Leptin Protects against 6-Hydroxydopamine-induced Dopaminergic Cell Death via Mitogen-activated Protein Kinase Signaling*

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The death of midbrain dopaminergic neurons in sporadic Parkinson disease is of unknown etiology but may involve altered growth factor signaling. The present study showed that leptin, a centrally acting hormone secreted by adipocytes, rescued dopaminergic neurons, reversed behavioral asymmetry, and restored striatal catecholamine levels in the unilateral 6-hydroxydopamine (6-OHDA) mouse model of dopaminergic cell death. In vitro studies using the murine dopaminergic cell line MN9D showed that leptin attenuated 6-OHDA-induced apoptotic markers, including caspase-9 and caspase-3 activation, internucleosomal DNA fragmentation, and cytochrome c release. ERK1/2 phosphorylation (pERK1/2) was found to be critical for mediating leptin-induced neuroprotection, because inhibition of the MEK pathway blocked both the pERK1/2 response and the pro-survival effect of leptin in cultures. Knockdown of the downstream messengers JAK2 or GRB2 precluded leptin-induced pERK1/2 activation and neuroprotection. Leptin/pERK1/2 signaling involved phosphorylation and nuclear localization of CREB (pCREB), a well known survival factor for dopaminergic neurons. Leptin induced a marked MEK-dependent increase in pCREB that was essential for neuroprotection following 6-OHDA toxicity. Transfection of a dominant negative MEK protein abolished leptin-enhanced pCREB formation, whereas a dominant negative CREB or decoy oligonucleotide diminished both pCREB binding to its target DNA sequence and MN9D survival against 6-OHDA toxicity. Moreover, in the substantia nigra of mice, leptin treatment increased the levels of pERK1/2, pCREB, and the downstream gene product BDNF, which were reversed by the MEK inhibitor PD98059. Collectively, these data provide evidence that leptin prevents the degeneration of dopaminergic neurons by 6-OHDA and may prove useful in the treatment of Parkinson disease.

The selective loss of dopaminergic neurons, particularly from the SNc, is a hallmark of idiopathic Parkinson disease (PD). Although the mechanism that triggers neurodegeneration in PD is unknown, there is evidence that decreased neurotrophin production and signaling in the brain may be a contributing factor (1, 2). Certain growth factors can indeed protect dopaminergic neurons from neurotoxic insults in both in vitro and in vivo PD models (3–5). Recently, neuroprotective activity has been ascribed to growth factors having primary functions that were at first thought to be limited to peripheral tissues, including erythropoietin (6) and granulocyte-colony stimulating factor (7). Investigation into the neuroprotective activity of these factors was partly spurred on by evidence that the localization of their receptors in the brain did not correlate with their known functions (8, 9). Leptin is another growth factor for which the broad distribution of its receptors in the brain does not correlate with its currently recognized roles in diet and energy metabolism (10–12).

Leptin is synthesized and released from adipocytes into the bloodstream and has significant biological activity in the central nervous system. Uptake of leptin into the brain occurs via the choroid plexus, which transfers leptin into the extracellular milieu of the brain (13), and concentrates it to ~0.7 nm (11.8 ng/ml) in the cerebral spinal fluid (14). The regulation of feeding and energy homeostasis are well known physiological actions that are mediated by leptin acting via the hypothalamus (15, 16) and the ventral tegmental area (17, 18). The wide expression of the leptin receptor in brain, however, suggests that leptin may have other functions in addition to the control of feeding. Two functionally distinct groups of dopaminergic neurons in the mesencephalon are rich in leptin receptor**

3 The abbreviations used are: SNc, substantia nigra pars compacta; 6-OHDA, 6-hydroxydopamine; BDNF, brain-derived neurotrophic factor; CREB, cAMP-regulated binding protein; pCREB, phosphorylated CREB; E17, embryonic day 17; ERK1/2, extracellular regulated kinases 1 and 2; GRB2, growth factor receptor-bound protein 2; JAK-STAT, Janus-tyrosine kinase activating the signal transducer and activator of transcription; MEK, mitogen extracellular kinase-regulated pathway; PD, Parkinson disease; PI3K, phosphatidylinositol 3-kinase; pERK1/2 or pERK, phosphorylated ERK1/2; shRNA, short hairpin RNA; XTT, 2,3-[2-[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-5-nitro)benzene sulfonic acid; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; TH, tyrosine hydroxylase; EMSA, electrophoretic mobility shift assay; wt, wild type; dn, dominant negative.

