Progress in Neuro-Psychopharmacology & Biological Psychiatry 34 (2010) 510–515

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Changes in hippocampal connexin 36 mRNA and protein levels during epileptogenesis in the kindling model of epilepsy

Siamak Beheshti [a,b], Mohammad Sayyah [b,*], Majid Golkar [c], Hoori Sepehri [a], Jalal Babaie [c], Behrouz Vaziri [d]

[a] Department of Animal Biology, School of Biology, University College of Science, Tehran University, Tehran, Iran
[b] Department of Physiology and Pharmacology, Pasteur Institute of Iran, Tehran, Iran
[c] Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran
[d] Department of Biotechnology, Pasteur Institute of Iran, Tehran, Iran

ARTICLE INFO

Article history:
Received 5 December 2009
Received in revised form 8 February 2010
Accepted 8 February 2010
Available online 12 February 2010

Keywords:
Amygdala kindling
Connexin 36
Epileptogenesis
Hippocampus

ABSTRACT

Objective: Identification of key molecular changes occurring during epileptogenesis provides better understanding of epilepsy and helps to develop strategies to modify those changes and thus, block the epileptogenic process. Gap junctional communication is thought to be involved in epileptogenesis. This communication can be affected by changes in expression of gap junctional protein subunits called connexins (Cxs). One of the main brain regions involved in epileptogenesis is the hippocampus in which there is a network of gap junctional communication between different cell types.
Method: Cx36 and Cx43 expressions at both mRNA and protein level were measured in rat hippocampus during epileptogenesis in the kindling model of epilepsy.
Results: Cx36 expression at both mRNA and protein level was upregulated during acquisition of focal seizures but returned to basal level after acquisition of secondarily-generalized seizures. No change in Cx43 gene and protein expression was found during kindling epileptogenesis.
Conclusion: These results further point out the significance of Cx36 as a target to modify epileptogenic process and to develop antiepileptogenic treatments.

1. Introduction

Epilepsy is the most common neurologic disorder after stroke (Porter and Meldrum, 2001). Currently available antiepileptic drugs suppress seizures without altering the underlying course of epilepsy (Herman, 2006). Epileptogenesis is the process by which parts of a normal brain are converted to a hyperexcitable, epileptic brain (Dichter, 2006). Identification of key molecular changes provides better understanding of epileptogenesis and points to targets that can be used to modify the epileptogenic process and develop antiepileptogenic treatments.

Seizure is a result of abnormal excessive synchronized electrical activity of a large group of neurons (Porter and Meldrum, 2001). Spreading of the abnormal activity of a small group of cells to adjacent cells involves large cell groups and results in generalization of the seizures (Fisher et al., 2005). Besides the synaptic contacts between the cells, gap junctional coupling is supposed to be involved in the generation or spreading of the seizures (Perez-Velazquez and Carlen, 2000; Carlen et al., 2000; Kohling et al., 2001; Traub et al., 2001, 2002; Nemani and Binder, 2005). Gap junctions are specialized cell–cell contacts between eukaryotic cells, composed of aggregates of transmembrane channels, which directly connect the cytoplasm of adjacent cells, allowing intercellular movement of ions, metabolites and second messengers (Condorelli et al., 2003; Sohl et al., 2005). Each channel consists of two hemichannels (termed connexons), each of which is composed of six subunit proteins called connexin (Cx) (Sohl et al., 2005). It has been shown that seizures can be blocked by gap junction blockers, and gap junction openers exacerbate seizure activity (Perez-Velazquez et al., 1994; Dudek et al., 1998; Carlen et al., 2000; Gadja et al., 2003, 2005; Nemani and Binder, 2005; Samoilova et al., 2008; He et al., 2009). A number of studies have also established that epilepsy is associated with changes in Cxs expression and intercellular coupling (Rouach et al., 2002). Apart from the results, in most of the epilepsy studies performed in human and animals regarding Cxs expression, the changes have been evaluated in the brain that is already epileptic (Naus et al., 1991; Khurgel and Ivy, 1996; Elisevich et al., 1997b; Sohl et al., 2000; Li et al., 2001; Aronica et al., 2001; Fonseca et al., 2002; Szente et al., 2002; Condorelli et al., 2003; Samoilova et al., 2003; Gadja et al., 2003, 2006; Collignon et al.,

