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MELATONIN PREVENTS LEARNING DISORDERS IN BRAIN-LESIONED NEWBORN MICE

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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, may be effective in preventing learning disabilities caused by perinatal brain injuries in human preterm infants.

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

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,b; 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-

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Abbreviations: CS, conditioned stimulus; P, postnatal day; PBS, phosphate buffer saline; S.D., standard deviation; S.E.M., standard error of the mean; USV, ultrasonic vocalization.

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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 (IFFA-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 from the midline in the lateral-medial plane and 1.5 mm anterior to the bregma in the rostro-caudal plane. Two 1-μl 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 mm^3 (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% menthyl 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 $100\times[T(CS+)-T(CS-)]/[T(CS+)-T(CS-)]$, 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