Expression of connexin 30 and connexin 32 in hippocampus of rat during epileptogenesis in a kindling model of epilepsy

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Abstract: Objective Understanding the molecular and cellular mechanisms underlying epileptogenesis yields new insights into potential therapies that may ultimately prevent epilepsy. Gap junctions (GJs) create direct intercellular conduits between adjacent cells and are formed by hexameric protein subunits called connexins (Cx). Changes in the expression of Cxs affect GJ communication and thereby could modulate the dissemination of electrical discharges. The hippocampus is one of the main regions involved in epileptogenesis and has a wide network of GJs between different cell types where Cx30 is expressed in astrocytes and Cx32 exists in neurons and oligodendrocytes. In the present study, we evaluated the changes of Cx30 and Cx32 expression in rat hippocampus during kindling epileptogenesis. **Methods** Rats were stereotaxically implanted with stimulating and recording electrodes in the basolateral amygdala, which was electrically stimulated once daily at afterdischarge threshold. Expression of Cx30 and Cx32, at both the mRNA and protein levels, was measured in the hippocampus at the beginning, in the middle (after acquisition of focal seizures), and at the end (after establishment of generalized seizures) of the kindling process, by real-time PCR and Western blot. **Results** Cx30 mRNA expression was upregulated at the beginning of kindling and after acquisition of focal seizures. Then it was downregulated when the animals acquired generalized seizures. Overexpression of Cx30 mRNA at the start of kindling was consistent with the respective initial protein increase. Thereafter, no change was found in protein abundance during kindling. Regarding Cx32, mRNA expression decreased after acquisition of generalized seizures and no other significant change was detected in mRNA and protein abundance during kindling. **Conclusion** We speculate that Cx32 GJ communication in the hippocampus does not contribute to kindling epileptogenesis. The Cx30 astrocytic network localized to perivascular regions in the hippocampus is, however, overexpressed at the initiation of kindling to clear excitotoxic molecules from the milieu.

Keywords: connexin 30; connexin 32; hippocampus; kindling

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1 Introduction

Epilepsy is the third most common neurological disorder after stroke and Alzheimer's disease^[1]. Yet treatment is

directed mainly at blocking the symptoms or stopping epileptic seizures, not the underlying causes. Epileptogenesis is a process by which normal brain tissue is transformed into a tissue capable of generating spontaneous recurrent seizures. Exploring the events occurring during this period would help in designing new strategies to prevent epilepsy.

In epilepsy, abnormal excessive synchronized electrical activity of a small group of neurons spreads to adjacent cells and even to a large group of cells and ultimately causes a seizure. Besides the synaptic contacts between cells, gap-junctional communication is involved in the generation or spread of seizures^[2,3]. Gap junctions (GJs) serve as intercellular channels providing direct cytoplasmic continuity and the intercellular movement of ions, metabolites and second messengers. They are constituted by the hexameric organization of protein subunits called connexin (Cx). Changes in the expression of Cxs during seizure activity has been proposed as a possible mechanism underlying neuronal synchronization^[4–9]. The hippocampus, the main component of the limbic system, plays an important role in epileptogenesis. It has a wide network of GJ communication between different cell types. It was recently reported that astroglial networks scale synaptic activity and plasticity in the hippocampus^[10]. Hippocampal astrocytes express both Cx30 and Cx43. While previous studies reported no change in the expression of hippocampal Cx43 during epileptogenesis^[6,7], studies on the other main astrocytic Cx, Cx30, are limited. Moreover, in most of these studies, the expression was evaluated after the induction of seizures and not during epileptogenesis^[7,11,12]. Cx32 is also abundant in hippocampal oligodendrocytes and interneurons^[4,5,13]. Despite several studies on changes in Cx32 expression in the epileptic state or consequent to seizures^[4,9,14–20], changes of this Cx during epileptogenesis have received little attention.