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MAPK Signaling in Leptin Neuroprotection

expression (12, 19). The medial group, comprising the ventral tegmental area, is known to help regulate food intake and reward pathways (18, 20). Located laterally are the dopaminergic neurons of the SNc, which are involved in the modulation of peripheral motor control and, more specifically, in PD. The lack of participation of the SNc in the control of appetitive behaviors and the presence of the leptin receptor suggests that leptin has a novel role in these dopaminergic neurons.

Leptin mediates intracellular signaling via the JAK-STAT pathway (21). The expression of JAK-2 and STAT3, the specific JAK-STAT proteins activated by leptin, are found in the SNc (22). The JAK-STAT pathway has many functions in neurons, including activation by ligands possessing neuroprotective activity in the brain, such as ciliary neurotrophic factor and erythropoietin (23). Considering the lack of any known physiological roles ascribed to leptin signaling in the SNc, it is plausible that leptin may influence the viability of dopaminergic neurons. We therefore investigated whether leptin itself is neuroprotective for dopaminergic neurons. Using both in vivo and in vitro models of PD, we found that exogenously administrated leptin can reverse dopaminergic cell loss and functional behaviors induced by the dopamine-neuron destroying toxin, 6-OHDA via the MEK/ERK signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Neurotoxin Exposure—Unless otherwise noted, chemicals were obtained from Sigma-Aldrich, including rat leptin. Culturing of cells, DNA laddering, and caspase activity were performed according to previously published procedures (6). Briefly, MN9D cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (HyClone, Logan, UT) using Primaria culture dishes (BD Biosciences). Neuronal differentiation was induced for 4 days using 1 mM n-butyrate before cells were used for experiments. Primary mesencephalic neuronal cultures were made from E17 rat pups as previously described (6).

The neurotoxin 6-OHDA-HBr was made fresh for each experiment in a non-oxidizing vehicle (4). Before toxin was added to the cultures, serum-containing media was removed and replaced with media without serum. Cells were exposed to 50 or 100 μM 6-OHDA for 30 min, followed by 2 washes of serum-free media, and fed for 24 h with serum-containing media. The leptin receptor antagonist used is a mutein form of rat leptin. Culturing of cells, DNA laddering, and caspase activity were performed according to previously published protocols (6). The expression plasmids used the pcDNA3.1(+) backbone. For JAK/GRB2 shRNA, inserted into the plasmid SpeI/EcoRV cloning sites, was the H1-RNA promoter followed by the gene-specific targeting sequence. For each of the targeted genes, a scramble sequence containing the same nucleotide composition but in a randomized order was also constructed and used as control. Additional controls also included the shRNA-targeting green fluorescent protein (5′-AACAGCTGCTAGGATTACA-3′). Upon construction, all inserts were verified by the sequencing reaction on both strands. For nucleofection-mediated transfection, 5–6 × 10⁶ cells were transfected with 2 μg of plasmid DNA using the manufacturer’s recommended cell type-specific settings, then plated into either 100-mm culture dishes (2 × 10⁶ cells/dish) or 96-well plates (6 × 10⁴ cells/well) (Primaria, BD Biosciences). The cells were induced for neuronal differentiation for 4 days before use. For each of the targeted proteins, two shRNA targeting sequences were used in separate experiments, and all targeting sequences listed below were confirmed in preliminary studies for their effectiveness of knocking down the targeted genes: Jak1: 1, TCAAGAAGACTGAGGTGAA; 2, AGAGGATGATGAGAGAAA; Jak2: 1, GAGATAGCTAGCTAGCTAGCTAG; 2, CAGACAGCTAGCTAGCTAGCTAG; and GRB2: 1, TGAATGAGCTAGCTAGCTAGC; 2, AGGCCGACTCATTGGGAGAAA. The Western blot data presented used the first sequence from the above.