Abbreviations: ACSF, Artificial Cerebrospinal Fluid; AD, After-discharge; AMV, Avian Myeloblastoma Virus; bp, base pair; cDNA, Complementary Deoxyribonucleic acid; CNS, Central Nervous System; Cx, Connexin; ECL, Enhanced Chemiluminescence; i.c.v, intracerebroventricular; IgG, Immunoglobulin G; kDa, Kilodalton; mRNA, messenger Ribonucleic Acid; O.D, Optical Density; PVDF, Polyvinylidene Fluoride; RT-PCR, Reverse Transcription Polymerase Chain Reaction; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; TBST, Tris Buffered Saline Tween.

* Corresponding author. Tel./fax: +98 21 66968854.
E-mail address: sayyahm2@pasteur.ac.ir (M. Sayyah).

0278-5846/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pnpbp.2010.02.006

and there are few studies performed on Cxs expression during epileptogenesis (Elisevich et al., 1997a).

The kindling model of epilepsy represents a model of the seizures, which are analogous to human complex partial (focal) seizures with secondarily generalization (McNamara et al., 1980). Kindling is also a well established animal model of epileptogenesis in which periodic tetanic electrical stimulation of a particular brain region leads to a permanent state of hyperexcitability (Goddard and McIntyre, 1969). In the kindling model, with repeated administration of an initially subconvulsive electrical stimulus, seizures generally evolve through five stages: (1) facial clonus (2) head nodding (3) forelimb clonus (4) rearing and (5) rearing and falling accompanied by generalized clonic seizures (Racine, 1972). The behavioral seizure stages 1 and 2 correspond to the activation of ipsilateral limbic structures including—but not necessarily limited to—the hippocampus, and are considered as focal seizures, whereas stages 3–5 represent secondarily-generalized motor seizures and correspond to the propagation of discharge to the contralateral limbic structures and then to the outside of the limbic system (Hewapathirane and Burnham, 2005). Kindling permits the investigator to evaluate any change that occurs at the cellular and molecular levels at any stage of the transformation of the normal brain to a hyperexcitable one.

In the brain, Cxs are expressed in a cell type-specific manner (Rash et al., 2001). Among Cxs, Cx36 and Cx43 are well represented throughout the CNS. Cx36 is mainly expressed in neurons, especially interneurons (Condorelli et al., 1998, 2003) while Cx43 is predominantly expressed in astrocytes (Theis et al., 2003). Hippocampus is one of the main brain regions involved in kindling and epileptogenesis (Racine et al., 1989) and there is a network of gap junctional communication between different cell types within the hippocampus (Sohl et al., 2000). There is no report regarding the possible alterations in hippocampal Cx36 and Cx43 expression during epileptogenic process.

In the present study, the extent of expression of Cx36 and Cx43 at mRNA and protein level was investigated in the rat hippocampus during epileptogenesis in the amygdala-kindling model of epilepsy.

2. Methods

2.1. Materials

Ketamine and xylazine were from Rotex Medica (Germany) and Chanelle (Ireland), respectively. RNX-PLUS Reagent, Agarose, Acrylamide and Bis-Acrylamide were purchased from CinnaGen (Iran). Hot Start Taq DNA polymerase, 2× RNA Loading Dye, Page Ruler™ Prestained Protein Ladder and Protein Loading Buffer Pack were purchased from Fermentas (Lithuania). Protease Inhibitor Cocktail, RNase free DNase I, and 1st strand cDNA synthesis kit were from Roche (Germany). Enhanced Chemiluminescence (ECL) Advance Western Blotting Detection Kit was from Amersham (UK). Monoclonal anti-connexin 36 was from Zymed (USA). Monoclonal anti-connexin 43 was from Upstate (USA). Monoclonal anti-α-tubulin and anti-mouse IgG peroxidase conjugates were from Sigma-Aldrich (USA). Other chemicals were from Applichem (Germany) and Sigma-Aldrich (USA).

