MELATONIN PREVENTS LEARNING DISORDERS IN BRAIN-LESIONED NEWBORN MICE

M. BOUSLAMA, a,b J. RENAUD, a,b P. OLIVIER, a,b R. H. FONTAINE, a,b B. MATROT, a,b P. GRESSENS a,b AND J. GALLEGO a,b*

aINSERM, U676, Robert Debré Hospital, 48 bvd Sérurier, 75019 Paris, France
bParis 7 University, Denis Diderot Medical School, IFR02 and IFR 25, 10 av de Verdun, 75010 Paris, France

Abstract—Perinatal brain injuries often result in irreversible learning disabilities, which manifest in early childhood. These injuries are chiefly ascribable to marked susceptibility of the immature brain to glutamate-induced excitotoxicity. No treatments are available. One well-characterized model of perinatal brain injuries consists in injecting the glutamate analog ibotenate into the brain of 5-day-old mice. The resulting excitotoxic lesions resemble the hypoxic–ischemic gray-matter lesions seen in full-term and near-term newborns, as well as the white-matter lesions of preterm newborns. We previously reported that these lesions disrupted odor preference conditioning in newborn mice. The aim of this study was to assess the effectiveness of the neuroprotector melatonin in preventing learning disabilities in newborn mice with ibotenate-induced brain injury. In postnatal day (P) 6–P7 pups, we tested psychomotor reflexes, spontaneous preference for maternal odors as an index of memory, ultrasonic vocalization responses to stroking as an index of sensitivity to tactile stimuli, and conditioned preference for an odor previously paired with stroking as an index of learning abilities. Without melatonin, conditioning was abolished, whereas spontaneous odor preference, psychomotor reflexes, and sensitivity to tactile stimuli were normal. Thus, abolition of conditioning was not associated with sensorimotor impairments. Histological analysis confirmed the efficacy of melatonin in reducing white-matter lesions induced by ibotenate. Furthermore, treatment with melatonin protected the ability to develop conditioning. Thus, melatonin, which easily crosses the blood–brain barrier and has been proven safe in children (Weiss et al., 2006), may be effective in preventing learning disabilities caused by perinatal brain injuries in human preterm infants. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: excitotoxicity, prematurity, white matter, gray matter, conditioning, neuromodulation.

Many motor and cognitive disorders of early childhood, most notably learning disabilities, are caused by perinatal brain injuries (Wood et al., 2005) that are chiefly ascribable to marked susceptibility of the immature brain to glutamate-induced excitotoxicity (Johnston, 2005). The incidence of such learning disorders is increasing in developed countries, in part due to increases in the number of pre-term deliveries and in survival rates of preterm newborns (Hack et al., 2005; Hintz et al., 2005; Marlow et al., 2005). No treatments are available for the prevention of perinatal brain injuries and their functional consequences, which is therefore a focus of active research.

Mouse models of perinatal brain injuries have proven useful for identifying potential neuroprotective agents (Northington, 2006). One well-characterized model consists in injecting the glutamate analog ibotenate intracerebrally in 5-day-old mice (Ikonomidou et al., 1989a; Olney et al., 1989; Gressens et al., 1997; Dommergues et al., 2000; Johnston, 2005). Ibotenate activates NMDA and metabotropic receptors and produces brain lesions that resemble the hypoxic–ischemic gray-matter lesions of full-term and near-term newborns, as well as the white-matter lesions of preterm newborns (Ikonomidou et al., 1989a,b; Olney et al., 1989; Gressens et al., 1997; Dommergues et al., 2000; Johnston, 2005). Several treatments administered intraperitoneally have been found to reduce the size of ibotenate-induced brain lesions in mouse pups, suggesting potential neuroprotective effects in human newborns (Husson et al., 2002; Bouslama et al., 2006). Among these treatments, melatonin is particularly promising, since it easily crosses the blood–brain barrier and has been proven safe in children (Weiss et al., 2006). However, its effectiveness in preventing cognitive disorders in newborn mice (or other species) has not been examined. Establishing that melatonin preserves function in pre-clinical studies is a crucial step toward evaluating the usefulness of melatonin in human newborns.

