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Prophylactic treatment with melatonin before recurrent neonatal seizures: Effects on long-term neurobehavioral changes and the underlying expression of metabolism-related genes in rat hippocampus and cerebral cortex

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ABSTRACT

Although it has been suggested that the protective effect of melatonin against seizure-induced neurotoxicity involves inhibition of neuronal lipid peroxidation, current data concerning the exact molecular mechanism are still limited. This study was undertaken to investigate the changes in neurobehavioral, cognitive and lipid metabolism-related gene expressions in both hippocampus and cerebral cortex of rats subjected to recurrent neonatal seizures, and the effects of melatonin treatment before seizure (55 mg/kg, 1 mg/ml). 6-day-old (P6) SD rats were randomly divided into four groups of control (CONT, the same below), melatonin treated control (Mel), recurrent neonatal seizure (RS) and melatonin and RS combination treatment (Mel + RS). Neurological behavioral parameters of brain damage (plane righting reflex, negative geotaxis reaction reflex, Cliff avoidance reflex, forelimb suspension reflex) were observed on P31. Morris water maze test was performed during P29–P35. Then the protein levels of ACAT1, Cathepsin-E and Ca^{2+}/calmodulin-dependent protein kinase II (CAMK II) in hippocampus and cerebral cortex were detected by western blot method. As expected, RS group showed a significant delay or reduce of the four reflexes, as well as bad performance in the Morris water maze test. Flurothyl-induced neurobehavioral toxicology was blocked by pre-treatment with melatonin. In parallel with these behavioral changes, gene expression by western blot method demonstrated that rats pretreated with melatonin (Mel + RS) showed a significant down-regulated expression of ACAT-1, Cathepsin-E and up-regulated CAMK II in hippocampus and cerebral cortex when compared with RS group. Our findings provide support for ACAT-1/Cathepsin-E as well as CaMK II being potential targets for the treatment of neonatal seizure-induced brain damage by melatonin.

1. Introduction

Clinically, there are 20–25% patients develop therapeutic failure with conventional anti-epileptic drugs (AEDs) (Anovadiya et al., 2012). To make matters worse, these AEDs often result in serious physiological and psychological harm to patients. Therefore, alternative treatments for epilepsy, including melatonin and ketogenic diet have attracted intensive attention in clinical practice. Given the better clinical compliance than ketogenic diet, melatonin has recently been used as a feasible treatment for pediatric epilepsy (Rogovik and Goldman, 2010).

Melatonin is manufactured by the pineal gland. Seizures could induce markedly increased levels of melatonin in childhood refractory epilepsy and febrile seizures (Ardura et al., 2010; Paprocka et al., 2010; Jain and Besag, 2013). Giving melatonin to pediatric patients suffering from severe epileptic disorders was shown to be anticonvulsive (Uberos et al., 2011). Also, 10 mg daily melatonin treatment at bedtime decreased diurnal seizures in a study of ten patients aged 9 to 32 years with intractable epilepsy (Goldberg-Stern et al., 2012). Consistently, the majority of experimental data also indicates anticonvulsant properties of the hormone (Petkova et al., 2014; Mareš et al., 2013; Forcelli et al., 2013). On the other hand, however, proconvulsant effects of melatonin were also reported both in clinical and experimental studies (Sheldon, 1998; Musshoff and Speckmann, 2003). This contradiction suggests that more experimental studies are required before to recognize melatonin as a potential add-on therapy in children with epilepsy.

Previous studies that showed anticonvulsant action of melatonin were mainly conducted in adult animals (rats, mice, hamster and guinea pigs). However, the effects of melatonin have been seldom examined in developmental animals. Rogério F et al. once reported that melatonin at doses of 1–50 mg/kg decreased neuronal death on neonatal rat

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motoneurons after sciatic nerve transaction (Rogério et al., 2002). Recently, Forcelli PA et al. demonstrated that melatonin (0–80 mg/kg) prior to PTZ potentiated the anticonvulsant efficacy of phenobarbital (Forcelli et al., 2013). However, both studies did not further investigate the neurobehavioral changes and the underlying molecular mechanisms.

As long as developmental seizure-induced neurobehavioral changes are concerned, previous studies including ours mainly focused on cognitive deficits by Morris water maze analysis (Ni et al., 2009). However, we have also found that neonatal seizures could induce not only long-term cognitive deficits but also changes of neurobehavioral development reflexes (Ni et al., 2012). Accordingly, in this study both neurobehavioral reflexes and cognitive abilities were examined.

It has been established that the melatonin possesses broad-spectrum free radical scavenging and antioxidant activities, and prevents seizure-induce neuronal lesions by limiting NO.-induced lipid peroxidation (Skaper et al., 1999; Kabuto et al., 1998). Our preliminary studies using flurothyl-induced recurrent neonatal seizure model and penicillin-induced developmental epilepsy model have shown that peroxidation-related lipid metabolism signals may be involved in ketogenic diet's neuroprotective efficacy (data not published). Furthermore, we have recently found up-regulated acetyl-CoA acetyltransferase 1 (ACAT1) in rat hippocampus following developmental seizure-induced long-term brain damage which could be inhibited by E-64d pretreatment (Ni et al., 2013). In addition, Jeong HJ et al. previously reported that KA increased the mRNA expression of Cathepsin E in hippocampus using microarray and subsequent RT-PCR method, which was suppressed by ketogenic diet (Jeong et al., 2010).