In the kindling model of epilepsy, the seizures are analogous to human complex partial (focal) seizures with secondary generalization^[20]. Kindling is 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. In the kindling model,

with repeated administration of an initially sub-convulsive electrical stimulus, seizures generally evolve through five stages. Stages 1–3 are generally considered to be focal seizures, whereas stages 4–5 are secondarily generalized motor seizures. Kindling permits the investigator to evaluate any changes that occur at the cellular and molecular levels at any stage of the transformation of a normal brain to an epileptic one. In the present study, we examined the changes of Cx30 and Cx32 expression in rat hippocampus at the transcriptional and translational levels, at the beginning, in the middle (after acquisition of focal seizures), and at the end (after establishment of generalized seizures) of kindling.

2 Materials and methods

2.1 Animals Male Wistar rats (280–320 g, Pasteur Institute of Iran) were used throughout this study. All animal experiment protocols were approved by the Review Board and Ethics Committee of Pasteur Institute and conformed to European Communities Council Directive of November 1986 (86/609/EEC).

2.2 Stereotaxic surgery and kindling procedure The rats were stereotaxically implanted with stimulating and recording electrodes in the basolateral amygdala of the right hemisphere, according to the previously described method^[6]. After 7 days of recovery, the afterdischarge (AD) threshold in the amygdala was determined by a 2-s, 100-Hz monophasic square-wave stimulus of 1 ms per pulse^[6]. Two groups of animals were stimulated once daily at the AD threshold until they showed behavioral seizure stage 2 for two consecutive days (partially-kindled rats with focal seizures) or behavioral seizure stage 5 for three consecutive days (fully-kindled rats with generalized seizures) according to the Racine classification^[20]. A third group received only a single AD threshold stimulation. For each of these groups, a sham group (electrode implanted without stimulation) was used.

2.3 Tissue collection The animals were decapitated 24 h after the last stimulation and the brain was removed and incubated in chilled artificial cerebrospinal fluid (ACSF) consisting of the following (in mmol/L): 124 NaCl, 4.4 KCl, 2 CaCl₂, 2 MgCl₂, 1.2 KH₂PO₄, 25 NaHCO₃ and 10

glucose (pH 7.3). The hippocampus was dissected and frozen immediately in liquid nitrogen and stored at -80°C . The rest of the brain was placed in 10% formalin at room temperature, cut into 10- μm sections, and qualitatively examined for electrode position using a stereoscopic microscope (Olympus, Japan). Data from animals in which the electrode was in the wrong place were not included in the results.

2.4 Gene expression assay

2.4.1 Tissue preparation The frozen hippocampal samples were pulverized completely and mixed with 200 μL chilled phosphate-buffered saline (in mmol/L: 137 NaCl, 2.7 KCl, 4.3 $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 1.4 KH_2PO_4), vortexed for 30 s, centrifuged, and the supernatant divided into aliquots. An appropriate volume of a protease inhibitor cocktail (Roche, Germany) according to the manufacturer's guideline was added to those samples, which were allocated for immunoblotting.

2.4.2 RNA extraction Total cellular RNA was isolated from the hippocampus by a modification of the guanidine isothiocyanate phenol-chloroform method^[21] using RNX-PLUS reagent (Fermentas, Ukraine). The RNA was treated with 10 U RNase-free DNase I (Roche, Germany) to avoid DNA contamination. The integrity of the RNA samples was determined using denaturing agarose gel electrophoresis. The concentration and purity of the RNAs were determined by spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE). The mean absorbance ratio at 260/280 nm was 1.94 ± 0.0 and at 260/230 nm was 1.98 ± 0.1 .

2.4.3 cDNA synthesis The reverse transcription reaction was performed with a first-strand cDNA synthesis kit (Qiagen, Germany) using Oligo-dT primer, AMV reverse transcriptase and 1 μg total RNA as template, according to the

manufacturer's instructions. The concentration of synthetic cDNA was measured at 260 and 280 nm. DNA samples with A260/A280 ratios >1.5 were selected for quantitative analysis.

