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Cx36 in the mouse hippocampus during and after pilocarpine-induced status epilepticus

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ABSTRACT

Gap junctions play an important role in the synchronization activity of coupled cells. Hippocampal inhibitory interneurons are involved in epileptogenesis and seizure activity, and express gap junction protein connexin (Cx) 36. Cx36 is also localized in the axons (mossy fibers) of granule cells in the dentate gyrus. While it has been documented that Cx36 is involved in epileptogenesis, there are still controversies regarding the expression levels of Cx36 at different developmental stages of human and animal models of epileptogenesis. In this study, the expression of Cx36 was investigated in the mouse hippocampus at 1 h, 4 h during pilocarpine-induced status epilepticus (PISE) and 1 week, 2 months after PISE. We found that Cx36 was down-regulated in neurons at different time points during and after PISE, whereas it was increased significantly in the stratum lucidum of CA3 area at 2 months after PISE. Double immunofluorescence indicated that Cx36 was localized in parvalbumin (PV) immunopositive interneuron in CA1 area and in mossy fibers and their terminals in the stratum lucidum of CA3 area. It suggests that decreased expression of Cx36 in interneurons may be related to less effective inhibitory control of excitatory activity of hippocampal principal neurons. However, the increased Cx36 immunopositive product in mossy fibers at the chronic stage after PISE may enhance the contacts between granule cells in the dentate gyrus and pyramidal neurons in CA3 area. The two different changes of Cx36 may be implicated in the epileptogenesis.

1. Introduction

In the central nervous system, gap junction-mediated communication is the most common type of electrical coupling between different neurons as well as between neurons and other types of brain cells such as astrocytes. Gap junctions are conductive channels formed by connexins (Cxs) that connect the interiors of coupled cells. Their large internal diameter allows the exchange of small molecules, intercellular ions, metabolites, second messengers and electrical signals. Gap junctions synchronize activity of coupled cells and are thought to play important roles in intercellular signaling in brain development, morphogenesis, metabolism, cell survival and death (Rouach et al., 2002). Direct electrical communication through gap junctions can alter neuronal activity over a much shorter interval than propagation through chemical synapses. Gap junction provides a mechanism of communication between adjacent cells and play vital roles in pathologic conditions of the central nervous system such as inflammation, epilepsy and ischemia (Dere and Zlomuzica, 2012; Eugenin et al., 2012; Simon et al., 2014). The hippocampus consists of CA1, CA3 areas and the dentate gyrus (DG). It plays an important role in epilepsy and electrographic seizures appear to originate from hippocampus and propagate to the amygdala and neocortex (Curia et al., 2008).

The inhibitory interneurons in the hippocampus have been reported playing crucial roles in the generation of synchronous population discharge patterns (Buzsaki et al., 1992; Freund and Buzsaki, 1996; Hormuzdi et al., 2001). The primary cause of seizure activity is loss of sensitive interneurons (Dinocourt et al., 2003; Knopp et al., 2008; Thind et al., 2010) and subsequent impairment of GABA-mediated inhibition in the hippocampus (Cossart et al., 2001; Sayin et al., 2003; Sloviter, 1987). However, it remains unknown on the phenotypic alterations of interneurons in epileptogenesis and seizure activity. The inhibitory GABAergic interneurons express the calcium-binding proteins such as

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parvalbumin (PV) or calbindin (CB). CB immunopositive product is also localized in the mossy fibers (axons of granule cells) of the stratum lucidum (SL) in CA3 area (Dumas et al., 2004; Tang et al., 2006). The reduction of PV immunopositive neurons in the hippocampus has been reported both in the animal model (Tang et al., 2006) and patients (Wittner et al., 2005) with temporal lobe epilepsy. Among the inhibitory interneurons, PV-expressing cells participate in the control of pyramidal cell excitability (Laura et al., 2015; Sessolo et al., 2015) and are involved in the regulation and controlling of seizure activity (Dutton et al., 2013; Kuruba et al., 2011; Tang et al., 2006).

Morphological studies showed that PV-immunopositive GABAergic interneurons were interconnected by gap junctions (Fukuda and Kosaka, 2000). Among the 20 types of gap junction proteins, only a few Cxs are expressed in mature neurons (Dere and Zlomuzica, 2012). Cx36 was observed between interneurons using electron microscopy and immunolabelling techniques (Belluardo et al., 2000; Condorelli et al., 1998; Deans et al., 2001; Priest et al., 2001; Rash et al., 2001). Electrophysiological studies confirmed that Cx36 was expressed in multiple subtypes of interneurons in hippocampus (Connors and Long, 2004) and mostly in PV and CB immunopositive interneurons (Ma et al., 2011; Priest et al., 2001). Moreover, a recent study indicated the existence of mixed electrical–chemical synaptic transmission from mossy fibers to CA3 pyramidal cells (Vivar et al., 2012). In hippocampus, Cx36 gap junctions have been observed not only at dendrodendritic or dendrosomatic neuronal contacts, but also at the locations between mossy fiber terminals in CA3 area (Hamzei-Sichani et al., 2007; Nagy, 2012). The homogeneous gap junctions between mossy fiber terminals and neurons may offer efficient cellular channels for finely regulating cell-to-cell communication.