EMSAL—Electrophoretic mobility shift assay (EMSA) was performed as described previously (30). Nuclear proteins were extracted from cultured cells, and the protein content in nuclear fractions was measured using the Bradford method.
(Bulletin 1177, Bio-Rad). To perform EMSA, the CRE sense
(5’-GAACCGTGTGACGTTACGC-3’) and antisense (5’-
GCGTAACGTCACACGGTT-3’) oligonucleotides were
end-labeled with [γ-32P]ATP using T4 polynucleotide kinase,
purified using the QIAquick Nucleotide Removal Kit (Qiagen),
and then annealed. The DNA-protein binding reaction was
performed in a total volume of 20 μl containing the binding assay
buffer (10 mM Tris–HCl, 50 mM NaCl, 0.5 mM DTT, 0.5 mM
EDTA, 1 mM MgCl2, 5% glycerol, pH 7.6), 0.0175 pmol of
labeled double-stranded probe (>10,000 cpm), 10 μg of nuclear
protein, and 1 μg of poly(dI–dC). After incubation for 20 min at
room temperature, the reaction mixture was subjected to elec-
rophoresis for 2 h in a non-denaturing 4% polyacrylamide gel
in 0.25× TBE (22.5 mM Tris borate and 0.5 mM EDTA). The gel
was dried and subjected to autoradiography. The specificity of
CRE-binding activity was determined by performing both com-
petition assays and supershift assays. For the competition assays, 100-fold molar excess of unlabeled double-stranded oli-
gonucleotide of the CRE binding site was used as a specific
competitor. For supershift assays, 2.5 μg of antibody against
CREB was added 30 min before the addition of the oligonucleo-
otide probe.

In some experiments, we investigated the functional role of
CREB in leptin neuroprotection using a CRE decoy oligonu-
cleotide. Neuronal differentiated MN9D cells were transfected
for 6 h with CRE decoy oligonucleotide (5’-TAGCTGCAGAGAGCI-
CTCTCTGACGTA-3’) or control CRE mismatch sequence (5’-
TAGCTGCAGAGAGCGCTCTCTGACGTA-3’) at the concentration of 0.1 μmol/liter (all linkages phospho-
rhothioate protected, Bio-Synthesis Inc., Lewisville, TX). Trans-
fection was done using Lipofectamine 2000 (0.3 μmol/liter)
according to the manufacturers’ instructions.

In Vivo Studies—All animal experiments were approved by
the University of Pittsburgh Institutional Animal Care and Use
Committee and carried out in accordance with the National
Institutes of Health Guide for the Care and Use of Laboratory
Animals. Male C57/BL6 mice (Jackson Laboratory, Bar Harbor,
ME) weighing 25g were placed in a stereotoxic device (Kopf,
Tujunga, CA) while under 1.5% isoflurane anesthesia. Animals
first received either 0 or 6.25 μl of leptin in physiological saline
or saline alone (5 μl), into the right substantia nigra at a
depth of 3.7 mm from the dural surface. Thirty minutes later, 3 μg of 6-OHDA (in 1.4 μl of saline) or saline alone was injected into the right striatum. Some animals received 2.0 μl of PD98059 (0.2 mM) or Me2SO (0.2
mM) in saline into the right lateral ventricle 30 min before
6-OHDA at coordinates 0.3-mm rostral and 1.0-mm lateral to
bregma, at a depth of 2.5 mm.

Behavioral Testing—Previously published protocols were
followed (6). Apomorphine-induced rotations were monitored
1 week before lesioning and then at 3 and 8 weeks following
6-OHDA lesioning. Mice were given a subcutaneous injection
of apomorphine (0.1 mg/kg in physiological saline) (31) and
then placed individually in plastic beakers (diameter: 13 cm)
and videotaped from above for 30 min. Analyses of completed
(360°) left and right rotations were made offline.

For spontaneous turning, the corner test procedures of
Zhang et al. were used (32). Each trial consists of the preference,
right or left, an animal spontaneously rears when encountering
a 30-degree corner. Mice were tested 1 week before lesioning
and then at 8 weeks following lesioning. Each testing period
consisted of 10 trials.

Measurements of catecholamine levels were made by remov-
ing the striata of mice and immediately freezing them on dry
ice, and stored at −80 °C, 3 and 8 weeks after lesioning. High
performance liquid chromatography with electrochemical
detection was used to measure dopamine and its metabolites
(33). Catecholamine concentrations are expressed as pico-
moles per mg of fresh striatal tissue.

Stereological Cell Counting—The Bioquant Image Analysis
program was used to count SNC dopaminergic neurons that
were immunopositive for TH. The Bioquant Software is inter-
faced with a stage encoder to interpret x-, y-, and z-axis move-
ment of the microscope stage. The entire SNCs from a given
section was captured with a color charge-coupled device cam-
era, and grid squares of 50×50 μm were generated over the
region of interest. The optical dissector method requires that
100–150 cells be counted in a given structure to estimate the
total number (34, 35). The number of points needed within the
outlined area was empirically determined by prior studies: a
25×25 μm counting frame and a focus depth of 25 μm in a
40-μm section (a dissector) should be used. An average of nine
dissectors per section yields the desired number of counted
neurons throughout the SNCs at the levels of the dorsal hip-
pcampus. The rostral-caudal length of the SNC was 4 mm, and
every fourth section was counted. The total number of neurons
was calculated using the optical dissector, equal to the quotient
of the total number of neurons counted and the product of the
fractions for sampling section frequency (SSF, fraction of sec-
tions counted), area section frequency (ASF, sampling area/
area between dissectors), and thickness section frequency (TSF,
dissector depth/section thickness), or n = Σneurons counted ×
1/SSF × 1/ASF × 1/TSF). For our study, SSF = 1/4 sections,
ASF = 25×25 mm/50×50 mm, and TSF = 25 mm/40 mm.