2.4.4 Real-time PCR and comparative threshold cycle method

Cx30 and Cx32 were chosen as target genes and α -tubulin and GAPDH were used as internal reference genes. All primers (Table 1) were designed using Primer Express v.3.0 (Applied Biosystems, Foster City, CA). The specificity of the primers for their target sequences was checked on the NCBI website (www.ncbi.nlm.nih.gov/blast). The SYBR Green I real-time PCR assay was carried out in final reaction volume of 25 μL with 12.5 μL SYBR Green I Master mix (Applied Biosystems, Paisley, UK), 100 nmol/L forward and reverse primers and 300 ng cDNA. Thermal cycling was performed on the ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) using the following cycling conditions: 10 min at 95°C as the first denaturation step, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each complete amplification stage was followed by a dissociation stage at 95°C for 15 s, 60°C for 30 s and 5°C for 15 s. The extent of gene expression was calculated using comparative threshold cycles. The mean threshold cycle (mC_T) was obtained from duplicate amplifications during the exponential phase of amplification. The geometrical mean of the two reference gene C_T values was subtracted from the mC_T value of the target genes to obtain ΔC_T . The $\Delta\Delta\text{C}_T$ value for each sample was calculated from the corresponding ΔC_T values:

$$\Delta\Delta\text{C}_T = \Delta\text{C}_T (\text{test sample}) - \Delta\text{C}_T (\text{control sample}).$$

The calculated $\Delta\Delta\text{C}_T$ was converted to a ratio using the formula ($\text{Ratio} = 2^{-\Delta\Delta\text{C}_T}$)^[22]. Dissociation curve analysis

Table 1. Primers used for real-time PCR

Target	Forward primer 5'→3'	Reverse primer 5'→3'	Amplicon (bp)
Cx30	AATGTGGCCGAGTTGTGTACC	AAGCTGGTGATGGCATTCTGAC	161
Cx32	CGGCATCTGCATTATCTCAAC	CAGCAGCTTGTTGATCTCATTCTG	163
α -tubulin	CTGGAACCCACAGTTATTGATGAAG	GGCATAGTTATTGGCAGCATCCTTC	159
GAPDH	AGTCAAGGCTGAGAATGGGAAG	CATACTCAGCACCAGCATCACC	160

was performed for each amplification reaction to detect any primer dimers or non-specific PCR products. Before using the comparative threshold cycle method, the amplification efficiency of each gene was determined from the standard curve drawn by plotting the logarithmic input amount of template DNA *versus* the corresponding C_T values. The corresponding real-time PCR efficiencies were calculated according to the slope of the standard curve and the following equation: Efficiency = $[10^{(-1/\text{slope})}] - 1$ [23]. Data evaluation was carried out using the ABI PRISM 7300 Sequence Detection System and SDS software v.1.2.3 (Applied Biosystems, UK).

2.5 Immunoblotting The second part of the homogenized hippocampus was removed from -80°C and centrifuged at 14 000 g at 4°C for 10 min. The supernatant was collected and the total protein concentration was determined using Bio-Rad DC protein assay reagents. Samples were dissolved in protein loading buffer and denatured for 5 min at 95°C prior to loading. Equal amounts of protein from each animal (5 $\mu\text{g}/\text{lane}$ for α -tubulin, 10 $\mu\text{g}/\text{lane}$ for Cx30 and Cx32) were resolved by denaturing SDS-polyacrylamide gel electrophoresis with 12% acrylamide and transferred to a PVDF membrane (Pharmacia Amersham, UK) by electroblotting (Mini trans blot electrophoretic transfer cell, Bio-Rad). The membrane was blocked in TBST buffer (100 mmol/L Tris base, 150 mmol/L NaCl, and 0.2% Tween 20) containing 2% ECL Advance blocking agent at room temperature for 60 min, rinsed briefly with TBST buffer and then incubated for 60 min with the following primary antibodies: mouse monoclonal anti-Cx30 and Cx32 (Invitrogen, USA) diluted 1:200 000, and mouse monoclonal anti- α -tubulin (Sigma-Aldrich, Germany) diluted 1:200 000.

