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Leptin treatment prevents long-term abnormalities in cognition, seizure threshold, hippocampal mossy fiber sprouting and ZnT3/CB-D28k expression in a rat developmental "twist" seizure model

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ABSTRACT

The mechanism of linking neonatal seizures with long-term brain damage is unclear, and there is no effective drug to block this long-term pathological process. Recently, the fat-derived hormone leptin has been appreciated for its neuroprotective function in neurodegenerative processes, although less is known about the effects of leptin on neonatal seizure-induced brain damage. Here, we developed a "twist" seizure model by coupling pilocarpine-induced neonatal status epilepticus (SE) with later exposure to penicillin to test whether leptin treatment immediately after neonatal SE would exert neuroprotective effects on cognition, seizure threshold and hippocampal mossy fiber sprouting, as well if leptin had any influence on the expression of zinc transporter 3 (ZnT3) and calcium homeostasis-related CB-D28k in the hippocampus. Fifty Sprague-Dawley rats (postnatal day 6, P6) were randomly assigned to four groups: control (n = 10), control with intraperitoneal (i.p.) injection of leptin (Leptin, n = 10), pilocarpine-induced neonatal SE (RS), and RS i.p. leptin injection (RS + Leptin). At P6, all the rats in the RS group and RS + Leptin group were injected with lithium chloride i.p. (5 mEq/kg). Pilocarpine (320 mg/kg, i.p.) was administered 30 min after scopolamine methyl chloride (1 mg/kg) injection at P7 to block the peripheral effect of pilocarpine. From P8 to P14, the animals in the Leptin group and RS + Leptin group were given leptin (4 mg/kg, i.p.). The Morris water maze test was performed during P28-P33. Following routine seizure threshold detection and Timm staining procedures, Western blot analysis was performed for each group. Pilocarpine-induced neonatal SE severely impaired learning and memory abilities, reduced seizure threshold, and induced aberrant hippocampal CA3 mossy fiber sprouting. In parallel, there was a significantly down-regulated protein level of CB-D28k and an up-regulated protein level of ZnT3 in the hippocampus of the RS group. Furthermore, leptin treatment soon after neonatal SE for seven consecutive days counteracted these hyperexcitability-related alterations. These novel findings established that leptin has a neuroprotective role in the model of cholinergic neonatal SE and highlights ZnT3/CB-D28k associated-Zn (2 +)/Ca (2 +) signaling as a promising therapeutic target.

1. Introduction

The relationship between epilepsy and metabolism is receiving growing attention from both clinical and therapeutic practice. Leptin is a multi-potency adipocyte hormone, and in addition to its classic metabolic regulation, it can also regulate hippocampal neuronal morphology, activity-dependent synaptic plasticity and cognitive function (Morrison, 2009; Munzberg and Morrison, 2015). Leptin replacement therapy in patients with leptin deficiency caused by ob gene mutations leads to changes in brain structure and function, including in the hippocampus (Paz-Filho, 2016).

Lambrechts et al. recently evaluated changes in serum leptin levels in patients with refractory epilepsy treated with a ketogenic diet (KD), which is a low-carbohydrate, moderate-protein, high-fat diet. They showed that serum leptin decreased statistically during three months of KD treatment (Lambrechts et al., 2016). Experimental studies also showed that leptin significantly suppressed seizures via direct effects on glutamate neurotransmission in the hippocampus (Diano and Horvath, 2008; Xu et al., 2008). In leptin deficient ob/ob mice, Erbayat-Altay et al. demonstrated increased severity of pentylenetetrazol-induced seizures (Erbayat-Altay et al., 2008). The neuroprotective effects of leptin following kainic acid or pilocarpine-induced SE were also reported (Jayaram et al., 2013; Obeid et al., 2010). However, on the other hand, results opposite to the anticonvulsant or neuroprotective effects

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described above have also been described. For example, Aslan et al. reported proconvulsant activity of leptin on penicillin-induced epileptiform in rats (Aslan et al., 2010). Lynch et al. found that Male C57BL/6JRj mice pretreated with leptin had no clear convulsant-related effects, but exhibited proconvulsant activity with both NMDA and kainate-induced convulsions (Lynch et al., 2010). Cowley et al. revealed that the use of leptin increased the frequency of action potentials in proopiomelanocortin neurons (Cowley et al., 2001). These contradictory reports of the dual role of leptin in seizures suggest that there should be more experimental studies before applying leptin to patients with epilepsy.

The alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) receptors are known targets for leptin and are involved in hippocampal- dependent learning and memory, including effects on glutamate receptor trafficking, neuronal morphology and activity-dependent synaptic plasticity (Irving and Harvey, 2013). The signaling mechanism underlying these effects involves an increase in the insertion of synaptic GluR2-lacking AMPA receptors and thus increases in intracellular Ca(2+) levels ([Ca(2+)](i)) (Shanley et al., 2001; Moult and Harvey, 2009). Because the relative abundance of subunit mRNAs of AMPARs determines gating and Ca^{2+}/Zn^{2+} permeability in principal neurons and interneurons in rat CNS (Geiger et al., 1995), reducing Ca(2+) permeability of AMPA receptors via expression of Ca(2+)-impermeable GluR2(R) channels rescues vulnerable CA1 pyramidal neurons from forebrain ischemic injury (Liu et al., 2004), we thus hypothesized that leptin could modulate Ca(2+)/Zn(2+) signaling-dependent hippocampal synaptic plasticity, especially neuronal morphology and cognition.