Statistical Analysis—All results are expressed as mean ± S.E.
of at least 6–9 measurements per data point, from three inde-
pendent experiments. ∗ or # denotes p < 0.05, ** or ## p < 0.01,
and *** p < 0.001 obtained from analysis of variance and pro-
ected least significant difference.

RESULTS

Leptin Protects Dopaminergic Neurons from 6-OHDA Neuro-
toxicity in Vivo—To determine if leptin is neuroprotective for
dopaminergic neurons, we injected leptin intranigrally in a
murine PD model. The unilateral 6-OHDA neurotoxin para-
digm induces specific degeneration of the dopaminergic neu-
rions in the SNC. As shown in Fig. 1a, immunohistochemical
staining of the SNCs demonstrated that the 6-OHDA-induced
loss of TH-positive neurons is reversed by leptin pre-treatment.
Stereological quantification of the number of TH-positive neu-
rions 3 or 8 weeks after 6-OHDA lesioning showed that leptin-
treated animals had a greater number of immunopositive TH-
positive neurons in the SNC compared with animals treated
with vehicle (Fig. 1b).
Leptin Is Neuroprotective against 6-OHDA Toxicty in Dopaminergic Neurons in Vitro—We next investigated the signaling mechanisms underlying the neuroprotective effects of leptin using neuronal differentiated MN9D cells, a murine dopaminergic cell line that is susceptible to 6-OHDA toxicity. Leptin (10–50 μg/ml) protected MN9D cells from 6-OHDA (50–100 μM)-induced cell death in a concentration-dependent manner (Fig. 2a). The efficacy of leptin protection was greater against 50 μM 6-OHDA, with maximal effects at 20–30 μg/ml. The neuroprotection afforded by leptin was also dependent on the time and sequencing of leptin and 6-OHDA exposure; the greatest protection was achieved when leptin was added 1–6 h before exposure of MN9D cells to 6-OHDA (Fig. 2b).

Previous experience with MN9D cells has shown that 6-OHDA induces caspase-dependent cell death via the intrinsic apoptotic pathway (6). The ability of leptin to inhibit the appearance of apoptotic traits that accompany caspase-dependent apoptosis was therefore examined. When MN9D cells were exposed to 6-OHDA, release of cytochrome c into the cytosol was distinctly detectable in a time-dependent manner. In contrast, pretreatment of cultures with leptin abolished the appearance of cytosolic cytochrome c (Fig. 2c). Exposure of MN9D cells to 6-OHDA also increased the levels of the active, cleaved forms of caspase-3 and -9 (Fig. 2d) as well as the enzymatic activity of both enzymes up to 16 h after toxin. Again, leptin treatment completely inhibited the activation of both caspase-3 and -9 at all time points tested, up to 16 h following toxin exposure (Fig. 2, d and e). Genomic DNA degradation induced by 6-OHDA exposure was examined by two methods. First, extensive DNA laddering was found after exposure to 6-OHDA, which was completely absent when cells were pretreated with leptin (Fig. 2f). Leptin alone (20 μg/ml) did not induce any DNA damage. Second, TUNEL staining was used to identify MN9D cells that had undergone apoptotic DNA fragmentation during

Functional behavioral outcomes were also examined using behavioral paradigms that measure the functional status of the motor aspects of the lesioned nigrostriatal dopaminergic system. Apomorphine-induced asymmetrical rotations contralateral to the side of 6-OHDA injection were significantly decreased by leptin administration compared with vehicle-treated controls (Fig. 1c). Mice lesioned with 6-OHDA developed a preference for turning toward their right, which was also significantly attenuated in animals treated with leptin (Fig. 1d).