The antibodies were diluted in blocking buffer. After washing 4 times with TBST buffer ($1\times$ for 15 min and $3\times$ for 5 min), the membrane was incubated with peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, Germany; diluted 1:100 000 for Cx30 and Cx32 and 1:2 000 000 for α -tubulin) for 1 h, then washed 4 times with TBST buffer ($1\times$ for 15 min and $3\times$ for 5 min) and reacted with ECL Advance Western blotting detection reagents (Pharmacia Amersham, UK), for 4 min. X-ray film (Retina, USA) was exposed for 2–5 min and then developed to visualize the antibody binding. Bands were quantified by densitometry using Labworks analysis software (Ultra Violet Products, UK). The relative levels of Cx30 and Cx32 proteins were expressed as ratios (Cx30/ α -tubulin $\times 100\%$, Cx32/ α -tubulin $\times 100\%$).

2.6 Statistical analysis The data were analyzed by ANOVA with Tukey's *post-hoc* test and are presented as mean $\pm$ SEM. In all experiments, a P value <0.05 was considered statistically significant.

3 Results

Initial afterdischarges of 6.6 ± 0.6 s were recorded from the amygdala at the beginning of the kindling procedure. The duration of afterdischarges increased during kindling development and reached 34.6 ± 2.7 s after acquisition of focal seizures and 64.8 ± 4.4 s after acquisition of generalized seizures (Fig. 1).

3.1 Gene expression analysis Melting curve analysis for the Cx30, Cx32, GAPDH and α -tubulin gene fragments revealed a unique PCR product in each reaction. Melting temperatures of 80.5°C for GAPDH, 81.3°C for α -tubulin, 79.8°C for Cx30, and 79.0°C for Cx32 were obtained. The

Fig. 1. Representative electroencephalograms from the amygdala of a rat after development of focal (upper) and generalized (lower) seizures.

amplification curves for both reference genes (α -tubulin and GAPDH) crossed the threshold line at the same point. Mean threshold cycles of 21.95 for GAPDH and α -tubulin, 25.61 for Cx32, and 27.92 for Cx30 were obtained.

Initial single stimulation of the amygdala resulted in a significant increase in Cx30 gene expression in the hippocampus compared to the sham unstimulated group (Fig. 2A). The Cx30 overexpression remained after acquisition of focal seizures but the level of amplification was less than that after the first stimulation. Then Cx30 mRNA expression significantly decreased when the animals became fully

kindled and showed generalized seizures.

Regarding Cx32, although an increase in mRNA expression occurred after the first stimulation and after the acquisition of focal seizures, there were no statistical significances (Fig. 2B). However, the Cx32 mRNA significantly decreased after the acquisition of generalized seizures and reached a level much lower than that in the corresponding sham group.

3.2 Immunoblot analysis Cx30 protein level was significantly increased in the hippocampus after the first stimulation (Fig. 3A). No other change was noted after the devel-

Fig. 2. Connexin 30 (A) and connexin 32 (B) mRNA levels in the hippocampus of rats during kindling development. Connexin mRNA levels were normalized to those of α -tubulin and GAPDH mRNA. Data are expressed as mean $\pm$ SEM ($n = 5$). ** $P < 0.01$ and *** $P < 0.001$ compared to the respective control group.

Fig. 3. Connexin 30 (Cx30, A) and connexin 32 (Cx32, B) protein levels in the hippocampus of rats during kindling development. C: Representative immunoblots of Cx30, Cx32 and α -tubulin. Each immunoblot was performed in duplicate to increase the reliability of the measurements. I, test; II, sham. Connexin protein levels were normalized to that of α -tubulin. Data are expressed as mean $\pm$ SEM ($n = 5$). * $P < 0.05$ compared with the respective sham control group.

opment of focal and generalized seizures. Cx32 protein level did not undergo any significant changes during the kindling process (Fig. 3B).