The aim of the present study was to assess the effects of melatonin treatment on cognitive function in newborn mice with ibotenate-induced brain lesions. We previously reported that 6/7-day-old mice with ibotenate-induced brain lesions showed normal weights, breathing patterns, and preferences for maternal odors, suggesting apparently normal general status. In contrast, conditioned preference for odors previously paired with stroking (a surrogate of maternal care) was abolished, suggesting disruption of associative abilities (Bouslama et al., 2005, 2006). Here, we tested the effectiveness of melatonin in preventing these associative learning deficits. Furthermore, we examined whether the learning deficits in ibotenate-lesioned pups were associated with sensorimotor impairments. To do this, we used Fox-battery psychomotor tests suitable for use on P6 and P7 (Fox, 1965), and we analyzed respon-
siveness to tactile stimulation by measuring the ultrasonic vocalization (USV) response to stroking.

**EXPERIMENTAL PROCEDURES**

**Animals**

Mouse pups (N=118) were obtained from Swiss female mice (IFAA-CREDO, L’Arbresle, France) housed at 24 °C with a 12-h light/dark cycle and free access to food and water. Twelve litters were used. Melatonin and ibotenate were given on postnatal day (P) 5 (day of birth: P0). Behavioral tests were run on P6 and P7. Experimental protocols were approved by our institutional review board, met INSERM guidelines, and complied with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Every effort was made to minimize the number of animals used and their suffering.

**Treatments**

Excitotoxic brain lesions were caused by injecting ibotenate (Sigma, St.-Quentin Fallavier, France) on P5, as previously described (Marret et al., 1995; Gressens et al., 1997, 1998; Dommergues et al., 2000; Laudenbach et al., 2001; Tahraoui et al., 2001; Husson et al., 2002; Bouslama et al., 2006). Ibotenate was diluted in phosphate-buffer saline (PBS) containing 0.01% acetic acid (final pH, 5.15). Briefly, each lightly anesthetized pup was placed under a warming lamp. Ibotenate was injected into the neopallial parenchyma using a 25-gauge needle on a 50-μl Hamilton syringe (Massay, France), which was mounted on a calibrated microdispenser attached to a mechanically rigid holder. The needle was inserted 2 mm under the external surface of the scalp in the frontoparietal region of the right hemisphere, 1 mm anterior to the bregma in the rostro-caudal plane. Two 1-mm boluses (5 μg each) of ibotenate (Tocris, diluted in 0.02% acetic acid, 0.1 M, PBS) were injected 30 s apart. The needle was left in place for 20 s after the second bolus. The diffusion of ibotenate can be estimated between 0.5 and 1 mm³ (based on the injection of Toluidine Blue, data not shown). Previous studies established that the tip of the needle consistently reached the periventricular white matter (Husson et al., 2005) and that the 10–μg dose of ibotenate consistently caused brain damage, as further confirmed in the present study in a subsample of pups (see Lesion size determination below). Melatonin was diluted in PBS DMSO 40% (5 mg/kg; final pH, 7.35) and injected intraperitoneally using a 26-gauge needle on a 50-μl Hamilton syringe. All i.p. injections (melatonin or PBS DMSO 40%) were given 15 min before the intracerebral injection of ibotenate or PBS. The injections were carried out between 5 p.m. and 7 p.m.

**Design**

Within each litter, the pups were randomly allocated to intracerebral ibotenate or PBS. In the main study, within each of these two groups, the pups were randomly assigned to i.p. melatonin or PBS. Thus, we obtained four treatment groups: ibotenate+PBS, ibotenate+melatonin, PBS+PBS, and PBS+melatonin. In addition, an untreated group was examined to investigate possible effects of experimental manipulation. Thus, about 20% of each litter (i.e., one or two pups per litter) was randomly allocated to each treatment group, thus precluding any bias caused by between- or within-litter factors. In a complementary study, the ultrasonic response to stroking was assessed in ibotenate and PBS groups only.

