Restless legs syndrome (RLS) is a common neurologic condition involving iron and dopamine systems. We sought to create an animal model consistent with RLS based on current understanding of human pathology. We performed bilateral 6-hydroxydopamine (6-OHDA) lesioning in the A11 nucleus of C57BL/6 mice and deprived a subset of mice from dietary iron to observe whether these manipulations can increase motor activity. Iron levels in serum, brain, and especially spinal cord were significantly decreased after iron deprivation. Interestingly, 6-OHDA lesioning appeared to further reduce CNS iron stores. Pathologic examination demonstrated a 94% reduction in A11 tyrosine hydroxylase staining cells in mice injected with 6-OHDA but minimal effects on other areas. Locomotor activities were significantly increased in both the mice that were iron deprived and the A11-lesioned mice compared with controls. The combination of iron deprivation and A11 lesions further significantly augmented activity. Additionally, the mice in the combined iron-deprived and lesioned group were more aggressive. The increased activity in A11-lesioned mice with or without iron deprivation was normalized after treatment with the D2/D3 agonist ropinirole, as is seen in human RLS but was worsened by the D1 agonist SKF38393. This model could be consistent with human RLS, attention deficit hyperactivity disorder, or akathisia.

Key Words: Animal models, Dopamine, Iron, Restless legs syndrome.

INTRODUCTION
Restless legs syndrome (RLS) is a sensory-motor disorder with a prevalence of about 10% in predominately Caucasian populations (1). The primary feature of the disease is an urge to move the legs, and sometimes arms, frequently associated with an uncomfortable sensation, worsening of symptoms at night, worsening with inactivity, and transient relief with movement (2).

Although a complete pathophysiologic understanding of RLS remains elusive, dopaminergic dysfunction and iron deficiency (ID) are believed to play key roles. CNS iron dysregulation is strongly implicated by an inverse correlation between CSF serum ferritin levels and RLS symptoms (3). Human pathologic data also demonstrate reduced intracellular iron stores and altered iron homeostatic regulation (4). A dopaminergic system is implicated by some functional imaging studies (5), and the robust benefit that dopaminergic agonists provide for human RLS (6–8). Subcortical and spinal cord anatomy is suggested by clinical features (primary leg involvement), neurophysiology studies (9), and the frequent onset of RLS after spinal cord injury (10).

Involvement of a little studied area of the brain known as the diencephalic-spinal (A11) dopaminergic nucleus could potentially integrate these background features; it is a subcortical dopaminergic system that projects into the spinal cord, is near circadian control centers, and is involved in antinociception function (11). We previously demonstrated increased locomotor activity in a small number of rats after lesioning resulted in a 50% dopaminergic cell loss in the A11 nucleus (12). To further test this hypothesis, we established a model of 6-hydroxydopamine (6-OHDA)-lesioned mice in this area to determine whether any subsequent behavioral responses were consistent with RLS and evaluated the independent and synergistic effects of dietary iron deprivation. Finally, we tested several medications known to improve or exacerbate human RLS.

MATERIALS AND METHODS
Animal studies were approved by the Baylor College of Medicine Animal Use and Care Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Eighty C57BL/6 male mice were randomly divided into 4 eventual groups at 28 days after birth. The male C57BL/6 mice were group-housed in a climate-controlled animal colony with a reversed day/night cycle (12 hours/12 hours, 6 am–6 pm). All animals had free access to food and water for 24 hours each day. Five mice were housed per cage.

Beginning on day 28 after birth, half of the mice (n = 40) were given a low-iron diet (TD 80396, Harlan Teklad,
Madison, WI) of 3.5 mg of iron/kg and the others (40 mice) received a regular diet with 35 mg of iron/kg in the form of ferric citric. Weight, serum iron measurement, and behavioral studies were performed at baseline and at 1 month after dietary intervention and again 1 month after 6-OHDA lesions. The pharmacologic interventions were done subsequently over the next 2 months (Table 1).