4 Discussion

The results of this study showed overexpression of Cx30 at both the transcriptional and translational/post-translational levels in the hippocampus at the beginning of kindling epileptogenesis. This overexpression remained at the mRNA level (but not at the protein level) after acquisition of focal seizures but then was significantly down-regulated in epileptic animals. Regarding Cx32, only a significant decrease in gene expression was found in the hippocampus after the development of generalized seizures.

Cx30 and Cx32 expression has been studied in tissues from patients with epilepsy and animal models of epilepsy. Condorelli *et al.* reported that kainate treatment induces a strong and region-specific increase in the astroglial Cx30 mRNA level, suggesting a direct or indirect involvement of this connexin in the neuronal apoptotic process^[12]. Söhl *et al.* reported no change in the expression of Cx30, Cx43 and Cx32, at both the mRNA and protein levels in the hippocampus of young kainate-treated rats^[8]. Li *et al.* showed that after 2–6 h of exposure to bicuculline, Cx32 mRNA expression is upregulated 2–3 fold of the control level, and the protein level is significantly elevated thereafter^[4]. Szente *et al.* found significant elevation of the Cx32 mRNA level at the primary focus as well as at the mirror focus after 60 min of repeated ictal discharges induced by 4-aminopyridine^[14]. Yao *et al.* reported upregulation of Cx32 in the hippocampus of patients with intractable epilepsy^[17]. In contrast, Collignon *et al.* reported a decrease in Cx32 in hippocampal subregions in patients suffering from mesial temporal lobe epilepsy^[15]. These differences in Cx30 and Cx32 expression in different studies may be due to variations in the preparations used, methods of seizures induction, duration of seizure activity, time-point examined after seizures, and different brain regions and subregions used. Although these studies indicate a relationship between Cx30 and Cx32 GJ coupling and seizure activity,

the data were obtained from human and animal tissues that were already epileptic and did not give any information regarding the possible changes in Cx30 and Cx32 expression during the process of epileptogenesis. In other words, it is not clear whether the changes in Cx expression occurred in response to seizures and epileptic conditions, or were generated during the development of epilepsy. In the present study, we set out to determine the pattern of expression of hippocampal Cx30 and Cx32 during kindling, which is a model of epileptogenesis^[20].

In vivo Cx30 expression accounts for ~50% of astrocytic coupling in the hippocampus^[24]. Cx30 gene expression in the hippocampus increased to over two-fold of that in sham group at the beginning of the epileptogenic process. This significant overexpression persisted until the acquisition of focal seizures. Cx30 gene expression then significantly decreased in epileptic rats to a level much lower than that of sham (unstimulated) rats. At the post-transcriptional level, only at the start of epileptogenesis, i.e. after the first kindling stimulation, was overexpression found and no change in Cx30 protein abundance occurred afterward during the kindling process. Intercellular communication through GJs can be regulated at several levels, including changes in Cx transcription, translation, stability, post-translational processing, insertion/removal from the cell membrane, and channel gating^[24]. However, post-translational processes are thought to be the major factor in regulating Cx levels and functional coupling^[24]. We found an increase in the abundance of Cx30 protein at the start of kindling. A correlation between Cx protein expression and functional coupling has been demonstrated by many researchers^[25,26]. Although our results do not address whether overexpression of Cx30 protein is accompanied by an increase in functional coupling, enhancement of hippocampal junctional coupling by Cx30 hemichannels at the start of kindling epileptogenesis is plausible.

