Altered immunohistochemical distribution of hippocampal interneurons following traumatic brain injury

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Abstract

Aim: The aim of this study was to determine whether alteration of interneurons in the hippocampus might play a role in neuronal damage caused by brain trauma and whether immunohistochemical expressions in the hippocampus might change during the adaptive stage as early time point following traumatic brain injury (TBI).

Methodology: For the study, we used Sprague-Dawley (SD) rats and manufactured TBI animal model by creating cryogenic injury to specific brain tissue. Thereafter, immunohistochemical approaches were utilized to determine parvalbumin (PV) and calbindin D-28K (CB) expression in the hippocampus following brain trauma in a time course. All hippocampal tissues were analyzed qualitatively and quantitatively using immunoreactivity, cell counting and densitometry. Statistical significance was determined by one-way ANOVA and Bonferroni's test.

Results: At early time period following TBI, both PV and CB immunoreactivities decreased in the lesioned hippocampus. However, their expression levels were recovered to control levels as time passed by. On the other hand, PV immunoreactivity in contralateral hippocampus was transiently reduced whereas CB expression remained unchanged.

Interpretation: Results of this study revealed that altered distribution of interneuronal populations in the hippocampus might contribute to neuronal loss induced by abnormality of inhibitory neurotransmission at early time period following brain damage, thus leading to the development of epileptogenesis in patients with TBI.

Key words: Calbindin D-28K, Epileptogenesis, Parvalbumin, Post-traumatic epilepsy, Traumatic brain injury

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Introduction

It is well known that traumatic brain injury (TBI) is accompanied by a delayed pathophysiologic change in degenerated neurons when the brain tissue is directly damaged by external factors (Gaetz, 2004). These brain injuries can degrade various cognitive functions and impair motor ability of patients. In some patients, TBI may cause recurrent epileptic seizures due to sequela of brain damage (Kharlamov *et al.*, 2011). Although consequences of TBI differ by individuals, more than 50% of patients with recurrent epileptic seizure can progress to post-traumatic epilepsy (PTE) within one year (Willmore and Ueda, 2009). In addition, the morbidity rate of epileptic patients increases with the severity of brain damage (Weiss *et al.*, 1983, 1986).

On the other hand, some previous studies have demonstrated that the cause of epilepsy is due to excessive enhancement of neuronal excitability caused by abnormal inhibitory neurotransmissions (Guerrini, 2001; Meldrum, 2002; Rozycka and Trzeciak, 2003). Thus, the main cause of pathophysiological characteristics of epilepsy might be attributed to abnormal production of inhibitory neurotransmitter such as γ -aminobutyric acid (GABA). In addition, significantly down-regulated GABAergic interneuron due to diverse brain damage effects can result in declined control of neuronal excitability, thus leading to epileptogenesis following brain trauma (De Lanerolle *et al.*, 1989). However, in several studies a variety of experimental animal models have shown that GABAergic interneuron is increased in epileptic animals as compared to normal group. Moreover, "disinhibition theory" states that epilepsy occurs due to inhibition of GABA secretion by formation of many inhibitory synapses in GABAergic interneuron (Peterson, 1987). Despite these efforts, the basic mechanism of epileptogenesis following brain trauma has not been clarified yet, although epilepsy caused by TBI accounts for more than 5% of all epilepsy patients and more than 20% of symptomatic epilepsy patients (Pagni and Zenga, 2005; Garga and Lowenstein, 2006; Mani and Barry, 2006).

Calcium binding proteins (CBPs), including parvalbumin (PV) and calbindin D-28k (CB), that regulate the intracellular calcium ion in response to neuronal hyperexcitability are well known as specific markers for denaturation of various neurons (Fritschy *et al.*, 1999; Sutch *et al.*, 1999). These results seem to reveal that the functionalities of various GABAergic interneurons play an important role in the pathogenesis of epilepsy following TBI, suggesting that CBPs might be altered in this process. Therefore, the present study was carried out to determine the effects of GABAergic interneurons on the development and spread of epilepsy after TBI, using an experimental animal model at early time stage. Basic medical data was also examined to determine the role and function of these factors in the pathogenesis of epilepsy, following TBI.