Analysis of dopamine and its primary metabolites was measured from the primary target of the SNc, the striatum. As shown in Fig. 1e, a significant drop in both dopamine and 3,4-dihydroxyphenylacetic acid levels occurred 3 and 8 weeks post-lesioning, whereas homovanillic acid was significantly decreased 8 weeks after 6-OHDA lesioning as well. Treatment with leptin resulted in dopamine and 3,4-dihydroxyphenylacetic acid concentrations that were significantly elevated compared with vehicle treatment at both 3 and 8 weeks following lesioning. Similarly, leptin significantly raised homovanillic acid levels 8 weeks after 6-OHDA.

Leptin protects the nigrostriatal dopaminergic system from 6-OHDA-induced degeneration in mice. a, brain sections from the midbrain showing TH immunoreactivity in the SNc 3 weeks after injection of saline, 6-OHDA preceded by vehicle or 6-OHDA preceded by leptin. Arrows indicate the SNc region lesioned by 6-OHDA. b, the numbers of TH-positive neurons in the ipsilateral SNc (injected side) were counted using non-biased stereological methods 3 and 8 weeks after the injections indicated. Controls were injected with saline. c, apomorphine-induced circling before 6-OHDA injection (Pre) or 3 and 8 weeks after the injections indicated is shown as the number of turns in the first 30 min after intraperitoneal injection of apomorphine. d, spontaneous turning using the corner test before 6-OHDA injection (Pre) or 8 weeks after the injections indicated. The mean numbers of right turns out of 10 trials/session are reported. e, high performance liquid chromatography-electrochemical detection data of catecholamine levels from the ipsilateral striatum 3 or 8 weeks following the indicated injections. Catecholamines are expressed as picomoles/mg of fresh, wet tissue. n = 11–12 per group for all experiments. *, p < 0.05; **, p < 0.01 versus control; #, p < 0.05; ##, p < 0.01 versus 6-OHDA plus vehicle treatment.
of leptin-exposed MN9D cells showed that pERK1/2 levels were increased in a time-dependent fashion (Fig. 3a). Because 6-OHDA alone can also alter pERK1/2 signaling, we compared the activation of pERK1/2 by both 6-OHDA and leptin and found that, although 6-OHDA (50 μM) alone induced a transient (1–2 h) increase in pERK1/2, the addition of leptin induced pERK1/2 that occurred much earlier and was sustained for at least 4 h (Fig. 3b). Experiments using the pharmacological inhibitors of MEK, PD98059 (10 μM) or U0126 (3 μM), revealed that MEK was critical to the ability of leptin to induce ERK1/2 phosphorylation (Fig. 3c) and maintain cell viability when challenged with 6-OHDA (Fig. 3d).

To further determine if ERK1/2 activation is essential for leptin neuroprotection, MN9D cells were transfected with either a wild-type (wt-MEK) or dominant negative MEK (dn-MEK), followed by neuronal differentiation for 4 days. At 2 h after leptin treatment (30 μg/ml), pERK1/2 was examined by immunoblotting. dn-MEK-transfected cells showed significantly reduced phosphorylation of ERK1/2 to a level comparable to treatment of non-transfected cells with PD98059 (Fig. 3e). When cell viability of the transfecteds was measured 24 h after 6-OHDA exposure (50 μM, 30 min), wt-MEK was not protective in the absence of leptin (Fig. 3f). In contrast, dn-MEK abolished the ability of leptin to protect MN9D cells from 6-OHDA toxicity (Fig. 3f). Thus, an active form of MEK is required by leptin for neuronal protection from 6-OHDA toxicity in dopaminergic cells.