**Psychomotor reflexes**

We used three Fox-battery tests (Fox, 1965) that were suitable for use on P6 and P7. For all tests, each pup was placed on a non-slip soft surface made of foam. The maximum time for each test was set at 20 s.

**Righting reflex.** Each pup was placed on its back on the foam. We recorded the time needed by the pup to regain its feet.

**Negative geotaxis.** Each pup was placed with the head facing downward on a 45% incline. We recorded the time needed for the pup to turn until it faced up the slope.

**Cliff-drop aversion reflex.** Each pup was placed on the edge of an elevated platform, with both front paws over the edge. We recorded the time needed for the pup to crawl away from the edge.

**Maternal odor preference test**

Spontaneous preference for home-litter odor was tested on P6 and P7, between 9:00 a.m. and noon. Two plastic boxes (7 cm high, 12 cm long, and 8 cm wide) were placed 2.5 cm apart and covered with a single metallic mesh floor. One box was filled with home litter and the other with clean litter. A Plexiglas plate (2.5 cm by 12 cm) was secured to the mesh above the space between the two boxes and delimited a neutral zone. The temperature on the mesh was maintained at 31–33 °C by two heating lamps placed symmetrically about 30–40 cm above the boxes.

During each test, the pup was placed in the middle of the neutral zone (parallel to the edges of the boxes on either side. The experimenter measured the time spent over each odor, i.e. with at least its head toward the odor beyond the edge of the neutral zone, which defined odor preference. The space was sufficiently narrow (2.5 cm) that a movement of the head was sufficient to assess odor preference. As soon as the pup’s snout crossed one of the edges of the neutral zone, a timer was started to measure the time spent over the corresponding odor. The test was performed five times in each pup, changing the direction in which the pup was placed on the neutral zone between consecutive tests. The mesh and the neutral zone were wiped carefully between pups; they were not wiped between tests, to minimize pup manipulation and test duration.

**Conditioned odor preference for artificial odors paired with stroking**

The above-described setup was used, using one box filled with pine shavings sprinkled with 1 ml of 97% menthol acetate (peppermint odor, Aldrich, Steinheim, Germany) and the other with pine shavings sprinkled with 1 ml of 97% Limonen (lemon odor, Aldrich). We used a classic conditioning paradigm previously validated in newborn mice (Bouslama et al., 2005, 2006). Within each of the four treatment groups, the pups were randomly assigned to the lemon conditioned stimulus (CS⁺) group or the peppermint CS⁻ stimulus. For acquisition, the pup was placed over the CS⁺ odor (lemon or peppermint) and stroked gently with a paintbrush for 30 s, which served as a surrogate for maternal care, as well as over the CS⁻ (the other odor) for 30 s without stroking. During acquisition, 10 CS⁺/unconditioned stimulus presentations alternated with 10 CS⁻ presentations. The first acquisition trial was CS⁺ in half the pups and CS⁻ in the other half. This sequence was repeated 10 times (total acquisition duration, 10 min). Immediately after acquisition, conditioning was assessed using five preference tests in each pup. Conditioning experiments were carried out on P6 and P7 between 1:00 pm and 6:00 pm. Conditioning scores were computed as 100×[(T (CS⁺)−T (CS⁻))/T (CS⁺)−T (CS⁻)], where T (CS⁺) and T (CS⁻) were the total times spent over the CS⁺ and the CS⁻, respectively.

