Altered expression of pannexin proteins in patients with temporal lobe epilepsy

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Abstract. The aim of the present study was to investigate the expression of the pannexin (Panx) proteins, Panx1 and Panx2, in the temporal lobe tissue of patients with temporal lobe epilepsy (TLE). Immunohistochemistry and western blotting methods were used to localize and quantify Panx1 and Panx2 in the surgically removed brain tissue of patients with TLE (n=37). The results were then compared with non-epileptogenic controls (n=9). Panx1 and Panx2 expression was detected in the temporal lobe cortex of patients with TLE and in the control tissues. Panx1 and Panx2 proteins were expressed in all layers of the epileptic cortex, but predominantly in layers II and III of the cortex in the control group. Panx1 protein expression was significantly higher in the temporal lobe cortex of the patients with TLE than in the controls ($P<0.05$; t-test); however, no significant differences were identified in the Panx2 expression levels between the patients and the controls ($P>0.05$; t-test). The expression of the two Panx proteins in the tissue layers of the epileptic cortex varied in the patients and controls. The results indicate that Panx channels may be involved in the pathogenesis of TLE.

Introduction

Temporal lobe epilepsies (TLEs) are a group of medical disorders that result in recurrent epileptic seizures arising from one or both temporal lobes of the brain.

The presence of gap junction proteins termed pannexins (Panx) was first reported by Panchin *et al* in 2000 (1) . These proteins release large signaling molecules, including ATP and arachidonic acid derivatives. Three subtypes of the Panx family have been identified as Panx1, 2 and 3 (2,3). Human and mouse Panx1 mRNA is ubiquitously expressed in normal tissues of humans and mice, respectively; however, human Panx2 is a brain-specific gene and Panx3 is expressed predominantly in osteoblasts and synovial fibroblasts in *in silico* evaluation. Furthermore, Panx1 has been shown to form functional hemichannels by itself or in conjunction with Panx2 (1,4).

Panx1 channel opening has been indicated to contribute to epileptiform seizure activity. Thompson *et al* (5) identified that Panx1 hemichannel opening is triggered by *N*-Methyl-D-aspartate stimulation and may be a significant target for the treatment of epilepsy. Furthermore, it has been demonstrated that reduced levels of extracellular glucose due to fasting or adhering to a ketogenic diet may induce Panx1 hemichannel-mediated ATP release from CA3 neurons (6). This in turn hyperpolarizes neuronal membrane potentials via ATP-sensitive potassium channels. The role of Panx2 in the pathology of epilepsy is less certain than that of Panx1. However, since Panx2 may participate in the functional hemichannels with Panx1, this subtype was also included in the present study in order to investigate whether the expression pattern changed in epileptic brain tissues.

To the best of our knowledge, the current study compared for the first time the expression pattern of Panx1 and Panx2 in brain tissues in patients with TLE and non-epileptic patients using immunohistochemistry and western blotting techniques, to aid in our further understanding of the pathogenesis of TLE.

Patients and methods

Patient selection. Epileptic brain tissue was obtained from 37 patients with TLE and from nine control patients with traumatic brain injury (Tables I and II) (7).

The patients with TLE had typical clinical manifestations and characteristic electroencephalography (EEG) findings. The diagnosis of the seizure type was confirmed according to the 1981 International Classification of Epileptic Seizures of the International League Against Epilepsy (8). Prior to surgery, the epileptic lesions were located in all patients by brain magnetic resonance imaging (MRI) and 24-h or video-EEG recordings. Sphenoidal electrode monitoring and intraoperative electrocorticography were performed to localize the epileptic lesion prior to resection.

For the control experiments, temporal lobe tissue from nine patients with traumatic brain injury was obtained from the files of the Department of Neurosurgery of Xiangya Hospital (Changsha, China). These patients had undergone surgery due

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Table I. Data for patients with temporal lobe epilepsy.