To show that leptin protection in vitro was not peculiar to the MN9D cell line, we also tested its ability to protect cultured primary dopamine neurons. Only 52.5 ± 14.6% TH-positive neurons remained at 48 h following exposure to 40 μM 6-OHDA alone. Pretreatment with leptin (10 μg/μl) for 6 h increased the number of surviving TH-positive neurons to 72.6% ± 18.8 (p < 0.05). Consistent with the results in MN9D cells, the addition of PD98059 diminished the neuroprotective effects of leptin in primary dopamine neurons by ~50% (data not shown).
Leptin Neuroprotection Occurs via Leptin Receptor-associated Signaling Molecules—We next wanted to determine whether leptin-mediated neuroprotection occurred via known leptin receptor-mediated signaling pathways. Results of Western blot analysis indicated that mainly the short forms of the leptin receptor are present in MN9D cells, whereas both the long and short forms of the receptor are expressed in the midbrain and striatum (Fig. 4a). To demonstrate the specific involvement of the leptin receptor in protection from 6-OHDA-mediated toxicity, we used a leptin mutein as an antagonist of leptin receptor activation. This leptin receptor antagonist is a mutant form of leptin containing three mutations (L39A/D40A/F41A), which can efficiently bind to the leptin receptor but is incapable of activating the receptor (24). Indeed, the leptin antagonist inhibited leptin-induced neuroprotection when it was co-incubated along with leptin (Fig. 4b). Next, we analyzed the contribution of two downstream effector proteins for leptin receptor, JAK2 and GRB2 (37), to the protective actions of leptin in dopaminergic neurons. Knockdown experiments were performed with shRNA-producing plasmids transfected into MN9D cells. Fig. 4c shows the successful knockdown of JAK1, JAK2, or GRB2. We found that knockdown of JAK2 or GRB2 abolished leptin-induced increases in pERK1/2 levels (Fig. 4d). Because JAK1 activity is not affected by leptin, its loss should not have any effect on pERK1/2 levels. Indeed, we found that knockdown of JAK1 did not alter pERK1/2 activation, nor did empty plasmid or scrambled JAK2/GRB2 sequences. The viability of transfected cells in response to 6-OHDA exposure was then tested. Transfectants showed equivalent sensitivity to 6-OHDA as plasmid controls; however, leptin did not rescue either JAK2 or GRB2 knockdown cells (Fig. 4e). These results suggest that activation of ERK1/2 by the leptin receptor-mediated signaling molecules JAK2 and GRB2 is required for leptin-mediated neuroprotection.

Leptin Induces the Activation and Nuclear Localization of CREB via an ERK1/2-dependent Mechanism—To further define leptin-induced neuroprotective signaling pathways, we examined the formation of activated CREB, a critical pro-survival signaling pathway in dopaminergic neurons that could...
potentially be linked to ERK1/2-mediated neuroprotection (38). After 30 min of exposure to leptin, an increase in pCREB levels was evident in MN9D cells, and this increase lasted for at least 4 h (Fig. 5a). Although 6-OHDA exposure (30 min) alone increased pCREB as well, the increase was transient and had completely disappeared after 30 min (Fig. 5b). In contrast, the addition of leptin produced a rise in pCREB that remained elevated for at least 4 h (Fig. 5b).

Because several different signaling pathways can induce CREB phosphorylation, we wanted to determine which pathways were contributing to leptin-mediated pCREB formation. Fig. 5c shows the results of using pharmacological inhibitors of known CREB-activating pathways. The four inhibitors used are known to prevent activation of protein kinase A (HA89), calcium-calmodulin-dependent protein kinase type 2 (KN62), PI3K (LY294002, LY), and MEK (PD98059, PD). Of these, only PD98059 prevented leptin-induced increase in pCREB, suggesting an essential role of MEK/ERK in pCREB activation. Immunofluorescent images of MN9D cells show that leptin increased the cytosolic level of pERK1/2 compared with vehicle-treated cells, whereas it induced a nuclear localization of pCREB (Fig. 5d). Treatment with PD98059 diminished the staining for both pCREB and pERK1/2 in leptin-treated cells (Fig. 5d). To further determine the role of MEK/ERK in leptin-induced activation of CREB, MN9D cells were transfected with either the wild-type MEK (wt-MEK) or the dominant negative mutant of MEK (dn-MEK) and then subjected to neuronal differentiation for 4 days. Controls consisted of mock or enhanced green fluorescent protein transfection. Transfection with wt-MEK did not significantly increase pCREB levels compared with controls (Fig. 5e). Instead, paralleling the results seen with the formation of pERK1/2, transfection with dn-MEK significantly reduced leptin-induced pCREB formation.