**Responsiveness to tactile stimuli**

In a complementary experiment, we assessed responsiveness to tactile stimuli in a separate group of 6-day-old pups. The pups...
were allocated at random to either ibotenate \((n=22);\) mean weight: 3.55 g (S.D. 0.59); and mean body temperature: 32.7 °C (S.D. 1.3) or PBS \((n=22);\) mean weight: 3.53 g (S.D. 0.49); and mean body temperature: 32.9 °C (standard deviation (S.D.) 1). The pups were tested on P6, between 10:00 a.m. and 6:00 p.m. A plastic box (7 cm high, 12 cm long, and 8 cm wide) was filled with clean litter to expose the pups to a neutral odor. The box was covered with a metallic mesh floor. A heating lamp was used to maintain temperature in the box at 26–27 °C, a cold temperature at which newborn mouse pups are more likely to produce USVs, compared with thermoneutrality. Each pup was extracted from the litter and placed on the mesh. Then, 10 30-s periods of stroking alternated with 10 30-s periods without stroking (total duration, 10 min). The sequence started with stroking in half the pups and with no-stroking in the other half. USVs were continuously recorded using an ultrasound bat detector D230 (Petterton Elektronik AB, Uppsala, Sweden). The microphone was placed 2 cm above the mesh floor. The frequency range of the detector (10–120 kHz) included the frequency range of USVs in newborn mice (30–90 kHz (Branchi et al., 2001)). Sounds were digitized at 200 kHz (16 bits, PCI 6143, National Instruments, Austin, TX, USA) and stored on disk using custom-written software (Labview, National Instruments). Total USV durations were calculated over consecutive 10-s periods. In a preliminary experiment, we checked that USVs were not contaminated by parasite ultrasonic sounds produced by friction of the brush during stroking. To this aim, we recorded USVs in four anesthetized pups (compared with four non-anesthetized pups) exposed to the above protocol. Stroking did not produce any ultrasounds, establishing the validity of USV recording during stroking.

**Lesion size determination**

Lesion sizes were determined in randomly chosen small samples of pups within the ibotenate+PBS, ibotenate+melatonin, and PBS+PBS groups \((n=6, n=7,\) and \(n=6,\) respectively). The pups were decapitated 5 days after intracerebral ibotenate injection on P5, and the brains were fixed in 4% formaldehyde for 5 days. After embedding in paraffin, 15-μm sections were cut in the coronal plane, from the frontal to the occipital pole. Every third section was stained with Cresyl Violet. Total lesion volume was measured using the NeuroLucida software-controlled computer system (MicroBrightField Europe, Magdeburg, Germany).

**Statistics**

All variables were evaluated using standard analyses of variance (ANOVA) with treatment as the between-subject factor (Superanova Software, Abacus Concepts, Berkeley, CA, USA, and Statview 5, SAS Institute Inc., NC, USA). In conditioning experiments, the time spent over the odors in the preference tests was analyzed with CS (CS+ versus CS−) and test number (1 through 5) as the within-subject factors. In the USV study, USV duration was analyzed with stimulation (stroking versus no stroking), test number (1 through 10), and periods within each 30-s test (three 10-s periods) as within-subject factors. Litter effects on treatments were not significant for either analysis, and this factor will not be mentioned further. To take into account the heterogeneity of correlations among repeated measurements, we adjusted the degrees of freedom using the Greenhouse-Geisser factor, which is a conservative correction procedure (Keselman and Keselman, 1984). Within-subject main effects and interactions are reported, together with \(P\) values based on these adjusted degrees of freedom. To examine the relationship between brain-damage severity and conditioning scores, we used the nonparametric Wilcoxon rank test and linear correlation coefficients. Values are means±standard deviation (S.D.) in the text and means±standard error of the mean (standard error of the mean (S.E.M.)) in the figures.

**RESULTS**

**Ibotenate-induced brain lesions and neuroprotection by melatonin**

Overall, mortality was low (<3% in all treatment groups). Ibotenate treatment induced neocortical gray-matter and white-matter injury (main effect for treatment: cortical gray matter, \(F(2,16)=234.64\) and \(P<0.0001;\) white matter, \(F(2,16)=50.47\) and \(P<0.0001;\) and partial comparisons shown in Fig. 1). Melatonin treatment reduced the white-matter lesions without affecting the gray-matter lesions (Fig. 1).

