2 h with 10 μM of either STAT3 decoy DNA or control DNA and were then treated for 24 h with 10 nM leptin. Cultures were then exposed to 50 μM glutamate for 6 h, and DHR fluorescence intensity was quantified. **, p < 0.01 compared with the value for vehicle plus glutamate-treated cultures, and to the value for cultures treated with STAT3 decoy DNA plus glutamate. c, hippocampal neurons were pretreated for 1 h with 500 nM LY294002 or vehicle and were then treated for 24 h with 10 nM leptin. Cultures were then exposed to 50 μM glutamate for 12 h, and TMRE fluorescence intensity was quantified. *, p < 0.05; **, p < 0.01 compared with the values for vehicle-treated cultures. #, p < 0.05 compared with the values for cultures treated with glutamate alone or LY294002 plus glutamate. d, hippocampal neurons were pretreated for 1 h with 500 nM LY294002 or vehicle and were then treated for 24 h with 10 nM leptin. Cultures were then exposed to 50 μM glutamate for 6 h, and DHR fluorescence intensity was quantified. *, p < 0.05; **, p < 0.01 compared with the value for control cultures exposed to glutamate.
Mitochondrial stabilization/protection appears to be an important mechanism by which leptin promotes the survival of hippocampal neurons under conditions of oxidative and excitotoxic stress. Previous studies have shown that neuronal deaths induced by glutamate, trophic factor deprivation, and iron each involve oxyradical production and mitochondrial dysfunction (18, 25, 32, 51–53). We found that leptin treatment significantly attenuates glutamate- and iron-induced decreases in mitochondrial membrane potential (TMRE fluorescence) and increases in mitochondrial reactive oxygen

FIGURE 6. Involvement of the JAK2-STAT and PI 3-kinase pathways, but not mitogen-activated protein kinases, in the cell survival-promoting action of leptin on hippocampal neurons. a, hippocampal neurons were pretreated with vehicle or 10 nM AG490 (JAK2 inhibitor) for 2 h and were then treated with 10 nM leptin for the indicated time periods, and cell lysates were subjected to immunoblotting with an antibody against phosphorylated Akt (p-Akt). Note that leptin increased levels of p-Akt and that AG490 blocked this effect of leptin. b, hippocampal neurons were pretreated with 2 μM of the MAPK inhibitor PD98059 for 2 h, followed by treatment with 10 nM leptin for 24 h and then exposure to 50 μM glutamate for 24 h. Neuron survival was quantified, and values are the means ± S.E. of determinations made in four cultures. **, p < 0.01 compared with the value for untreated cultures. #, p < 0.05 compared with the value for glutamate-treated cultures. c, hippocampal neurons were pretreated for 2 h with PI 3-kinase inhibitors LY294002 (500 nM) or wortmannin (200 nM). Neurons were then treated (or not) with 10 nM leptin for 24 h and then exposure to 50 μM glutamate for 24 h. Neuron survival was quantified, and values are the means ± S.E. of determinations made in four cultures. Note that the neuroprotective effect of leptin was significantly attenuated in cultures treated with LY294002 or wortmannin.

FIGURE 7. Hippocampal neurons in leptin receptor-deficient mice (db/db mice) exhibit increased vulnerability to kainate-induced damage. a, brain (left) and body (right) weights of wild-type (WT) and db/db mice. *, p < 0.01 compared with the value for WT mice. b, micrographs of Nissl-stained coronal brain sections from WT and db/db mice that had received an intrahippocampal infusion of either kainate or saline (control) 24 h prior to euthanasia. Note the greater amount of kainate-induced damage to CA1 and CA3 neurons in db/db mice compared with WT mice. c and d, results of quantitative determinations of numbers of undamaged CA1 (c) and CA3 (d) neurons in db/db mice compared with WT mice. Values are the mean and S.E. (6–8 mice per group). *, p < 0.05; **, p < 0.01 compared with the value for kainate-treated WT mice.
Neurotrophic Actions of Leptin in the Hippocampus

It was reported previously that leptin treatment can protect hippocampal neurons against kainate-induced damage (57). We found that STAT3 decoy DNA abolished the ability of leptin to stabilize mitochondrial membrane potential and suppress ROS production in neurons exposed to glutamate. When taken together with the data showing that leptin induces the expression of Bcl-xL and Mn-SOD, in a STAT3-dependent manner, our findings reveal a signal transduction pathway and two mito-protective proteins that mediate the neuroprotective actions of leptin. The up-regulation of Bcl-xL and Mn-SOD likely contributes to leptin-mediated mitochondrial stabilization because Mn-SOD is a key mitochondrial antioxidant enzyme (18, 54), whereas Bcl-xL stabilizes mitochondrial membranes (55). However, other mechanisms may contribute to the excitoprotective action of leptin. Indeed, several studies have provided evidence that leptin can modify neuronal excitability and responsiveness to glutamate, in the context of synaptic and behavioral plasticity. For example, leptin inhibited seizure-like activity in rat hippocampal neurons by PI 3-kinase-mediated activation of BK potassium channels (14) and induced long term depression at hippocampal CA1 synapses (56). In addition, leptin receptor-deficient rodents exhibit impaired hippocampal long-term potentiation and spatial memory ability (15).

It was reported previously that leptin treatment can protect cells in the developing rodent brain against necrotic death induced by the excitotoxin ibotenic acid (57). We found that leptin receptor mutant mice exhibited increased vulnerability to excitotoxic injury, whereas intraventricular administration of leptin in normal mice protected hippocampal neurons. These findings suggest that leptin can exert a cytoprotective action in hippocampal neurons under conditions relevant to the pathogenesis of several different neurodegenerative disorders. Over-activation of glutamate receptors and oxidative stress are believed to contribute to the death of neurons in epilepsy (43), stroke (58), Alzheimer disease (27), Parkinson disease (59), and amyotrophic lateral sclerosis (60). In addition to protecting neurons against direct exposure to glutamate, leptin increased the resistance of neurons to death induced by iron, a metal that induces membrane lipid peroxidation and renders neurons vulnerable to excitotoxicity (26). Leptin was also effective in protecting hippocampal neurons against death induced by trophic factor withdrawal, a model relevant to natural developmental cell death (24). However, whether leptin signaling plays a role in determining which neurons live and which die during the period of programmed cell death remains to be established.

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Leptin-mediated Cell Survival Signaling in Hippocampal Neurons Mediated by JAK STAT3 and Mitochondrial Stabilization

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