Using EMSA, we next showed that transfection of MN9D cells with a CRE decoy oligonucleotide or a dominant negative CREB (dn-CRE) could inhibit CREB-CRE binding activity under the treatment of leptin. The basal level of CREB-CRE binding was increased by the addition of leptin in mock-transfected cells (Fig. 5f, lanes 1 and 2). Transfection with a CRE decoy oligonucleotide diminished leptin-induced CREB-CRE binding, but a control oligonucleotide had no effect (Fig. 5f, lanes 3 and 4). Similarly, transfection with dn-CRE abolished leptin-induced CREB-CRE binding activity, whereas wt-CREB transfection had little effect (Fig. 5f, lanes 5 and 6). When MN9D cell transfectants were challenged with 6-OHDA, wt-CREB significantly enhanced cell viability, whereas CRE decoy oligonucleotide or dn-CRE significantly reduced cell viability (Fig. 5g). Furthermore, diminished cell viability could not be subsequently rescued by leptin for either CRE decoy oligonucleotide or dn-CRE transfectant (Fig. 5g). The enhanced CREB-CRE binding activity is thus a key signaling mechanism underlying leptin-mediated neuroprotection.
BDNF Induction by Leptin Is Mediated by CREB—BDNF is one of the major gene products induced by CREB signaling in brain tissues (39) and is also increased in the hypothalamus following leptin treatment (40). We analyzed MN9D cells to determine whether this occurs in dopaminergic cells. Western blot analysis of cultures treated with leptin showed a time-dependent increase in BDNF levels (Fig. 6a). Inhibition of leptin-induced BDNF expression occurred when cells were transfected with CRE decoy oligonucleotide, indicating that CREB activation was required for this effect by leptin (Fig. 6b). Moreover, because BDNF is a secretory protein, we quantitatively measured the levels of BDNF in concentrated culture media after MN9D cells were treated with leptin for 4 h. There was ~1.9-fold increase in BDNF levels in leptin-treated cultures over controls (Fig. 6c). Participation of the MEK/ERK/CREB pathway was confirmed when the MEK/ERK inhibitor PD98059 or transfection of the CRE decoy oligonucleotide resulted in a similar decrease in BDNF levels in leptin-treated cultures (Fig. 6c).

ERK1/2 Activation and ERK Dependence of CREB Activation in Dopaminergic Neurons of 6-OHDA-Lesioned Mice—It was important to determine if the results of the signal transduction experiments in vitro could be corroborated in vivo. Therefore, pERK1/2 and pCREB were examined by performing immunohistochemistry and Western blots using midbrain tissues from mice treated with or without leptin. Under control conditions, dopaminergic neurons of the SNc are moderately positive for pERK1/2 (Fig. 7a, panels A–C and J–L). Injection of leptin into the SNc resulted in a marked increase in the staining of pERK1/2 with mainly a cytosolic localization in TH-positive neurons (Fig. 7a, panels D–F, arrows in panels M–O). Intracerebroventricular injection of PD98059 prior to leptin injection abolished the enhanced pERK1/2 response (Fig. 7a, panels G–I and P–R). Western blot
pCREB-like immunoreactivity in the SNc was also examined. Dopaminergic neurons from control animals displayed low levels of diffuse pCREB staining (Fig. 7e, panels A–C and J–L). Leptin treatment caused a marked increase of pCREB with a nuclear localization in primarily, but not limited to, TH-positive neurons (Fig. 7e, panels D–F; arrows in panels M–O), while pretreatment with PD98059 reduced the enhanced nuclear signal for pCREB (Fig. 7e, panels G–I and P–R). Western blot analysis also showed that leptin significantly enhanced the level of pCREB in the midbrain, which was prevented by inhibition of the MEK pathway with PD98059 (Fig. 7f). Injection of 6-OHDA alone was not able to increase pCREB levels above control levels, whereas leptin significantly raised pCREB levels up to 6 h after 6-OHDA lesioning (Fig. 7g). To corroborate our cell culture results, we analyzed midbrain levels of BDNF following leptin injection. Western blot analysis showed a significant increase of BDNF at the 6-h time point that was abolished by PD98059 treatment (Fig. 7h).

Lastly, we wanted to further verify the in vivo significance of the MEK pathway in leptin-mediated neuroprotection in the PD murine model. We therefore examined whether the inhibition of the MEK pathway in vivo could alter leptin-mediated reversal of PD-like features. Indeed, leptin-mediated improvements in both the number of TH-positive neurons in the SNc (Fig. 8a) and behavioral asymmetry (Fig. 8, b and c) were lost with PD98059 pre-administration.

**DISCUSSION**

This study established, for the first time, the neuroprotective effects of leptin in experimental models of PD. Using both in vivo and in vitro methods, we demonstrated that, not only did leptin have significant protective effects by rescuing dopaminergic neurons from 6-OHDA toxicity, it also preserved the functioning of the dopamine system. Leptin reversed behavioral, biochemical, and histological parameters up to 2 months after 6-OHDA lesioning of the SNc, confirming the bioactivity of exogenously supplied leptin in an animal PD model. The MEK-ERK1/2 signaling pathway was found to be essential in leptin-mediated neuroprotection. Downstream of ERK1/2, activation of the transcription factor CREB closely paralleled pERK1/2 formation, and directly contributed to the prosurvival effects mediated by leptin. Knockdown of the leptin receptor adaptor signaling proteins JAK2 or GRB2 demonstrated the specific activation of the leptin pathway in pERK1/2 formation.