### 6-Hydroxydopamine Lesions

One month after dietary manipulation, the animals were anesthetized with rodent combo III (42.8 mg/mL ketamine, 8.6 mg/mL xylazine, and 1.4 mg/mL acepromazine [Ketam, Xylax, and Acepr]; Northwest Pharmacy 6002671) at the dosage of 50 μL/mouse. A total of 40 mice (20 fed with a normal diet and 20 fed with a low-iron diet) were then stereotaxically injected (mediolateral 0.35 mm, dorsal/ventral 4.5 mm, anteroposterior −1.95 mm) with 1 μL of 0.2% 6-OHDA in 0.01% ascorbic acid saline into the bilateral A11 nuclei (Fig. 1). A total of 40 control sham mice (20 fed with normal diet and 20 fed with an iron-deprived diet) were similarly injected with 0.01% ascorbic acid in 1 μL of saline.

### Locomotor Activity Measurements

Locomotor activities were carried out at different times throughout the study (1 month after iron deprivation, 1 month after 6-OHDA and PBS injection, and 30 minutes after each drug trial). Locomotor activities were measured using a VersaMax Activity Monitor model VMRXZY (Accuscan Instruments, Inc., Columbus, OH). It consisted of a set of four 18- × 18- × 18-inch Plexiglas boxes with vertical and horizontal laser sensors connected through an interface to a microcomputer. To assess spontaneous locomotor activity in a novel environment, individual mice were placed in the Activity Monitor for 30-minute periods to acclimate to the environment before the 60-minute locomotor assessments. One count corresponds to the consecutive interruption of 2 infrared beams. All of the locomotor activities were expressed as distance and the number of movements covered by the mice in a 60-minute period. The

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**TABLE 1. Study Time Line**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lesion-ID</th>
<th>Lesion-Control</th>
<th>Sham-ID</th>
<th>Sham-Control</th>
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<tbody>
<tr>
<td>Number Days 0–28</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
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<tr>
<td>Day 28* Iron deprivation begins</td>
<td>Regular diet</td>
<td>Iron deprivation begins</td>
<td>Regular diet</td>
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<td>Days 58–62 Weaning</td>
<td>Locomotor activities test</td>
<td>Serum iron measurement</td>
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<tr>
<td>Days 62–67 6-OHDA</td>
<td>Locomotor activities test</td>
<td>Serum iron measurement</td>
<td></td>
<td></td>
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<tr>
<td>Days 88–94 6-OHDA Saline</td>
<td>Weight measurement</td>
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<tr>
<td>Days 94–100 Drug intervention (ropinirole, SKF38393, haloperidol)</td>
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<tr>
<td>After Day 100 Killed and TH immunohistochemistry of diencephalon neurons</td>
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<td></td>
<td>Brain and spinal cord iron measurement</td>
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ID, iron deficiency; 6-OHDA, 6-hydroxydopamine; TH, tyrosine hydroxylase.

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**FIGURE 1.** A diagram showing 6-hydroxydopamine (6-OHDA) lesion sites in the mouse A11 nucleus. (A) Simulation of 6-OHDA injection site in a coronal section. (B) Simulation of 6-OHDA injection site in a sagittal section.
Lesion-control 20 34.25
Lesion-ID 20 23.29
Sham-ID 20 16.78

of iron deprivation and 1 month of A11 lesions, paraformaldehyde overnight, followed by 30% sucrose. Perfusion was completed, the brains and the spinal cords were weighed and digested in concentrated hydrochloric acid, using the same kit from DCL in a microtiter plate assay. At 4°C for 1 hour. The peroxidase was revealed by incubation with a rabbit TH antibody (AB152; Chemicon, Temecula, CA) at a dilution of 1:200 in PBS containing 0.1% Triton-X 100 overnight at 4°C. Tissue sections were pretreated with 0.1% hydrogen peroxide and 3% H2O2. The sections were then incubated with avidin-biotinylated horseradish peroxidase complex for 2 hours at room temperature and subsequently incubated with avidin-biotinylated horseradish peroxidase complex for 1 hour. The peroxidase was revealed by incubation with a solution of 0.03% 3,3′-diaminobenzidine tetrahydrochloride containing 0.015% hydrogen peroxide.