Materials and Methods

Experimental animals: All experiments utilized the progeny of Sprague-Dawley rats obtained from the Experimental Animal Center, Soonchunhyang University (Cheonan, South Korea). All animals were provided access to a commercial diet and water *ad libitum* under controlled temperature, humidity and lighting conditions (light/dark cycle 12:12, and $22 \pm 2^\circ\text{C}$, $55 \pm 5\%$). All animal protocols were approved by the Administrative Panel on Laboratory Animal Care of Soonchunhyang University (permit No. SCH15-0002). All efforts were made to avoid suffering of rats and minimize the number used during the experiments.

Induction of traumatic brain injury: The cryogenic TBI animal model was used as described in previous study (Siren *et al.*, 2006) with some modification. Briefly, ten-week-old male Sprague-Dawley rats were anaesthetized under an intraperitoneal injection of ketamine (90 mg kg^{-1}) and xylazine (4 mg kg^{-1}). The frontoparietal skull was exposed through a scalp incision under stereotaxic frame and a cryogenic lesion was placed on the right frontoparietal cortex (unilateral lesions) or on both frontoparietal cortices (bilateral lesions). For cryogenic lesions, a cone-shaped copper cylinder with tip diameter of 2 mm was cooled with liquid nitrogen (-183°C). The tip was stereotaxically placed in direct contact with the exposed frontoparietal skull and kept in place for 60 sec. Sham-operated animals underwent the same procedure without cooling the metal cone.

Tissue processes: Anesthetized animals (urethane 1.5 mg kg^{-1}) were perfused transcardially with PBS, followed by fixing with 4% paraformaldehyde in 0.1M PB for different time periods for a time course study (30 min, 1, 3, 6, 12, 24 hr and 3, 5, 7 days after cryogenic lesion and control). Brains were removed and post-fixed in the same fixative for 4 hr and rinsed in PB containing 30% sucrose at 4°C for 2 days. Thereafter, tissues were frozen and sectioned with a cryostat to $30 \mu\text{m}$ thickness. Consecutive sections were collected in six-well plates containing PBS. For cell counts, every sixth section in the series throughout the entire hippocampus of the same animal were used. To determine neuronal survival in hippocampus, counter staining of Nissle bodies was performed with cresyl violet solution after cryogenic lesion.

Immunohistochemistry: Immunohistochemical procedures were performed according to published studies (Kim *et al.*, 2008) with some modifications. Briefly, sections were first incubated with 3% bovine serum albumin in PBS for 30 min at room temperature. Sections were then incubated with the rabbit anti-parvalbumin (PV) IgG and/or the rabbit anti-calbindin D-28k (CB) IgG (1:1,000, Chemicon, Temecula, CA, USA) primary antibodies in PBS containing 0.3% triton X-100 overnight at room temperature. The sections were washed three times with PBS for 10 min each and sequentially incubated with biotinylated goat

anti-rabbit IgG (Vector, Burlingame, CA, USA) and ABC complex (Vector, USA) diluted 1:200 in the same solution as primary antiserum. Between incubations, tissues were washed with PBS three times for 10 min each. These sections were then visualized with 3,3'-diaminobenzidine in 0.1 M Tris buffer and mounted on gelatin-coated slides. Immunoreactions were observed under a Leica DMRB microscope (Germany), and images were captured using an Olympus DP72 digital camera and a DP2-BSW microscope digital camera software. To establish the specificity of immunostaining, a negative control test was carried out with pre-immune serum instead of primary antibody. The negative control resulted in the absence of immunoreactivity in any structures.