2.2. Animals

Adult male Wistar rats (300–350 g, Pasteur Institute of Iran) were used throughout the study. Animals were housed in groups of 2 and had free access to food (standard laboratory rodents chow) and drinking water. The animal house temperature was maintained at 23 ± 1 °C with an alternating 12-hr light/dark cycle (light on from 6 a.m.). All animal experiments were carried out in accordance with the European Communities Council Directive of November 1986 (86/609/EEC) in such a way to minimize the number of animals and their suffering. All the injections were done intraperitoneally (i.p.).

2.3. Kindling procedure and tissue preparation

The rats were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg). Then they were stereotaxically implanted with bipolar stimulating and monopolar recording stainless-steel Teflon-coated electrodes (A.M. Systems, USA, twisted into a tripolar configuration), in the basolateral amygdala (coordinates: A,−2.5 mm from bregma; L, 4.8 mm from bregma; V, 7.5 mm from dura) of the right hemisphere (Paxinos and Watson, 2005). The electrodes were fixed on the skull with dental acrylic. After 7 days of recovery period, after-discharge (AD) threshold was determined in amygdala by a 2-sec, 100 Hz monophasic square-wave stimulus of 1 ms per wave. The stimulation was initially delivered at 50 μA and then at 5-min intervals, increasing stimulus intensity in increments of 50 μA was delivered until at least 5 s of AD was recorded (Sayyah et al., 2007). Then two different groups of animals with known AD threshold (each group consisting of 5–7 rats) were stimulated once daily at AD threshold until they showed behavioral seizure stage 2 in two consecutive days (as partially-kindled rats) or behavioral seizure stage 5 in three consecutive days (as fully kindled rats) according to Racine classification (Racine, 1972). Two other groups of electrode-implanted, non-stimulated rats were used and considered as sham-operated control groups (sham 1 and sham 2 groups corresponds to the partially- and fully kindled rats, respectively).

In previous similar studies, the time courses of 3 h or two weeks were used to study the changes in Cxs expression (Elisevich et al., 1997a; Sohl et al., 2000). However, these time courses seem too early or late. Therefore, we chose a 24-hr time point, which was also the interval of our stimulation protocol, to evaluate the changes in Cxs expression at mRNA and protein level. Twenty four hours after showing last behavioral seizure stage of 2 or 5, all the animals and their corresponding controls were decapitated under deep ether anesthesia and their brain were removed immediately. The brains were incubated in chilled artificial cerebrospinal fluid (ACSF) with pH 7.3 consisted of the following composition (in mM): 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 10 Glucose. The hippocampi of the right hemisphere of the brains (the side that the electrodes were implanted in the amygdala) were removed and frozen immediately in liquid nitrogen and stored at −80 °C. The rest of the brains were placed in 10% formalin for at least 24 h at room temperature and they were then processed, cut into 10 μm thick slices and stained by the method described before (Sayyah et al., 2007). The stained slices were qualitatively analyzed for electrode position using a stereoscopic microscope (Olympus, Japan). The data of the animals, in which their electrode was in false placement, were not included in the results.

2.4. Gene expression study

The frozen hippocampi were removed from −80 °C and pulverized completely. About 200 μl of chilled phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, and 1.4 mM KH_2PO_4) was added to the pulverized tissues and vortexed for 30 s, then spinned and aliquoted in two separate microtubes equally. One of the so prepared samples was used for gene expression study and the second part for immunoblotting. An appropriate volume of a protease inhibitor cocktail according to the manufacturer's proposal was added to samples, which were allocated for immunoblotting.

Total cellular RNA was isolated from the hippocampus by a modification of the guanidine isothiocyanate phenol-chloroform method (Ausubel et al., 2002) using RNX-PLUS reagent then treated with 10 U RNase free DNase I (Roche, Germany) to avoid any DNA contamination. The integrity of the RNA samples was determined using denaturing agarose gel electrophoresis. The concentrations of the RNAs were determined spectrophotometrically (Nanodrop, USA). The mean 260/280 ratios were 1.94 ± 0.0, while those of 260/230 were 1.98 ± 0.1.