**Psychomotor reflexes were preserved in pups with ibotenate-induced brain lesions**

All pups given ibotenate displayed tonic–clonic seizures within the first 24 h following ibotenate injection. Body weight, temperature, and psychomotor reflexes were very similar across treatment groups (Table 1). Thus, ibotenate injections had no major effects on general status or psychomotor reflexes after 24 h.

**Spontaneous odor preference was preserved in pups with ibotenate-induced brain lesions**

Spontaneous preference for home-litter odor was not significantly affected by ibotenate treatment (Fig. 2A). In all treatment groups, the time spent over the home litter was significantly longer than the time spent over the clean litter (main effect for odor: \(F(1,91)=389.48\) and \(P<0.0001;\) and pairwise comparisons shown in Fig. 2A; group differences were not significant). Thus, neither olfactory function nor the motor abilities needed to express odor preference were impaired in pups with ibotenate-induced lesions.

**Conditioning was abolished in pups with ibotenate-induced brain lesions but was preserved by melatonin treatment**

Pups given intracerebral PBS spent significantly more time over the odor paired with stroking (CS+) than over the other odor (CS−, Fig. 2B), a result that demonstrated conditioning. Conditioning was completely abolished in the pups given ibotenate without melatonin (Fig. 4B, CS by treatment interaction, \(F(4,91)=15.82\) and \(P<0.0001;\) and pairwise comparisons shown in Fig. 2B). With melatonin treatment, significant conditioning developed despite ibotenate injection (CS+ versus CS−, \(P<0.05,\) Fig. 2B). Compared with i.p. PBS, i.p. melatonin did not improve conditioning in pups without ibotenate treatment. Thus, melatonin treatment preserved conditioning abilities in ibotenate-treated pups.

**Correlation between lesion size and conditioning score**

An overall correlation was found between white-matter lesion size and conditioning score \((R^2=0.50, P<0.0007,\) Fig. 3). However, correlations within treatment groups were not significant. The size of gray-matter lesions showed small interindividual differences among pups with
or without melatonin treatment, and no significant correlations were found between gray-matter lesion size and conditioning scores (Fig. 3).

**Responsiveness to stroking was preserved in pups with ibotenate-induced brain lesions**

Stroking elicited closely similar USV responses in ibotenate-lesioned and control groups (main effect for stroking, $F(1,40)=52.82$ and $P<0.0001$, Fig. 4). USV durations decreased markedly within each test (main effect of periods: $F(2,80)=62.51$ and $P<0.0001$). In the first three tests, USV duration returned to high levels by the beginning of the next test. Subsequently, USV duration decreased across trials (main effect of test: $F(1,9)=16.96$ and $P<0.0001$). Both within- and between-trial decreases in USV duration suggested habituation. Group differences were not significant in any of the analyses.

**DISCUSSION**

This study confirmed that ibotenate-induced brain lesions abolished conditioned preference for an artificial odor previously paired with stroking in newborn mice, whereas spontaneous preference for maternal odor was preserved. Furthermore, psychomotor abilities and ultrasonic responses to stroking were normal in pups with brain lesions, suggesting that abolition of conditioning was not associated with sensorimotor impairments. Importantly, melatonin treatment prevented the loss of conditioning ability in pups with induced brain lesions, supporting melatonin as a good candidate for treating perinatal brain injuries in human preterm infants.