Sample	Age (years)	Gender	Disease duration (years)	Lobectomy[a]	Types of seizures
E1	22	M	14	L	CPS, SGS
E2	27	M	21	R	CPS, SGS
E3	28	M	12	R	CPS, SGS
E4	20	M	18	R	SPS, SGS
E5	27	M	4	R	CPS
E6	39	F	37	R	CPS, SGS
E7	20	M	8	R	CPS, SGS
E8	30	M	18	L	CPS, SGS
E9	28	F	12	L	CPS, SGS
E10	27	M	11	R	SPS, SGS
E11	32	F	4	L	CPS
E12	26	M	3	R	CPS
E13	17	M	7	L	SPS, SGS
E14	22	M	18	R	CPS
E15	25	M	8	R	GTCS, SPS
E16	29	M	8	R	GTCS, TS
E17	32	M	4	L	CPS
E18	20	M	8	R	CPS, SGS
E19	25	M	6	R	GTCS, TS
E20	17	M	4	L	CPS, SGS
E21	24	M	3	L	SPS, SGS
E22	23	M	20	L	CPS, SGS
E23	29	M	25	R	GTCS, SPS
E24	30	M	2	L	CPS
E25	18	F	7	L	CPS
E26	30	F	12	R	SPS, SGS
E27	22	M	10	L	SPS, SGS
E28	30	M	5	L	SPS, SGS
E29	24	F	14	R	CPS, SGS
E30	22	M	17	R	SPS, SGS
E31	32	F	28	R	SPS, SGS
E32	23	M	12	R	SPS, SGS
E33	21	M	17	R	CPS, SGS
E34	35	F	20	R	CPS, SGS
E35	31	F	10	L	SPS, SGS
E36	32	M	20	L	CPS, SGS
E37	34	F	21	L	SPS, SGS

[a]Side of temporal lobe resection (right or left). CPS, complex partial seizure; GTCS, generalized tonic-clonic seizure; SGS, secondary general seizure; T, tonic seizures; SPS, simple partial seizure.

to severe brain trauma and had no history of epilepsy or other abnormal pathology and exposure to anti-epileptic drugs. The samples comprised temporal neocortical tissue adjacent to the trauma-induced lesion.

Written informed consent was obtained from the patients or their relatives with regard to the use of data and tissues for the purpose of research studies. The study complied with guidelines for the conduct of research involving human subjects as established by the National Institute of Health and the Committee on Human Research at Xiangya Hospital.

Tissue processing. The brain tissue from the epileptic patients and the patients with traumatic brain injury was obtained during surgery. Following dissection, the specimens were divided into two portions. One portion was immediately frozen in liquid nitrogen, stored at -80˚C and subsequently used for western blotting. The other portion was fixed in 4% paraformaldehyde, embedded in paraffin blocks, cut into 5.0-μM transverse sections on a Model RM2135 microtome (Leica, Wetzlar, Germany) and preserved for future use at room temperature.

Table II. Data for patients used as controls.

Sample	Age (years)	Gender	Lobectomy[a]	Etiological diagnosis	Adjacent tissue pathology
C1	39	M	L	Trauma	Normal
C2	32	F	R	Trauma	Normal
C3	28	M	R	Trauma	Normal
C4	40	M	L	Trauma	Normal
C5	22	F	R	Trauma	Normal
C6	27	M	L	Trauma	Normal
C7	21	M	R	Trauma	Normal
C8	10	M	L	Trauma	Normal
C9	34	F	R	Trauma	Normal

[a]Side of temporal lobe resection (right or left).

Immunohistochemistry. Temporal lobe tissue sections of patients with epilepsy were processed for Panx immunohistochemistry using avidin-biotin peroxidase methods. Following dewaxing and rehydration, endogenous peroxidases were inactivated by adding 1% H_2O_2 for 30 min. The sections were rinsed in 0.01 M phosphate-buffered saline (PBS) and incubated in 10% normal goat serum in PBS for 2 h to reduce any non-specific binding.