A role for leptin in neuronal survival has yet to be fully identified. In the periphery, leptin inhibits apoptosis in a variety of cell types, including lymphocytes (41) and hepatic stellate cells (42), and in cancer cell lines (43, 44). Indeed, although no functions in the central nervous system have been identified aside from obesity, there is evidence that leptin may be involved in neuronal survival mechanisms (45, 46). Moreover, leptin may emulate the dual functions that dopamine plays in its parallel roles of integrating reward and central motor pathways. The recent discovery that leptin regulates the mesoaccumbal pathway and modulates feeding in the ventral tegmental area establishes leptin as having a critical role in midbrain dopamine and motivational behavior (17, 18). Our findings that leptin is capable of promoting the survival of dopaminergic neurons con-
firms that leptin may be important in the striatonigral pathway, which is vital for motor control and is most affected in PD.

Leptin signaling via JAK-STAT in neurons activates several antiapoptotic pathways, including MEK/ERK (36). Leptin receptor activation increases ERK1/2 activity via the protein phosphatase SHP-2. Enhanced association of SHP-2 with the adaptor molecule GRB2 occurs following JAK2 phosphorylation of the leptin receptor at Tyr-985, allowing the docking and formation of the SHP-2-GRB2 complex (37). Interfering with either SHP-2 activity (36) or GRB2 availability, as we show in the current study, greatly diminishes the ability of leptin to increase ERK1/2 activity.

The prosurvival activity of ERK1/2 in neurons is well known (for review see Ref. 47), and ERK1/2 is involved in the survival of dopaminergic neurons (6, 48–50). One of the prosurvival downstream messengers activated by ERK1/2 is the transcrip-
MAPK Signaling in Leptin Neuroprotection

Our results showing the MEK/ERK pathway being critical for leptin neuroprotection in dopamine cells is in contrast to an earlier finding that PI3K/Akt is instead responsible (46). However, that study used a different neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, as well as testing only in a dopaminergic cell line. The difference in toxin and cell type is most likely the major factor causing this discrepancy in signal transduction between the two studies. We have also found the PI3K/Akt and not the MEK/ERK pathway to be vital for dopamine neuron protection by erythropoietin (6). Signaling by activated Akt is an important molecular mechanism in dopaminergic neurons in vivo. Transduction of murine nigral dopaminergic neurons with a virally delivered constitutively active Akt had significant neurotrophic and neuroprotective effects against 6-OHDA toxicity (57). The studies showing the importance of PI3K activation are complementary to our current study, and underscore the likelihood that multiple signaling pathways are essential for dopamine neuron survival.

Among other neurotrophic or antiapoptotic molecules relevant to dopamine neuron survival or function and whose expression is influenced by CREB includes BDNF (39). BDNF is of particular interest for PD, because there is significant loss of BDNF in the idiopathic PD brain, especially in the SNc (59, 60). Because BDNF can rescue dopaminergic neurons from 6-OHDA-induced toxicity (61), pCREB-mediated enhancement of BDNF is a potential mechanism that mediates leptin-induced neuroprotection. The ability of leptin to effectively control weight via the hypothalamus also correlates with its ability to enhance hypothalamic BDNF levels (40). Leptin did indeed increase BDNF in our study via a CREB-mediated pathway and is at least partly responsible for leptin’s neuroprotective properties.

Using a naturally occurring prosurvival molecule such as leptin to prevent or even reverse neurodegeneration is an attractive strategy. As an endogenous molecule that normally enters the central nervous system, leptin bioactivity and safety are cur-
Recently being investigated (e.g. Refs. 58 and 62). Consideration of possible side-effects, such as altered weight gain, must be addressed. Although we did not find any evidence of weight abnormalities (data not shown), clinical administration of leptin for the treatment of PD would require usage for a much longer period, and may unmask these or other side effects.

In summary, this study shows that leptin can rescue dopaminergic neurons and associated behavioral features from 6-OHDA toxicity. In view of these results and considering the leptin receptor distribution in the SNc, leptin is conceivably a viable new treatment for the prevention of neurodegeneration in PD.

Acknowledgments—Immortalized dopaminergic MN9D cells were used courtesy of Alfred Heller, University of Chicago. We also thank Melanie Cobb (University of Texas Southwestern Medical Center, Dallas, TX) for providing the MEK-containing plasmids, Jane Cavanaugh (Duquesne University, Pittsburgh, PA) for the CREB-containing plasmids, and Dr. Arieh Gertler of Protein Laboratories Reho-vot Ltd. for the leptin antagonist. We also thank Pat Strickler for secretarial support.

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