Neuroscience 311 (2015) 382–397

MELATONIN REDUCES EXCITOTOXIC BLOOD–BRAIN BARRIER BREAKDOWN IN NEONATAL RATS

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Abstract—The blood–brain barrier (BBB) is a complex structure that protects the central nervous system from peripheral insults. Understanding the molecular basis of BBB function and dysfunction holds significant potential for future strategies to prevent and treat neurological damage. The aim of our study was (1) to investigate BBB alterations following excitotoxicity and (2) to test the protective properties of melatonin. Ibotenate, a glutamate analog, was injected intracerebrally in postnatal day 5 (P5) rat pups to mimic excitotoxic injury. Animals were than randomly divided into two groups, one receiving intraperitoneal (i.p.) melatonin injections (5 mg/kg), and the other phosphate buffer saline (PBS) injections. Pups were sacrificed 2, 4 and 18 h after ibotenate injection. We determined lesion size at 5 days by histology, the location and organization of tight junction (TJ) proteins by immunohistochemical studies, and BBB leakage by dextran extravasation. Expression levels of BBB genes (TJs, efflux transporters and detoxification enzymes) were determined in the cortex and choroid plexus by quantitative PCR. Dextran extravasation was seen 2 h after the insult, suggesting a rapid BBB breakdown that was resolved by 4 h. Extravasation was significantly reduced in melatonin-treated pups. Gene expression and immunohistochemical assays showed dynamic BBB modifications during the first 4 h, partially prevented by melatonin. Lesion-size measurements confirmed white matter neuroprotection by melatonin. Our study is the first to evaluate BBB structure and function at a very early time point following excitotoxicity in neonates. Melatonin neuroprotects by preventing TJ modifications and BBB disruption at this early phase, before its previously demonstrated anti-inflammatory, antioxidant and axonal regrowth-promoting effects.

Key words: blood–brain barrier, brain development, ibotenate, periventricular white matter damage, melatonin.

INTRODUCTION

The main role of the blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier (BCSFB, i.e. choroid plexus) is to maintain a precisely regulated intracerebral (i.c.) microenvironment. Tight junctions (TJs) closely connect barrier cells, and, together with adherens junctions, influx and efflux transporters, metabolic enzymes and the extracellular matrix, contribute to cell polarity and selective barrier permeability. Pericytes and astrocytic end-feet associated with the endothelium within the neurovascular unit are also fundamental in regulating the BBB phenotype (Neuwelt, 2004; Abbott et al., 2006, 2010; Neuwelt et al., 2008; Li et al., 2014).

Less is known about these barriers in the developing brain. BBB and BCSFB integrity plays a key role in protecting the developing brain (Ek et al., 2012). Recent studies have demonstrated that TJs in the BBB and BCSFB are already effective at the earliest stages of development (Saunders et al., 2008). Indeed, the high protein concentration of embryonic CSF appears to be a consequence of transcellular transfer across the epithelial cells of the choroid plexus, rather than a consequence of TJ immaturity (Liddelow et al., 2013). TJs are impermeable even to small molecules, and selective protein transfer is useful for ventricular expansion and central nervous system (CNS) development (Ek et al., 2006; Johansson et al., 2008). On the other hand, BBB breakdown is an

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Abbreviations: BBB, blood–brain barrier; BCSFB, blood–cerebrospinal fluid barrier; CNS, central nervous system; HPS, Hemalum phloxin saffron; P5, postnatal day 5; PBS, phosphate buffer saline; TJ, tight junction.

http://dx.doi.org/10.1016/j.neuroscience.2015.10.044

important contributing factor to injury in many neurological disorders in adults and infants. The extreme vulnerability of the developing brain seems mainly to stem from its susceptibility to toxins, drugs and deleterious endogenous compounds that are released during perinatal injuries and attain the neuropil during the developmental processes of cell division, differentiation, migration and synaptogenesis. Under pathological conditions such as cerebral ischemia–reperfusion (IR) or in the presence of an activated excitotoxic cascade, damage to cerebrovascular endothelial cells causes alterations in BBB function that could exacerbate neuronal injury and death. Elucidating early changes in BBB transport and permeability is key to understanding BBB function after cerebral ischemia and excitotoxic damage in newborns (Dammann and Leviton, 2004).