The sections were then incubated overnight with a Panx antiserum at 4˚C. Panx1 (1:150, rabbit polyclonal antibody; Abcam, Cambridge, UK) and Panx2 (1:250, rabbit polyclonal antibody; Abcam) antisera were diluted in PBS containing 2% normal goat serum. Following rinsing, the sections were incubated in biotinylated secondary antiserum (rabbit anti-goat IgG, 1:200; Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. Following a further rinsing, the sections were incubated in avidin-biotin peroxidase complex (1:200; Vectastain Elite ABC; Vector Laboratories) in PBS for 1 h.

To visualize the peroxidase labeling, the sections were processed with 0.06% diaminobenzidine tetrahydrochloride and 0.006% H_2O_2 diluted in 0.075 M PBS for 10 min. Following rinsing, the sections were dehydrated and placed under a cover slip. Visual field images were obtained layer by layer from every section using an Nikon TE2000 automatic microscope (Nikon, Tokyo, Japan) and Nikon DS-Fi1 (Nikon) pathology system. The full featured photo of the cortex was captured by PT Gui Pro 9.0.4 (New House Internet Services B.V., Rotterdam, The Netherlands).

Western blotting. A western blot analysis was performed to compare Panx immunoreactivity in the TLE and control groups. Tissue samples were cut into small sections, homogenized in radio-immunoprecipitation assay lysis buffer [0.05 mol/l Tris-HCl (pH 7.4), 0.15 mol/l NaCl, 1% Triton X-100, 1% (w/v) sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)], including 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 12,000 x g at 4˚C for 5 min. The protein concentration of the lysates was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The extracts (40 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were divided into two sections according to the location of molecular weight markers to detect Panx2 (69 kDa) and β-actin (42 kDa), which was used as a loading control. The PVDF membrane was blocked with 5% milk prepared in tris-buffered saline with 0.05% Tween-20 (TBST) then incubated for 1 h at room temperature.

Following extensive washing with TBST, the membranes were incubated in Panx2 (1:200, rabbit polyclonal antibody, Abcam, UK) and β-actin (mouse monoclonal IgG, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies for 12 h at 4˚C. Following three 10-min washes with TBST, each blot was incubated with a secondary antibody (goat anti-rabbit IgG for Panx2 blot and goat anti-mouse IgG for β-actin blot; Beyotime Company, Haimen, China) at a dilution of 1:1,000 for 1 h. Following three further washes with TBST and one wash with TBS (15 min each), the Panx2 protein and β-actin were detected using an enhanced chemiluminescence detection kit according to the manufacturer's instructions (Thermo Scientific Pierce, Waltham, MA, USA).

Panx1 was assayed by incubating the PVDF membranes with β-actin in stripping buffer [700 μl 14.4 mmol/l β-mercaptoethanol, SDS 1 g, 0.5 mol/l Tris-HCl (pH 6.8) and 12.5 ml in 100 ml ultra-pure water] for 30 min. The blot was then re-hybridized with the anti-Panx1 primary antibody (1:100, rabbit polyclonal antibody; Abcam) for 12 h at 4˚C. Following washing with TBST three times, the blot was incubated with goat anti-rabbit IgG (1:1000, Beyotime Company). Following washing three times with TBST and once with TBS, the Panx1 level was detected by enhanced chemiluminescence as described.

Statistical analysis. Statistical analysis was performed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). Data were presented as the mean ± SD and any differences between active and control groups were determined using χ^2 tests and t-tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. No significant differences were identified in the ratios of age, gender and lobectomy among

Table III. Clinical characteristics of the subjects.

	TLE group	Control group	P-value
Age	26.29±5.33	28.11±9.53	0.443[b]
Gender			
Male	27	6	0.706[c]
Female	10	3	
Lobectomy[a]			
Left	16	4	0.948[c]
Right	21	5	

[a]Side of temporal lobe resection (right or left); [b]t-test; [c]χ^2 test. TLE, temporal lobe epilepsy.