In this study, we investigated the effects of leptin treatment soon after neonatal SE on cognition, seizure threshold and hippocampal mossy fiber sprouting, as well if leptin had any influence on the expressions of zinc transporter 3 (ZnT3) and calcium homeostasis-related CB-D28k in the hippocampus of Sprague-Dawley rats submitted to cholinergic SE on postnatal day 6.

2. Materials and methods

2.1. Animal preparation

Fifty Sprague-Dawley rats on postnatal day 5 (P5) were obtained from the Chinese Academy of Sciences, Shanghai Experimental Animal Center, China. The animals were treated in accordance with the guidelines set by the National Institutes of Health for the humane treatment of animals. Adequate measures were taken to minimize pain and the number of animals used. All animals were randomly assigned to two groups: the pilocarpine hydrochloride (Sigma-Aldrich Chemical, WI, USA)-induced status epilepticus group (RS, n = 30) and the control group (n = 20). They were further divided into the control group without leptin (Control, n = 10), the control group plus intraperitoneal (i.p.) leptin treatment (Leptin, n = 10), the RS group without leptin treatment (RS) and the RS plus i.p. leptin treatment group (RS + Leptin). At P6, animals in the RS group and the RS + Leptin group received i.p. injections of lithium chloride (5 mEq/kg). Pilocarpine (320 mg/kg, i.p.) was administered 30 min after scopolamine methyl chloride (1 mg/kg) injection at P7 to block peripheral effects of pilocarpine. The rats that achieved the standard for seizures were randomly assigned to the RS group and RS + Leptin group (n = 10/each group). Murine leptin was obtained lyophilized from Preprotech Inc. (Rocky Hill, NJ) and reconstituted in 0.01 M PBS buffer. From P8 to P14, the animals in the Leptin group and the RS + Leptin group were given leptin (4 mg/kg, 1 ml/kg, i.p.). Control rats were injected with the same volume (1 ml/kg) of vehicle (0.01 M PBS). The four groups of animals were in the same feeding conditions.

2.2. Induction of status epilepticus

This model was conducted in accordance with the methods previously described by Torolira et al. (2016). Briefly, all the 30 rats in the RS group and the RS + Leptin group were injected with lithium chloride (5 mEq/kg, or 212 mg/kg, i.p.) at P6. Then, after 24 h (P7), animals were injected with pilocarpine (320 mg/kg, i.p.). Scopolamine methyl chloride hydrobromide (1 mg/kg, i.p.) was injected (1 mg/kg, i.p.) 30 min before the injection of pilocarpine to antagonize the peripheral effect of pilocarpine. After the injection, the seizures were observed. The standard of successful modeling is the emergence of limb clonus with vocalization (stage 3). The survival rate was 80% (24/30). The successful animals were randomly assigned to RS and RS + Leptin groups, each a group of 10 rats. The remaining 4 were reserved for proteomics to be tested and not included in the present study.

2.3. Morris water maze test

To evaluate visual spatial learning and memory abilities, five rats from each group were randomly selected and tested in the Morris water maze from P28 to P32 (n = 5/group) according to the procedure described previously (Ni et al., 2016). In short, for the place navigation test, the video/computing system automatically records the escape latency (the duration for each rat to find the platform in the water maze). For the space probe test, the platform was removed from the pool the following day after the navigation test (P33). Each rat was placed in water for 60 s, and the number of times crossing the platform quadrant was recorded.

2.4. Seizure threshold

Seizure susceptibility was studied by exposing the rats to penicillin using a procedure modified from one previously described (Tian et al., 2016). Based on the induced seizures using lithium chloride-pilocarpine, all ten rats from each group were injected with penicillin (5.1×10^6 U/kg/d, i.p.) on postnatal day 34. The time to the first seizure after penicillin injection was recorded, which was the seizure latency (min) (seizure threshold). The observation time was 1 h.

2.5. Timm staining

At the end of the seizure threshold test on P34, five rats from each group were randomly selected and given i.p. injection of chloral hydrate at a dose of 1 ml/100 g. After the anesthesia was complete, each group was perfused through the heart with 0.9% saline followed by 0.4% sodium sulfide and 4% paraformaldehyde in PBS in the order of 80 ml, 100 ml, 80 ml and 60 ml. The method used for Timm staining (n = 5/each group, P34) has been described previously (Ni et al., 2016). The mossy fiber sprouting was scored by a semi-quantitative scale that evaluated the terminal sprouting in the CA3 region. The person scoring the Timm staining was blind to treatment group.

2.6. Western blot analysis

The randomly selected five rats from each group were sacrificed by intraperitoneal injection of 4% chloral hydrate (1 ml/100 g). The hippocampi were rapidly removed into the already marked pre-cooled EP tube on dry ice and then quickly stored in a −80 °C refrigerator. The Western blot method has been described previously (n = 5/group, P34) (Tian et al., 2016). Briefly, after blocking, the polyvinylidene fluoride membrane blots were incubated with one of the following antibodies: goat anti-ZnT3 polyclonal antibody (1:1000, Santa Cruz), rabbit anti-CB-D28k polyclonal antibody (1:1000, Abcam) or rabbit anti-β-actin polyclonal antibody (1:3000, Bioworld Technology) in TBST contain 5% nonfat dry milk overnight at 4 °C. After washing with TBST 3 times, the blot was incubated with the secondary antibody for approximately