Animal models of perinatal brain injury have proven useful for understanding its pathophysiology and identifying potential neuroprotective agents (Northington, 2006). A well-characterized murine model of perinatal excitotoxic injury consists of the i.c. injection of the glutamate analog ibotenate in 5-day-old mouse pups (Marret et al., 1995). Ibotenate activates NMDA and metabotropic receptors, and leads to brain lesions that simulate both the hypoxic–ischemic gray matter lesions that characterize human full-term and near-term newborns and the white matter lesions that are typical of preterm newborns (Gressens et al., 2005; Johnston, 2005). Several treatments administered intraperitoneally (i.p.) have been found to reduce ibotenate-induced lesions in mouse pups, indicating potential neuroprotective effects that could be extended to humans (Husson et al., 2002; Bouslama et al., 2006). Melatonin, a hormone that modulates the entrainment of the circadian rhythms of several biological functions (Hardeland et al., 2012), easily crosses the BBB (Reiter et al., 2014), and has been proven to be neuroprotective either through its antiapoptotic and vasoconstrictive effects (Kaur et al., 2008), or by limiting the excitotoxic cascade (Tutunculer et al., 2005). Both melatonin and its metabolites are powerful antioxidants (Pei et al., 2003; Tai et al., 2010; Galano et al., 2013; Zhang and Zhang, 2014). Recent studies have shown a beneficial role for this molecule in preventing BBB disruption in adult models of traumatic brain injury, stroke and subarachnoid hemorrhage (Kaur and Ling, 2008; Kabadi and Maher, 2010; Dehghan et al., 2013) (Chen et al., 2014). Finally, melatonin has also been proven safe for use in children (Fulia et al., 2001; Gitto et al., 2001; Weiss et al., 2006).

The aim of the present study was (1) to characterize acute variations in BBB and BCSFB permeability and alterations in TJ proteins in the developing brain after exposure to an excitotoxic challenge, and (2) to evaluate the effects of melatonin on BBB function in the developing animal.

EXPERIMENTAL PROCEDURES

In order to understand the time-course of alterations in the BBB and BCSFB after ibotenate injection and the neuroprotective effects of melatonin, three different time points were chosen: 2, 4 and 18 h after the insult (+2, +4 and +18 h). An additional time point 24 h after injection was also used to investigate alterations of the BCSFB in the choroid plexus. The long-term effects of ibotenate and melatonin on brain lesions were evaluated in animals sacrificed at P10.

The experimental paradigm is shown in Fig. 1.

Animal preparation

All experimental procedures were performed with prior approval from the Ethics Committee of the Institut National de la Santé et de la Recherche Médicale (INSERM), and in accordance with NIH guidelines for the humane handling of animals. Female Sprague–Dawley rats with a 4 day-old litter (10 pups per litter) were obtained from Charles River (St. Germain-sur-l'Arbresle, France). Rats were given food and water *ad libitum* and housed in a temperature/light-controlled animal care facility. Every effort was made to minimize the number of animals used and their suffering.

Treatments

Excitotoxic brain lesions were caused in postnatal day 5 (P5) rat pups by injecting them i.c. with 5 mg/mL ibotenate (Tocris 0285, Bristol, UK) diluted in phosphate buffer saline (PBS), as previously described (Marret et al., 1995; Gressens et al., 1997; Dommergues et al., 2000; Laudenbach et al., 2001; Tahraoui et al., 2001; Husson et al., 2002). Ibotenate was injected into the neopallial parenchyma using a 25-gauge needle and 50-μl Hamilton syringe (Massay, France), mounted on a calibrated microdispenser attached to a rigid mechanical holder. The needle was inserted intracranially 2 mm under the external surface of the scalp into the frontoparietal region of the right hemisphere, 2.5 mm from the midline in the mediolateral plane and 4 mm anterior to the bregma in the rostrocaudal plane. Two 1-μl boluses (5 μg each) of ibotenate were injected. This dose has been shown to consistently cause brain damage in P5 mice (Husson et al., 2005). Two sham groups were also included, with only needle injury or PBS injections. Melatonin (Sigma M5250) 5 mg/kg was diluted with PBS 1×-DMSO 5% and then injected i.p. Control pups were injected i.p. with PBS 1×-DMSO 5%. All i.p. injections were administered 10 min after ibotenate injections.

Determination of lesion size

Lesion size was determined in 10 pups per group of both sexes. The pups were decapitated 5 days after i.c. ibotenate injection (P10), and their brains were fixed in 4% formaldehyde for 5 days and then embedded in paraffin. The size of neocortical and white matter lesions can be defined by the length on three orthogonal axes: the lateral–medial axis (in a coronal plane), the radial axis (also in a coronal plane, from the pial surface to the lateral ventricle), and the fronto-occipital axis (in a sagittal plane). In previous studies (Marret et al., 1995; Pansiot et al., 2010) we found an excellent correlation among the measurements from the three axes of the excitotoxic lesions. Based on these findings, we cut serial sections of the entire brain in the coronal plane at 15-μm intervals in