Pharmacologic Intervention

One month after surgery, the animals were challenged with several drugs known to improve or exacerbate human RLS. These drugs included the D2/D3 dopamine receptor agonist ropinirole (0.1 mg/kg), the D1 agonist 2,3,4,5-tetrahydro-7,8-dihydropinyl-1H-3-benzazepine (SKF-38393) (5 mg/kg), and the D2 antagonist haloperidol (0.5 mg/kg). All drugs were injected in the peritoneal cavity (SKF-38393) (5 mg/kg), and the D2 antagonist haloperidol (0.1 mg/kg). The primary efficacy point for movement loss in a cryostat (Microtome Cryostat HM 505; MICROM International GmbH, Walldorf, Germany). From the series, the adjacent sections (pair) were collected at systematically placed 30-μm intervals through the whole region. To examine whether the substantia nigra and ventral tegmental areas were affected by 6-OHDA injection in the A11 region, the mesencephalon was also cut at 30-μm thickness, and the slides were picked up at systematically placed 210-μm intervals through the entire mesencephalon region.

Immunohistochemistry

Tyrosine hydroxylase (TH) immunohistochemistry sections were pretreated with 0.1% hydrogen peroxide and washed 3 times for 5 minutes in 0.1 mol/L PBS, followed by incubation with a rabbit TH antibody (AB152; Chemicon, Temecula, CA) at a dilution of 1:2000 in PBS containing 0.1% Triton-X 100 overnight at 4°C. Subsequently, the sections were treated with a biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) at a dilution of 1:200 in PBS for 2 hours at room temperature and subsequently incubated with avidin-biotinylated horseradish peroxidase complex for 1 hour. The peroxidase was revealed by incubation with a solution of 0.03% 3,3′-diaminobenzidine tetrahydrochloride containing 0.015% hydrogen peroxide.

The loss of dopaminergic cells was assessed by comparing the numbers of TH-positive cells in the lesion sites. This was done by an investigator blinded to lesion/iron status. Stained sections were viewed under the Zeiss light microscope at a magnification of 20×. TH-positive cells in the A11 nucleus were counted in all pair sections, whereas TH-positive cells in substantia nigra and ventral tegmental areas were counted in 7 pair sections from each animal, by a stereologic cell quantitation technique. For unbiased cell counting, the dissector technique was used to estimate the number of TH-positive neurons in animal midbrain according to the Calaier principle (13).

Statistics

All the data are expressed as means ± SE. Statistical analyses of data were performed by Student t-test or one-way analysis of variance followed by multiple comparisons between groups. The primary efficacy point for movement

Serial coronal sections of diencephalic regions containing all groups of dopaminergic cells were cut at 30-μm thickness in a cryostat (Microtome Cryostat HM 505; MICROM International GmbH, Walldorf, Germany). From the series, the adjacent sections (pair) were collected at systematically placed 30-μm intervals through the whole region. To examine whether the substantia nigra and ventral tegmental areas were affected by 6-OHDA injection in the A11 region, the mesencephalon was also cut at 30-μm thickness, and the slides were picked up at systematically placed 210-μm intervals through the entire mesencephalon region.

Iron Measurements in Serum, Brain, and Spinal Cord

A serum iron assessment was obtained by tail vein at different times along the study course. Serum was collected and stored at −20°C until use. The serum iron levels were determined calorimetrically by using a kit from DCL (lot 20633; Diagnostic Chemical Limited, Oxford, CT) with modifications for the use in a microtiter plate assay. At autopsy, brain (striatum and cortex) and whole spinal cords were weighed and digested in concentrated hydrochloric acid. Tissue iron concentrations (micrograms of iron per gram of tissue) were determined spectrophotometrically by using the same kit from DCL in a microtiter plate assay.

Cryostat Section

The mice were killed after 3 months of behavioral testing. They were deeply anesthetized with rodent combo III intraperitoneally and fixed by transcardial perfusion with 50 mL of ice-cold 0.1 mol/L PBS (pH 7.4). When the perfusion was completed, the brains and the spinal cords were removed. The A11 regions were fixed in 4% paraformaldehyde overnight, followed by 30% sucrose.
RESULTS

Iron Measurement in Serum and CNS

After 1 month of the specialized diet, there was a 46 ± 2.4% reduction of serum iron in the iron deprivation mice compared with the normal diet mice (n = 40 per group, \( p < 0.01 \)) (Table 2).