Based on ultrastructural investigations, it is now well documented that Cx30 is widely expressed in astrocytes as a component of astrocyte/astrocyte as well as astrocyte/oligodendrocyte GJs and in perivascular regions localized to astrocytic end-feet^[27]. Expression of Cx30 in hippocampal

astrocytes has been reported and it plays a role in GJ communication between astrocytes in the hippocampus^[27,28]. Recently, Gosejacob *et al.* demonstrated the heterogeneous expression of Cx30 in the normal mouse hippocampus^[29]. A high level of expression is found around blood vessels in the stratum radiatum. Modest levels of Cx30 are found at blood vessels in the CA1 area as well as the stratum oriens near the pyramidal cell layers. In the dentate gyrus, robust Cx30 immunoreactivity is found in the hilus with low staining in the molecular layer. In all areas of the hippocampus, GJ plaques composed of Cx30 are found at blood vessels. It has been suggested that blockade of glial GJs can impair spatial buffering and result in the accumulation of extracellular potassium and glutamate, which in turn increase neuronal excitability and lead to seizures. In Cx43- and Cx30-knockout mice, the threshold of epileptiform discharge is reduced^[30] and hippocampal synaptic transmission and neuronal excitability increase due to decreased clearance of astroglial glutamate and potassium^[10]. Thus, augmentation of Cx30 protein expression at the beginning of the kindling process might be a compensatory response for neuronal discharges in order to buffer the milieu, propagate excitatory stimuli to neighboring cells, and thereby modulate hippocampal excitability and epileptogenesis. In order to test this proposal, further complementary studies on Cx30 channel gating during kindling epileptogenesis and on the effect of specific Cx30 blockers on kindling rate are warranted.

Cx32 is found between neurons as well as oligodendrocytes (oligodendrocyte/oligodendrocyte and oligodendrocyte/astrocyte GJs)^[5]. In the hippocampus, Cx32 has been detected in the majority of pyramidal neurons of the CA fields and cells of the dentate gyrus^[31]. In the CA1 subfield, Cx32 is expressed by parvalbumin-positive inhibitory interneurons and Cx32 GJs form between interneurons, within oligodendrocytes, or both, and play a role in inhibitory transmission in the hippocampus^[32]. It has recently been reported that Cx32-Cx43 knockout mice develop seizures^[16]. On the other hand, Cx32 protein is overexpressed in the hippocampus of patients with intractable epilepsy^[17]. Paradoxically, a significant decrease in Cx32 protein in

the hippocampus of epileptic patients has been reported^[15]. Here, we found significant downregulation of Cx32 at the transcriptional level in the hippocampus after the completion of kindling in rats. However, no change was detected at the protein level during kindling. This result is in agreement with the study performed by McCracken *et al.*, who reported no change in Cx32 protein expression in the dorsal hippocampus following single stimulation^[9]. Gajda *et al.* reported overexpression of cortical Cx32 mRNA at the epileptic foci after repeated seizures induced by 4-aminopyridine^[18]. It has also been reported that inhibition of Cx32 mRNA expression and protein synthesis by intracerebroventricular injection of a specific antisense oligodeoxynucleotide decreases audiogenic seizures^[19]. We did not find any change in Cx32 protein level during kindling epileptogenesis, so it is unlikely that Cx32 GJ communication is affected during kindling development. In order to address the specific expression of Cx30 and Cx32 in each region of the hippocampus, additional experiments such as *in situ* hybridization and immunohistochemistry are needed. Nonetheless, because of the punctate staining pattern of Cxs, which makes assignment to specific cells or cell types difficult or even impossible, immunohistochemistry is not enough and reporter genes are nowadays used to study Cx expression.

This study indicated that Cx30 in the astrocytic network localized to perivascular regions in the hippocampus is overexpressed at the start of kindling. This upregulation might have a neuroprotective role and decrease neuronal excitability by spatial buffering and scavenging excitotoxic molecules from the milieu. Further complementary experiments on epileptogenesis in knockout animals as well as using specific gene expression inhibitors such as antisense oligodeoxynucleotides are suggested to extend the results of this study.

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