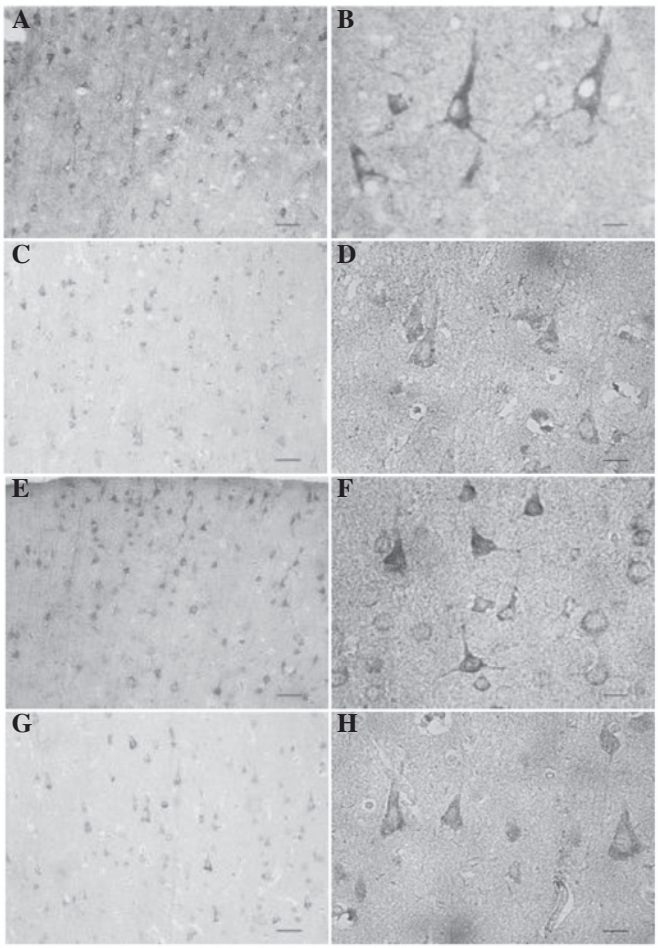

Figure 1. Microphotographs of immunohistochemistry. Distinct localization of Panx1 and Panx2 in layer III of control (A, B, E, F) and epileptic (C, D, G, H) temporal lobe cortex. The pyramidal cell bodies highly expressed Panx1 and Panx2 (A-D) in layer III of the temporal lobe in both groups. Scale bar, (A, C, E and G) 50 μm; (B, D, F and H) 10 μm. Pannexin, Panx.

the two groups of patients who donated tissues for the study (Table III). The TLE group included 27 male and 10 female patients with a mean age of 26.3 years (range, 17 to 39 years). The duration of epilepsy (time since onset of symptoms) ranged from 2 to 37 years (mean, 12.6 years). In total, 26 cases were categorized as partial seizures, secondarily generalized as tonic, clonic or tonic-clonic seizures, seven cases as complex partial seizures and four cases as other seizure types. The control group included six male and three female patients with brain trauma, between 10 and 40 years of age (mean age, 28.1 years).