After lesioning, there was a significant reduction in the serum iron, spinal cord iron, and brain iron in lesion-ID and sham-ID groups compared with lesion-control and sham-control groups (Table 2). The lesion-ID group showed a significantly greater CNS iron loss than the sham-ID group. In fact, even in the normal iron diet groups, the mice with A11 lesions demonstrated lower CNS iron stores than those who had saline injections. Interestingly, iron deprivation in the A11-lesioned animals resulted in much more relative iron loss in the spinal cord than in the brain.

Histology

The section analysis of TH-positive staining neurons in A11 region demonstrated a significant decrease in both lesion-ID (4.2 ± 1.3 cells) and lesion-control groups (5.2 ± 1.5 cells) compared with both sham-ID (66 ± 11 cells) and sham-control groups (65 ± 10 cells). Overall, a 94 ± 8.2% reduction of TH-positive neurons in the A11 region was observed.
found in mice after the 6-OHDA lesion (p < 0.01) (Figs. 2, 3). The numbers of TH-positive neurons in other dopaminergic regions (A12, A13, A14, and A9) were not significantly different in the 6-OHDA-lesioned mice, although there were slight trends suggesting minimal diffusion of the toxin (Figs. 2, 3). There was also no significant difference in cell counts between the lesion-ID and lesion-control groups, suggesting that 1 month of iron deprivation does not alter viability against 6-OHDA toxicity.

**Measurements of Locomotion**

All animals were measured for locomotor activities at 2 months after iron deprivation and 1 month after 6-OHDA or saline injections in A11 nuclei. Iron deprivation alone significantly increased our primary efficacy point, total locomotor activities (p < 0.01) (Fig. 4). 6-OHDA lesioning in A11 nuclei alone significantly increased total locomotor activities (p < 0.01). The combination of iron deprivation and 6-OHDA lesion significantly augmented total locomotor activities compared with iron derivation or 6-OHDA lesioning alone.

**Intervention with Dopaminergic Drugs**

Ropinirole, a D2/D3 agonist shown to improve human RLS, significantly reduced most movement measures in the combined lesion-ID group compared to the other 3 groups (Fig. 5A). Importantly, there was no significant motor effect of ropinirole in the lesion-control and sham-ID group versus each other or against the sham-control group. The D1 agonist SKF-38393 actually increased movement in all iron-deprived and A11-lesioned groups, especially in the combined lesion-ID group (Fig. 5B). In marked contrast, movement was reduced in the sham-control group. Haloperidol, a nonspecific dopamine antagonist, increased all movements in all groups (Fig. 5C). Interestingly, motor activity increased the most in the sham-control group.

**General Behavior**

Among the 4 groups, there were no differences in weight, survivability, or any other overt systemic illness. The mice in the lesion-ID group demonstrated much more aggression and attack behavior. This effect was not quantified except that 4 mice in this group were injured by others in the same group. Aggressive behaviors were not observed in the other 3 groups. There was no evidence of mice eating their own stool or attacking other mice for food as part of an iron craving (R. P. Allen, personal communication, 2006). Unlike our previous rat model, the mice did not demonstrate a nonfatigu-ing exaggerated startle response. No overt periodic limb movements in sleep were observed, but these would be difficult to see as mice cover their legs during sleep, and we did not otherwise evaluate them for periodic limb movements in sleep except for simple observation.

**DISCUSSION**

In our study, we sought to create a model consistent with RLS in mice on the basis of current theories of RLS pathogenesis. We demonstrated that increased locomotion was seen in both A11-lesioned mice and dietary iron-deprived mice compared with controls. Combined A11-lesioned and iron-deprived mice demonstrated significantly greater activity and aggressive behavior. The hyperactivity in the model was normalized after administration of a D2/D3 agonist; however, a D1 agonist actually increased movement in the model despite significantly reducing movement in the controls. Application of a D2 antagonist, haloperidol, increased the locomotor activity in all groups, especially controls.