**Neuroprotection by melatonin**

Histological findings from the present study confirmed that pre-treatment with melatonin protected against excitotoxic lesions induced by ibotenate in newborn mice (Husson et al., 2002). The ibotenate-induced white matter lesion is characterized by oligodendroglial death and axonal breakdown, both secondary to microglial activation and astrocyte death (Tahraoui et al., 2001). Melatonin treatment reduced the white-matter lesions without affecting the gray-matter lesions. Previous studies showed that melatonin did not prevent lesion induction but, instead, induced a secondary decrease in the white-matter lesion (Husson et al., 2002). Within 24 h after ibotenate and melatonin injections, a marker of axonal growth was expressed throughout the site of injection, showing that the resolution of the lesion was accompanied by axonal regrowth or sprouting (Husson et al., 2002). Whether melatonin actually promoted the axonal regrowth or sprouting or just attenuated the section of the right hemisphere from a pup treated with i.p. PBS followed by intracerebral ibotenate: note the typical neuronal loss in layers 2–6 (arrow) and the periventricular white-matter cystic lesion. (B) Brain from a pup treated with i.p. melatonin followed by intracerebral ibotenate. Melatonin protected the white matter against ibotenate-induced lesions without affecting the cortical gray matter. LV: lateral ventricle; CC: corpus callosum; ic: intracerebral. (C) Lesion sizes. Values are means±S.E.M.

![Fig. 1. Melatonin protects against ibotenate-induced white-matter lesions. Cresyl Violet sections showing brain lesions induced by ibotenate. The pups were injected on P5 and studied on P10. (A) Coronal section of the right hemisphere from a pup treated with i.p. PBS followed by intracerebral ibotenate: note the typical neuronal loss in layers 2–6 (arrow) and the periventricular white-matter cystic lesion. (B) Brain from a pup treated with i.p. melatonin followed by intracerebral ibotenate. Melatonin protected the white matter against ibotenate-induced lesions without affecting the cortical gray matter. LV: lateral ventricle; CC: corpus callosum; ic: intracerebral. (C) Lesion sizes. Values are means±S.E.M.](image-url)
the existing inhibition on these processes is not known. Furthermore, immunohistochemistry and TUNEL staining showed that melatonin delayed the ibotenate-induced microglial activation, without blunting it, and moderately reduced neuronal cell death (Husson et al., 2002). Whether synaptic transmission was influenced by the melatonin treatment in the present conditions is not known. Melatonin effects were blocked by specific melatonin-receptor antagonists and by activators of adenylate cyclase, which is normally inhibited by activation of the melatonin-receptor signaling pathway (Husson et al., 2002). Thus, the neuroprotective effects of melatonin were due to specific effects on specific receptors rather than to general antioxidant properties (Husson et al., 2002). The present study thus confirmed the efficacy of melatonin in reducing the size of ibotenate-induced white-matter lesions. As a rule, learning abilities cannot be directly inferred from lesion size. However, our conditioning data established that histological effects in melatonin-treated pups were accompanied by effective prevention of learning deficits.

Motor and sensory abilities were spared in brain-lesioned pups

All three psychomotor tests (righting reflex, negative geotaxis and cliff-drop aversion reflex) yielded closely similar results in brain-lesioned pups and PBS controls, suggesting normal motor and somatosensory functions in ibotenate-treated animals. We previously reported that important markers of neonatal status, such as respiratory and defense responses to hypoxia, were unaltered in brain-lesioned pups (Bouslama et al., 2006). The close similarity of body temperatures across treatment groups indicated that ibotenate-induced brain lesions had no major effect on autonomic system functions. Spontaneous preference for home litter was fully preserved in brain-lesioned pups, suggesting normal olfaction. The ultrasonic response to stroking was normal, indicating that perception of tactile stimuli was spared. In 5-day-old mouse pups, sensory information is limited to somatosensory inputs, mainly thermotactile in nature, and to chemical sensations, among which olfaction predominates.