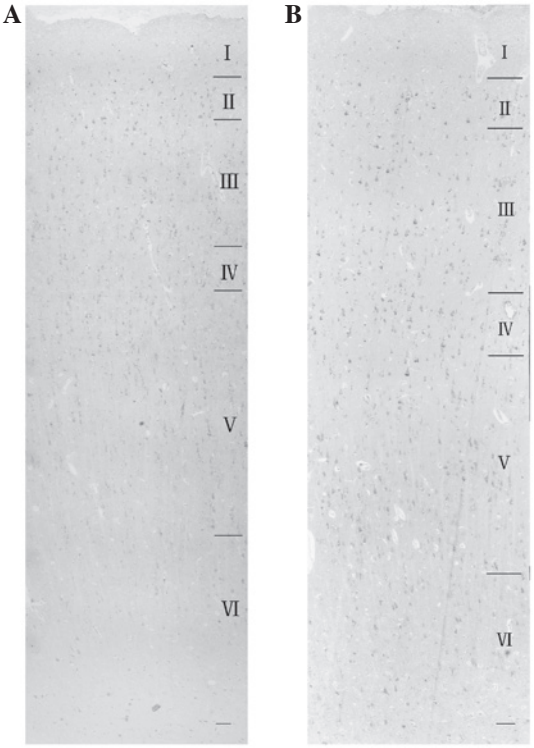

Figure 2. Distinct localization of (A) Panx1 and (B) Panx2 in the epileptic temporal lobe cortex. Panx1 and Panx2 proteins were expressed in all the six layers of the epileptic cortex in the temporal lobe formation. Expression was lowest in layer I. Scale bar, 100 μm (A and B). Pannexin, Panx.

Panx expression. Panx1 and Panx2 expression was detected in the temporal lobe cortex of patients with TLE and in the control tissues. In the control group, Panx1 and Panx2 proteins were expressed predominantly in formation layers II and III of the cortex of the temporal lobe formation (Fig. 1). In the TLE group, Panx1 and Panx2 were expressed in all the six layers of the epileptic cortex, but less in layer I than in the other five layers (Fig. 2).

At a higher magnification, the cell bodies of pyramidal cells were shown to be immunoreactive for Panx1 (Fig. 1B and D) and Panx2 (Fig. 1F and H) in layer III. In the TLE group, the nuclei of several small-sized cells exhibited moderate immunoreactivity (Fig. 1D and F).

The expression level of Panx protein in the brain tissue of the TLE patients was subject to semi-quantitative analysis using western blotting (Fig. 3A). The variance between the two groups of patients was examined by calculating the ratio of the optical density of Panx to β-actin (Fig 3B and C). The Panx1/β-actin ratio was 0.80±0.01 for the TLE samples and 0.39±0.08 for the control samples ($P<0.05$). Thus strong upregulation of Panx1 immunoreactivity was present in all samples from TLE patients compared with the control group.

The Panx2/β-actin ratio of the TLE samples was 0.67±0.02 and the corresponding ratio for the control samples was

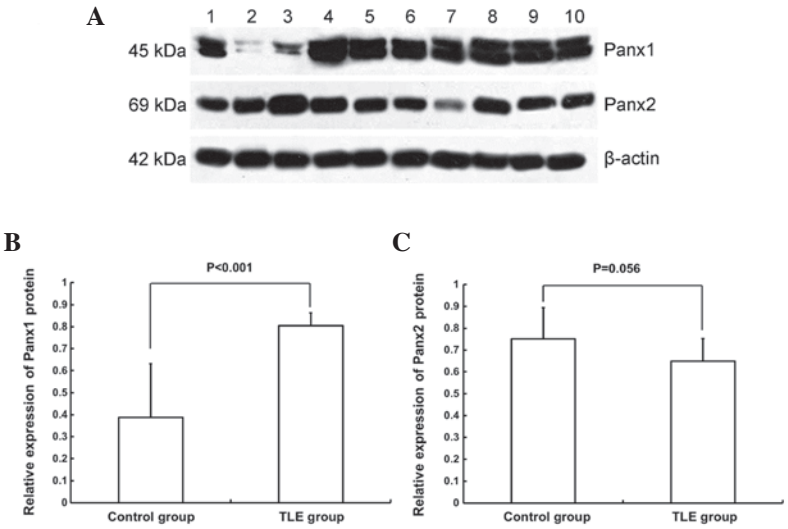

Figure 3. Semi-quantitative western blot analysis of Panx expression in the temporal lobe tissue of TLE patients. (A) Lanes 1-3 indicate protein levels in the control tissues, and lanes 4-10 indicate the protein levels of TLE patients. Immunoblot signals were developed by enhanced chemiluminescence. The two bands are likely to be the 43 and 47 kDa isoforms of Panx1 present in mouse heart tissue lysate although this was not specifically investigated. (B and C) The average ratio of Panx to β-actin for each group of patients. Error bars are the SD. Pannexin, Panx.

