Neuroscience 286 (2015) 203–215

IN VIVO EFFECTS OF BUMETANIDE AT BRAIN CONCENTRATIONS INCOMPATIBLE WITH NKCC1 INHIBITION ON NEWBORN DGC STRUCTURE AND SPONTANEOUS EEG SEIZURES FOLLOWING HYPOXIA-INDUCED NEONATAL SEIZURES

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Abstract—Neonatal seizures caused by perinatal asphyxia and hypoxic–ischemic encephalopathy can be refractory to conventional anticonvulsants. This may be due to the depolarizing effects of gamma-aminobutyric acid (GABA) achieved by the activity of the Na^+-K^+-$2Cl^-$ cotransporter (NKCC1). The aim of this study is to evaluate the long-term effects of bumetanide, a NKCC1 inhibitor, on hippocampal neurogenesis and seizure susceptibility in hypoxia-induced neonatal seizure model. Wistar rats were subjected to hypoxia-induced neonatal seizures at postnatal day 10 (P10). Following acute seizures, the rats were treated with intraperitoneal injection (i.p.) of bumetanide at a dose of 0.5 mg/kg for 3 weeks. In later adulthood, hypoxia-induced seizures increased the number of newborn dentate gyrus cells (DGCs), promoted mossy fiber sprouting (MFS) and reduced the apical dendritic complexity of newborn DGCs 1 month after the insults. In addition, these seizures resulted in long-lasting consequences, such as spontaneous electroencephalography (EEG) seizures, though spatial learning impairments were not seen. Bumetanide treatments significantly enhanced cell proliferation and dendritic development of newborn DGCs after neonatal seizures, accompanied by the decreased seizure activity. However, systemic administration of bumetanide resulted in much lower brain concentrations, and was incompatible with NKCC1 inhibition in blood–brain barrier (BBB)-protected brain tissue. Our results suggested that bumetanide might have long-term effects in suppressing seizure activity, and altering the neurogenesis after neonatal seizures. These effects of bumetanide may be mediated by the targets outside the BBB-protected central nerve system (CNS) or CNS-located target(s) other than NKCC1.

Key words: epilepsy, hypoxia, neurogenesis, neuronal plasticity, NKCC1.

INTRODUCTION

Neonatal seizures are devastating conditions due to diverse etiologies, with perinatal hypoxia–asphyxia being the most common (Tekgul et al., 2006). Neonatal seizures are associated with poor long-term neurodevelopment, resulting in conditions such as cerebral palsy, mental retardation and learning disorders (Brunquell et al., 2002; Ronen et al., 2007). In addition, neonatal seizures are often unresponsive to conventional medications and lead to epilepsy later in life (Brunquell et al., 2002; Ronen et al., 2007). The poor response of neonatal seizures to conventional antiepileptic drugs remains a significant clinical problem. Therefore, elucidating the mechanisms of altered plasticity induced by neonatal seizures may reveal new therapeutic targets.

It is well documented that experimental seizures in adult animals can result in aberrant hippocampal neurogenesis which is thought to contribute to, rather than counteract, abnormal brain activity (Hester and Danzer, 2013; Rotheneichner et al., 2013). In a neonatal brain, depolarizing and excitatory effects of GABA, due to high intracellular concentrations of chloride achieved by robust activity of Na^+-K^+-$2Cl^-$ cotransporter (NKCC1), may be one reason for the distinct consequences on neurogenesis in the context of adult and neonatal seizures (Haut et al., 2004; Rakhade and Jensen, 2009).

Although a number of studies have examined the effect of the NKCC1 inhibitor, the effect of bumetanide

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Abbreviations: ANOVA, analysis of variance; BBB, blood–brain barrier; BrdU, bromodeoxyuridine; CB, control + bumetanide; CNS, central nerve system; CRH, corticotrophin releasing hormone; DCX, doublecortin; DG, dentate gyrus; DGCs, dentate gyrus cells; EEG, electroencephalography; GABA, gamma-aminobutyric acid; GCL, granular cell layer; GFAP, glial fibrillary acidic protein; HPA, hypothalamic–pituitary–adrenal; HY, hypoxia; HYB, hypoxia + bumetanide; HYS, hypoxia + saline; i.p., intraperitoneal injection; MFS, mossy fiber sprouting; ML, molecular layer; NeuN, neuronal nuclei; NKCC1, Na^+-K^+-$2Cl^-$ cotransporter; P, postnatal day; PVN, paraventricular nucleus; SH, sham.

http://dx.doi.org/10.1016/j.neuroscience.2014.11.031
0306-4522/

on neonatal seizure activity in different animal models and the anticonvulsant effect of bumetanide *in vivo* remain inconclusive (Dzhala et al., 2005; Koyama et al., 2012; Cleary et al., 2013; Loscher et al., 2013; Puskarjov et al., 2014). Furthermore, whether bumetanide exerts antiepileptic effects by altering aberrant neurogenesis in neonatal seizures is unknown. The present study investigated the effects of bumetanide on the hippocampal neurogenesis in neonatal rats subjected to induced hypoxic seizures, as well as the long-term consequences of bumetanide on brain development.