The decrease of the ultrasonic response to stroking within each 30-s test and across tests was highly suggestive of habituation, which is characterized by decreasing strength of a response to a stimulus that is presented repeatedly (Groves and Thompson, 1970). Under this interpretation, habituation, a major form of non-associative learning, seems to have been spared in brain-lesioned pups in our study (whereas conditioning was lost). However, non-specific factors, such as fatigue, may produce a decreased response that may mimic habituation. The possibility that fatigue accounted for the decrease in USV with repeated stroking cannot be eliminated based on our data.

Assessment of associative abilities in newborn mice

In the conditioning study, the acquisition phase consisted in pairing an artificial odor (the CS+) with a tactile stimulus designed to resemble maternal care. Mother-seeking behavior is naturally present in newborn mice (and all new-

**Table 1. Treatment groups**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PBS</th>
<th>IBO</th>
<th>PBS</th>
<th>MEL</th>
<th>MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p.</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>—</td>
<td>PBS</td>
<td>IBO</td>
<td>IBO</td>
<td>PBS</td>
</tr>
<tr>
<td>N</td>
<td>16</td>
<td>20</td>
<td>20</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>3.73±0.56</td>
<td>3.54±0.64</td>
<td>3.49±0.62</td>
<td>3.58±0.50</td>
<td>3.41±0.71</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>34.10±1.23</td>
<td>34.57±0.97</td>
<td>34.21±1.29</td>
<td>34.18±1.17</td>
<td>33.89±1.68</td>
</tr>
<tr>
<td>RR (s)</td>
<td>2.00±3.44</td>
<td>1.65±1.75</td>
<td>2.35±3.40</td>
<td>2.45±3.47</td>
<td>2.08±3.21</td>
</tr>
<tr>
<td>NG (s)</td>
<td>9.62±7.85</td>
<td>8.65±8.01</td>
<td>9.50±7.88</td>
<td>7.95±7.13</td>
<td>10.03±7.60</td>
</tr>
<tr>
<td>CDA (s)</td>
<td>5.91±6.44</td>
<td>5.07±5.95</td>
<td>6.20±6.59</td>
<td>6.91±6.99</td>
<td>6.58±7.04</td>
</tr>
</tbody>
</table>

Weights and body temperatures were measured on P6. Righting reflex (RR), negative geotaxis (NG) and cliff-drop aversion (CDA) were measured on P6 and P7. Age had no significant effect on scores, as a main effect or in interaction with treatment. The values on P6 and P7 were averaged. Values are means±S.D. IBO: intracerebral injection of ibotenate on P5. MEL: i.p. injection of melatonin 15 min before ibotenate (or PBS) injection. Differences between groups were not significant.

**Fig. 2.** Spontaneous and conditioned odor preference. (A) Preference for home-litter odor (*P*<0.001 in each group; group differences were not significant). (B) Conditioned odor preference (i.e. longer time over the CS+) developed in the control groups, whereas it did not in the treatment groups. i.c.: intracerebral; IBO: ibotenate; MEL: melatonin. Values are means±S.E.M. *** *P*<0.001; ** *P*<0.01; * *P*<0.05.
borns of altricial species), and pups rely chiefly on odor to locate their mother. When exposed to the paired (CS+) and unpaired (CS−) odor during the test, the pups exhibited a preference for the CS+, a result that demonstrated conditioning. Because lemon was the CS+ and peppermint the CS− in half the pups, and the reverse in the other half, the conditioned preference for the CS+ cannot be ascribed to pre-existing preference for one of the two odors.

We used odors and stroking as the conditioned and unconditioned stimuli, respectively, because somatosensory inputs and olfaction are well developed in newborn mice. Odors also play a critical role in generating physiological and behavioral responses to extra-uterine stimuli in preterm infants (Schaal et al., 2004). Visual or auditory stimuli, which are often used as conditioned stimuli in adult animals, were not suitable for our study, since mice acquire hearing around P12 and vision around P14. Our study confirms that early associative abilities can be assessed in newborn mice despite the immaturity of most of the sensory functions, using developmentally appropriate conditioning protocols.