0.75±0.05. This difference was not statistically significant (P>0.05).

Discussion

In the present study, the expression patterns of Panx1 and Panx2 in the brain tissues of epileptic patients and controls were compared. To the best of our knowledge, this was the first time changes in Panx expression have been studied in human epileptic brains.

Panx1 and Panx2 are expressed in the central nervous system (1,4,9,10). The expression of Panx1, but not of Panx2, in *Xenopus* oocytes forms functional hemichannels (1,4). In addition, coinjection of these two Panx RNAs has been shown to result in hemichannels with functional properties that vary from those formed by Panx1 only. The functional characteristics of homomeric Panx1 versus heteromeric Panx1/Panx2 channels, and the varying expression patterns of Panx1 and Panx2 in the brain, indicate that Panx form cell type-specific channels that have distinct properties and that may serve different functions.

In the present study, it was demonstrated that the Panx1 and Panx2 proteins were expressed predominantly in layers II and III of the cortex in the control group. Previous studies have shown that Panx1 and Panx2 mRNAs are present in all regions of the cerebral cortex of adult rats; however, the intensity of expression varies in the individual layers. For example, cells in layers II/III and V have been shown to exhibit a stronger signal for Panx mRNAs than those in layers I, IV and VI (4). This was not quite consistent with the results of the present study in the control groups. This apparent difference between the results was likely to be due to two reasons. The first was that the subjects studied varied; one subject was a rat while the other was human. The second reason may be due to different methods being adopted; the previous study conducted *in situ* hybridization in an animal, while the present study used immunohistochemistry and western blotting in patients.

Cortical neurons are important in epilepsy. Layers III, V and VI of the cortex are the layers in which pyramidal cells are predominantly distributed and from which association fibers arise, and layer IV is the main target of thalamocortical afferents from thalamic type C neurons and from intra-hemispheric corticocortical afferents. Increased excitability and inward rectification in layer V cortical pyramidal neurons have been recorded in epileptic mutant mice (11). In chronically injured epileptogenic rats, excitatory synaptic connectivity is enhanced in layer V pyramidal neurons. This is accompanied by increased total axonal length and increased density of synaptic boutons (12). There is also evidence that epileptic discharges may be initiated from layer V and VI neurons (13). In the present study, Panx1 and Panx2 were expressed in all six layers of the epileptic cortex, with the lowest expression observed in layer I. Ectopic expression of Panx in the epileptic cortex indicates that Panx proteins may be involved in the function of neurons in layers IV, V and VI of the epileptic cortex.

The study of Panx1, as shown by western blot analysis, confirmed previous results obtained with Co^{2+}-treated brain slices (14). However the Panx2 findings are at variance. With the Co^{2+}-treated brain slices there was a 1.5-fold increase in Panx1 and a 1.4-fold increase in Panx2 mRNA; significant post-translational modifications of Panx1 protein were also observed after Co^{2+} treatment. While the present study identified significantly increased Panx1 levels in the TLE group, the change in Panx2 protein levels was not significant. With the exception of the difference between the species of the subjects studied and the research methods, this inconsistency was most likely due to the choice of the brain slice model vs. TLE patients to detect changes in Panx expression, which possibly reflects differences in the functional mechanisms of Panx in TLE and in acute seizure activity induced by Co^{2+}. Therefore, further studies are required to verify the most likely mechanism.

These experiments provide the first evidence for the expression of Panx protein in temporal lobe tissues of patients with

TLE. The present study observed increased Panx1 expression and the ectopic expression of Panx1 and Panx2 in temporal lobe tissue in epilepsy. These results may be associated with the functional changes of cells in the epileptic cortex. The results support the conclusion that Panx channels are important in the formation of epilepsy.

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