Inconsistent expression levels of Cx36 have been reported in human epileptic tissues and tissues from different animal models of epilepsy (Beheshti et al., 2010; Belousov, 2012; Belousov and Fontes, 2013; Collignon et al., 2006). These differences may be related to the developmental stages of the diseases. In animal models of the human temporal lobe epilepsy, epileptogenesis typically goes through 3 stages, i.e. chemical-induced status epilepticus (acute stage), silent period and occurrence of spontaneously recurrent seizures (Liu et al., 2007; Wu et al., 2015). We therefore hypothesized that progressive changes of Cx36 may be involved in epileptogenesis. In the present study, we aimed to investigate if progressive changes of Cx36 occurred in the hippocampal interneurons and mossy fibers at the acute (within 1 day after pilocarpine-induced status epilepticus), latent (1 week) and chronic (2 months) stages of epileptogenesis, in order to establish the relationship between changes of Cx36 and epileptogenesis.

2. Materials and methods

2.1. Pilocarpine treatment

Adult male Swiss mice weighing 25–30 g were used. Mice were given a single subcutaneous injection of methyl-scopolamine nitrate (1 mg/kg) (Sigma) 30 min before the injection of either pilocarpine in the experimental group or saline in the control one. In the experimental group, mice were given a single *i.p.* injection of pilocarpine at 300 mg/kg (Sigma) and experienced status epilepticus. The selection of the exposed animals for study was done according to Racine scale (Racine et al., 1972). Only those with Racine scale ≥Score 3 were chosen for experimental group. The animal experiment was approved by the Institutional Animal Care & Use Committee of Tan Tock Seng Hospital-National Neuroscience Institute. Efforts were made to minimize animal suffering and to use the minimal number of animals throughout the study.

2.2. Immunohistochemical staining for neuronal nuclei (NeuN), Cx36 and PV

A total of 30 mice were used for the immunocytochemical study. Six mice were euthanized at each of the following survival intervals, i.e. at 1 h and 4 h during pilocarpine-induced status epilepticus (PISE), and at 1 week and 2 months after status epilepticus (SE). Six mice with saline instead of pilocarpine injection were taken as the control. Under deep anesthesia with sodium pentobarbital, the mice were perfused transcardially with 0.9% saline 8 min initially, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 10 min. The brain of each mouse was then removed and kept overnight in 30% sucrose in 0.1 M PB. Serial coronal sections at 40 μm were cut in a cryostat. A set of 6 serial sections were prepared and placed individually in 6 different wells of a 24–well tissue culture dish for control, NeuN, PV, Cx36, Cx36 + PV, Cx36 + CB immunocytochemical reaction.

Free-floating sections were immunostained by using the ABC method. In brief, sections were first blocked in 4% normal goat serum for 2 h and incubated overnight at 4 °C in rabbit antibodies against Cx36 (4 μg/ml) (Life Technologies/Invitrogen, No.36-4600), mouse antibody against NeuN (1: 5000), (Chemicon, Temecula, CA), mouse antibody against PV (1: 2000) (Millipore, Temecula, CA), respectively. Sections were then washed in 0.1 M Tris-buffered saline containing 0.1% Triton-X 100 (TBS-TX, pH 7.4) and placed for 2 h in biotinylated secondary antibodies. After three washes in TBS-TX, the sections were placed in avidin-biotin-peroxidase complex (ABC) (Vector, Burlingame, CA) in TBS-TX for 2 h. They were then washed in 0.1 M Tris buffer (TB, pH 7.6) and reacted in a solution of 0.012% H_2O_2 and 0.05% 3,3′-diaminobenzidine (DAB, Sigma, St. Louis, MO) in TB for 3–5 min. Sections were washed, mounted, dehydrated and coverslipped. In the negative control, the primary antibodies were omitted.

2.3. Double immunofluorescence labeling of Cx36 and PV and CB

Double immunofluorescence labeling was carried out to co-localize Cx36 (8 μg/ ml) with PV (1:500) or CB (1:500) (Millipore) respectively. Tissue sections of the dorsal hippocampus from 6 control and 6 experimental mice at 2 months after PISE were incubated overnight in a mixture of two primary antibodies. Sections were then washed in TBS-TX and placed for 4 h in anti-rabbit IgG-Alexa594 (abcam) (for Cx36) (red), followed by incubation in anti-mouse IgG-Alexa488 (abcam) (for PV and CB) (green) for 2–3 h. Sections were mounted, coverslipped and observed with a confocal laser scanning microscope (Olympus Fluoview V500, Japan).

2.4. Western blot

The hippocampus with the similar survival intervals mentioned above was homogenized in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Triton X-100 and 1 mM PMSF. Homogenate was centrifuged (15,000*g*, for 15 min) at 4 °C and supernatant was quantified by the Bradford assay (Bio-Rad). Aliquots containing 50 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots electrophoresed by 12% SDS- polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Millipore). After blocking with 5% skimmed milk for 1 h, membranes were incubated with primary antibodies of rabbit anti-Cx36 (1 μg/ ml) at 4 °C overnight followed by incubation with corresponding HRP-conjugated secondary antibodies. The membranes were finally carried out with SuperSignal1 West PICO Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL, USA). All results were from five independent experiments. β-actin was used as a reference protein according to our previous studies (Tang et al., 2006).

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