**Abolition of conditioning in newborn mice with brain injury**

Conditioning was completely abolished in pups with ibotenate-induced brain lesions, in keeping with earlier results (Bouslama et al., 2006). Importantly, our results establish that loss of odor conditioning in ibotenate-treated pups was not ascribable to motor or sensory impairments. As noted above, righting, negative geotaxis, and cliff-drop aversion reflexes were normal in pups with ibotenate-induced brain lesions. Furthermore, tests of spontaneous preference tests for maternal odors (which yielded nearly identical results in brain-injured and control pups) showed that expression of odor preference was preserved in the pups with brain lesions. Finally, ultrasonic responses to tactile stimuli, under the same conditions as in the conditioning experiments, were closely similar in brain-injured and control pups, suggesting similar perception of stroking. Taken together, these results strongly support impairment of associative abilities, but not of motor or sensory function, in pups with ibotenate-induced brain lesions.

**Protection of learning by melatonin in newborn mice**

In contrast with brain-injured pups treated with PBS, those treated with melatonin showed normal conditioning. Melatonin did not significantly improve conditioning in pups that had no brain lesions (PBS+melatonin group). Furthermore, the 24 to 48-h interval between melatonin administration (P5) and behavioral testing (P6–P7) indicates that the protection afforded by melatonin cannot be ascribed to acute effects. Finally, melatonin did not affect the total time...
spent over either the CS+ or the CS−, which indirectly reflected the global activity level during the test for conditioning. This observation further supports absence of potential acute melatonin effects on behavior at the time of behavioral testing. Taken together, these results indicate that melatonin prevented the learning disabilities associated with excitotoxic brain lesions. However, several aspects of the beneficial effects of melatonin have not been addressed in the present study. In particular, the effects of the time of injection (15 min before the intracerebral injection of ibotenate in the present study) and of the age of the pups were not investigated. The dose of melatonin (5 g/kg) was determined based on a previous study that showed that this value corresponded to the maximal neuroprotection.

The beneficial effects of melatonin in ibotenate-treated pups probably stemmed from protection against white-matter lesions. White-matter lesions can damage both the commissural fibers that allow interhemispheric communication and the association fibers that connect neocortical areas, thereby leading to learning disabilities (Filley, 1998).

Clinical implications
Melatonin is a good candidate for clinical studies in human newborns at risk for developing brain lesions. In several animal models, melatonin protects the developing brain against white-matter damage (Husson et al., 2002; Welin et al., 2007), which is the hallmark of brain damage in preterm infants. Safety data are available from the widespread use of melatonin in adults to treat jet-lag syndrome and other sleep disorders, as well as for premedication (Penev and Zee, 1997; Bersani and Garavini, 2000; Naguib and Samarkandi, 2000); from use in children with various neurologically disabling disorders to improve sleep patterns and learning disabilities (Gordon, 2000); and, recently, from administration to neonates with sepsis (Gitto et al., 2004). In none of these studies were any major side effects reported. There is general agreement that short-term melatonin therapy in safe in neonates, even at pharmacological doses (Jan et al., 2007). The present dose (5 mg/kg intraperitoneally) was within the range of doses previously used in infants (e.g. 10 i.v. injections of melatonin, 10 mg/kg each (Gitto et al., 2005)). However, long term effects of early melatonin treatments have not been investigated. Preclinical studies in developing animals may be mandatory to analyze these possible long-term effects. The present study provides further experimental support for considering that melatonin is a promising candidate for treating preterm human infants with white-matter damage.

Acknowledgments—This work was supported by four nonprofit sources: the INSERM, the Paris 7 University, the French Ministry of Research (grants to M.B. and J.R.) and the Fondation Garches. We are grateful to Frédéric Dambricourt for developing the USV processing software and to Leslie Schwendimann for her excellent technical assistance.

REFERENCES

(Accepted 18 September 2007)
(Available online 19 September 2007)