Animal models cannot assess the subjective urge to move required for a clinical diagnosis of RLS. Nevertheless, the model, including drug effects, is consistent with the motor component of human RLS but could also be consistent with that in other hyperactive states such as anxiety, akathisia, or attention deficit hyperactivity disorder. The drug effects are especially provocative. Clinical RLS also robustly improves with D2/D3 agonists. The lack of improvement with ropinirole in A11-lesioned animals with normal iron intake suggests that the pharmacologic effect is more complicated than simple replacement of lost neuronal endogenous dopamine with an agonist. The dramatic difference between D2 family and D1 family receptor stimulation suggests that the lesioned tracts directly subserve D2 family receptors that may be in some unknown reciprocal inhibition with a D1 receptor group. This finding is consistent with the opposite effects that iron deprivation has on spinal D2 and D1 receptor activity (14). The clinical effects of isolated D1 agonists on human RLS are not known.

The dopamine antagonist haloperidol anecdotally worsens clinical RLS in at least some patients, but this finding is not consistent and formal studies are lacking. Anecdotally, this response is often delayed. The lack of model-specific increased activity from haloperidol, which tended to increase more in the control groups, may reflect the large dose used and the fact that there was very little additional dopamine to block in these pathways that incurred a 94% cell loss, such that the controls actually had more potential for change.
The increased aggression is interesting and only occurred in the combined A11-lesioned and iron-deprived group. This finding could possibly reflect the response to an unpleasant sensation or urge to move, the effects of sleep deprivation (not assessed), increased anxiety, or some direct effect on aggressive or behavioral disinhibition. Human RLS is associated with increased anxiety, which could represent a phenotypic correlate (15).

The most interesting histologic finding is the relationship between dopamine cell loss and iron stores. Dietary iron restrictions reduced CNS tissue stores of iron as expected. Lesioning of the A11 nucleus, however, appeared to augment this loss, especially in the spinal cord. Relatively reduced CNS iron was also seen in the A11-lesioned mice with normal dietary intake. This finding is especially noteworthy as the human pathology in RLS demonstrates reduced brain stores of iron that are currently unexplained (4). We cannot exclude the possibility that 6-OHDA directly inhibits iron acquisition independent of dopamine cell loss, but find this unlikely for 2 reasons: 1) the time lag between the 6-OHDA administration and the CNS iron measurement, and 2) the cord, which was subserved by the lesioned cells, was more iron deficient than the brain.

Several theories have been posited to explain how the primary iron reduction seen in RLS could impair dopaminergic function. Dopaminergic systems can be theoretically impaired during ID by 1) lowering dopamine production via reduced TH activity that requires iron, 2) downregulation of dopamine type 2 receptors that require iron, and/or 3) destabilizing dopaminergic synapses via Thy-1 downregulation, which also requires iron for its activity (16, 17). The possibility that a primary dopaminergic dysfunction could cause CNS iron loss has never been postulated and requires further study in humans and animal models.

The acquisition and regulation of iron in the brain is a complex age-related and region-dependent process that is tightly controlled at the blood-brain barrier. ID in early life also alters neuronal development, has been associated with behavioral abnormalities, including reduced attention, hyperactivity, and an increased risk of akathisia in humans (18–20), and has produced a variety of histologic and behavioral abnormalities in rats (21). The exact relationship between iron and dopamine in human RLS is not known.

In summary, our model, as any prospective animal model, cannot claim to meet the 4 cardinal criteria for RLS. The increased volitional movement seen in our model and response to medications are consistent with human RLS but also with those seen in other hyperkinetic states such as attention deficit hyperactivity disorder, which is associated with RLS. To our knowledge, this is also the first brain lesion to result in increased normal movement while animals are awake. Future behavioral assessments could better define this behavior by inclusion of sleep studies, such as the evaluation of periodic limb movements in sleep, anxiety assessments, specific tests for aggression, learning, and pain sensitivity thresholds.

REFERENCES