EXPERIMENTAL PROCEDURES

Animal models

The animals used in this study were litters of Wistar rats (P10, Experimental Animal Center, China Medical University, China). They were randomly assigned to the following groups: sham group (SH), hypoxia group (HY), hypoxia + saline group (HYS) and hypoxia + bumetanide group (HYB). Litters were housed with their dams until weaning at P21, after which they were group-housed and had access to water and food *ad libitum*. Rats were maintained with a 12-h light/dark cycle. All experiments were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). Efforts were made to minimize the number of animals used and their suffering.

To induce hypoxia-seizures, rats were exposed to graded global hypoxia for 15 min in an airtight chamber on P10, as described previously (Jensen et al., 1991). Oxygen concentration was maintained at 7% for 8 min, 6% for 4 min, 5% for 2 min and 4% for 1 min before the termination of hypoxia. The chamber was then uncovered and exposed to room air. Age-matched control rats were placed in the same chamber for a similar amount of time, but not subjected to the hypoxia treatment. All rats were returned to their dams within one hour. Seizure severity was classified into five levels by Racine's scale (Racine et al., 1972). I, facial movement; II head nodding; III, unilateral forelimb clonus; IV, bilateral forelimb clonus; V, tonic clonic seizure, rearing and failing. The rats in which seizure severity reached a level V with a frequency of more than five times in the airtight chamber were used in the following experiment (Koh et al., 2004).

Drug administration

Bumetanide was dissolved in 0.9% saline and the pH was adjusted to 9.0. Rats in the HYB group received bumetanide (0.5 mg/kg, intraperitoneal injection (i.p.)) once a day for 3 weeks (Mazarati et al., 2009) and in the HYS group rats received equal volume injections of saline (Fig. 1).

BrdU labeling

Bromodeoxyuridine (BrdU, Sigma, Saint Louis, USA) was dissolved in 0.9% saline with an adjusted pH value of 7.2. To investigate the rate of cell differentiation, one cohort of rats (n = 5 per group) received BrdU (100 mg/kg, i.p.) once a day on P11–15 and were sacrificed on P40. In order to observe the proliferation of newborn cells in later adulthood, another cohort of rats (n = 5 per group) were injected with BrdU (100 mg/kg, i.p.) once a day on P37–39, and they were sacrificed 24 h after the last injection without going through the water maze test (Fig. 1).

Electrode implantation and electroencephalography (EEG) recording

To record the brain's electrical activity, electrodes were implanted on rats (n = 3–4 per group) on P32. The rats were anesthetized with sodium pentobarbital (50 mg/kg), and then fixed into the stereotaxic apparatus. The electrodes were bipolar twisted silver steel and embedded in the skull with dental cement. These electrodes were implanted into the right hippocampal CA3 (3.8 mm posterior to bregma, 3.4 mm lateral, 3.6 mm ventral to the dura mater) and the nasal point, according to the coordinates derived from the atlas of Paxinos and Watson. After 3 days of recovery, spontaneous EEG seizures in the dentate gyrus were recorded in freely moving animals. The frequency and mean duration of these spontaneous EEG seizures during an EEG recording session were examined for 2 h (Sugaya et al., 2010). The EEG signals were digitized with LabScribe2 digital acquisition software (iWorx, Dover, Delaware, USA) and stored for offline analysis.

Morris water maze

Spatial learning was analyzed with a match-to-place version of the Morris water-maze on P37–39 (four trials per day) (Lee et al., 2001; Umit et al., 2004). At the end of the testing period (P39), a probe trial of 60s without the platform was used to assess how well the rats remembered the location of the platform. All of the swimming routes were monitored with SLY-WMS video tracking system (Shuolinyuan, Beijing, China).

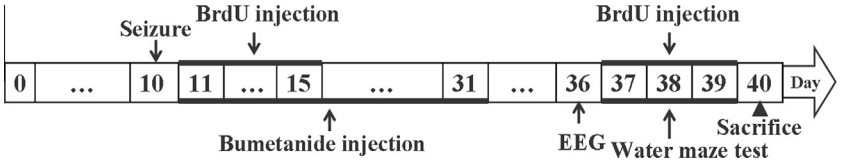

Fig. 1. Study design. The arrows indicate the timing of seizure, BrdU injection, bumetanide injection, EEG recording, water maze test and sacrifice after birth. The bold black line indicates the duration of these administrations. BrdU: bromodeoxyuridine, EEG: electroencephalography.