These results, combined with the important function of melatonin in the epileptic phenomena, prompted us to further explore whether pretreatment with melatonin could alleviate the deleterious neurobehavioral changes, which are integral components of developmental brain injury-induced excitotoxicity, as well as the expression changes of lipid metabolism related proteins ACAT1 and Cathepsin-E both in hippocampus and cerebral cortex. Besides, the protein level of Ca^{2+}/calmodulin-dependent protein kinase II (CAMK II) was also detected, which had been found to be the modulation target of melatonin in audiogenic seizure model of Krushinsky-Molodkina rats (Savina et al., 2005).

2. Materials and methods

2.1. Chemical and reagents

Flurothyl (bis-2, 2, 2-triflurothyl ether) and melatonin were purchased from Aldrich-Sigma Chemical (WI, USA). A rabbit anti-ACAT-1 polyclonal antibody was purchased from Cayman Chemical. A rabbit polyclonal antibody against Cathepsin-E and a rabbit polyclonal antibody against CaMK II were purchased from Abcam, and horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz.

2.2. Animals and experimental design

The experiments were carried out on Sprague-Dawley rats with balanced sex. Litters were culled to six pups at postnatal day 1–2 (postnatal day 0 is a day of birth) and randomly assigned to an experimental protocol. Attempts were made to minimize the number of animals used. The animals were weaned on postnatal day 21 (P21) and after this age they were housed on a standard light–dark cycle. The experimental protocol was in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Each experimental and control group contained 6 pups from 4 or 5 litters. The pups in each litter were divided into four groups: control (CONT, the same below), melatonin treated control (Mel), recurrent neonatal seizure (RS) and melatonin and RS combination treatment (Mel + RS). The experiments started at postnatal day 6(P6) which were chosen on the basis of comparison of brain development in infants and rat pups. The level of maturation corresponding to full-term human newborn is attained around postnatal 10, therefore, the P6 rats correspond to human perinatal administration (Tchekalarova et al., 2005).

The procedure of seizure induction using liquid volatile flurothyl has been described in detail previously (Ni et al., 2012). The experimental rats had 45 induced seizures during the nine consecutive days, from P6 to P14. Rats had 5 seizures/day for nine consecutive days, with a minimum of 30 min between seizures. Control rats were placed into the container for an equal amount of time without exposure to flurothyl. In the two melatonin treated groups, each rat was pretreated with melatonin before seizures were induced (55 mg/kg, 1 mg/ml, i.p.). Melatonin at this dose has been shown to have anticonvulsant effects (Moezi et al., 2011; Mareš et al., 2012). Melatonin was initially dissolved in ethanol until an adequate mixture (5% v/v, ethanol/saline) was obtained.

2.3. Neurobehavioral and cognitive tests

Neurological behavioral parameters of brain damage (plane righting reflex, negative geotaxis reaction reflex, Cliff avoidance reflex, forelimb suspension reflex) were observed on P31 according to the procedure previously described (Qin et al., 2011). Cognitive deficit was evaluated from P29 to P35 by Morris water maze test (Ni et al., 2012).

2.4. Western blot analysis

Protein levels were detected by western blot method on P35. The hippocampus and cerebral cortex from each group (number = 6) were used for western blot analysis as described by Wang (Wang et al., 2008). Polyvinylidene fluoride membrane blots after blocking solution TBS-T were incubated with one of the following antibodies: a rabbit anti-ACAT-1 polyclonal antibody (1:200), a rabbit polyclonal antibody against Cathepsin-E (1:500), a rabbit polyclonal antibody against CaMK II (1:1000) in Tris buffered saline containing 0.2% Tween-20 (TBST) and 3% nonfat dry milk for 3 h. The blot was washed several times with TBS-T and then incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 2 h (1:10,000). Specific bands were visualized on a film Kodak X-Omat LS using the ECL detection system Amersham. The relative changes of the intensity of each immunoreactive band were evaluated with Sigma Scan Pro 5 and were normalized to a loading control GAPDH.

2.5. Statistical analysis

Escape latency was analyzed using two-way ANOVAs (treatment as a between subject factor and training day as a within subject factor) for repeated measures. Subsequent comparisons were done using post hoc tests. The frequency of passing through the platform quadrant of spatial probe test in the water maze, and the protein levels were analyzed with one-way ANOVA followed by post hoc comparisons. The behavioral measures were analyzed by non-parametric Kruskal–Wallis test. Data was presented as the mean ± S.D. and statistical significance was considered as a $P < 0.05$.

3. Results

3.1. Neurological reflexes

The effects of neonatal seizure and pretreatment by melatonin on neurological development may be presented by different neurological reflexes. As a result, RS group showed a significant delay or reduce of negative geotaxis reaction reflex (Chi-Square = 11.99, P = 0.0074), Cliff avoidance reflex (Chi-Square = 15.62, P = 0.0014), as well as plane righting reflex (Chi-Square = 9.49, P = 0.023), and forelimb suspension reflex (Chi-Square = 12.86, P = 0.005) using Kruskal–Wallis