Quantification of data and statistical analysis: For quantification of each immunostaining, cell count was determined in the hippocampus according to previous studies (Kim *et al.*, 2007a; An and Kim, 2011). Briefly, immunostaining images (10 sections per animals) were captured in the same region (500×500 μm). Images were sampled from at least five different points within each hippocampal section. Thereafter, the number of each primary antibodies-positive cells within the sampled image was counted. All immunoreactive cells were counted regardless the intensity of labeling. Cell counts were performed by two different investigators who were blinded to the classification of tissues. In addition, hippocampal subregions (CA1, CA2-3 and DG) were delineated with a 2.5 × objective lens. Each image was normalized by adjusting the black and white range of the image using Adobe PhotoShop v. 8.0. Thereafter, 10 areas per rat (250 μm^2 for each area) were selected. Intensity measurements are presented as mean number of a 256 gray scale using NIH Image 1.59 software. Values of background staining were obtained from the corpus callosum. Optical density values were corrected by subtracting the average values of background noise obtained from five image inputs. All data obtained from the quantitative measurements were analyzed using one-way ANOVA to determine statistical significance. Bonferroni's test was used for post hoc comparisons. *P*-value less than 0.05 and 0.01 was considered statistically significant (Kim *et al.*, 2007b).

Results and Discussion

Previous studies have reported that traumatic brain injury is the main cause of mortality and disability in children and adult (Caveness, 1976; Kharlamov *et al.*, 2011; Many and Barry, 2006). Although many studies have determined the relationship between TBI and PTE (Coulter *et al.*, 1996; Golarai *et al.*, 2001; Prince *et al.*, 2009), the complex biological mechanism of epileptogenesis after brain injury remains controversial. Therefore, in this study, alteration of hippocampal interneurons (known to be critically involved to epileptogenesis) was investigated following TBI to understand abnormal neurotransmission at early stage. The results showed that immunohisto chemical distribution of parvalbumin (PV) and calbindin D-28k (CB) protein changed at specific time points after

brain injury. These alterations revealed various expression patterns at different time.

To examine functional alteration of interneurons in the hippocampus following TBI, we used cryogenic TBI animal model and induced brain lesion to frontoparietal cortex by cooling cooper cone-shape cylinder (Fig.1). Immunoreactivities of PV-positive interneurons were observed in diverse interneuronal populations, including basket neurons, the axo-axonic neurons, and some interneurons near pyramidal cell layer and the dentate subgranule cell layer of the entire hippocampus (Figs. 2B1-B3). CB expression was mainly detected in the dentate granule cell layer and some interneurons of CA1 and CA2-3 (Fig. 3B1-B3). These expressional patterns of PV and CB immunoreactivity closely matched with the previous reports of immunohistochemical distribution of CBPs (Celio, 1990). This is because PV is mainly contained in GABAergic interneurons such as basket cells and axo-axonic cells whereas CB is contained in granule cells located in the dentate gyrus and some neural cells (Toth and Freund, 1992; Hwang *et al.*, 2004 a,b).

After 30 min of TBI, PV immunoreactivity was significantly reduced in CA1, CA2-3 and the hilar region of dentate gyrus than control (TBI 30 min, 4.5% in CA1, 5.8% in CA2-3, 6.1% in DG; control, 11.8% in CA1, 20.5% in CA2-3, 18.7% in DG; *P* < 0.01, Figs. 2B1-B3, C1-C3, G and Table.1) (Kim *et al.*, 2007b). Immunodensity analysis for PV expression also revealed significantly reduction at 30 min after TBI compared to that in the control groups (TBI 30 min, 75.2% in CA1, 78.4% in CA2-3, 71.2% in DG; control, 100%; *P* < 0.01, Fig. 2F and Table.1) (Kim *et al.*, 2007b). The expression of CB-positive interneurons also decreased, similar to PV immunoreactivity in the entire hippocampus (TBI 30 min, 15.8% in CA1, 7.8% in CA2-3, 67.2% in DG; control, 42.7% in CA1, 21.7% in CA2-3, 98.7% in DG; *P* < 0.01 and 0.05, Fig. 3B1-B3, C1-C3, G and Table 2) (Kim *et al.*, 2007b). In addition, its immunodensities were reduced (TBI 30 min, 65.3% in CA1, 56.7% in CA2-3, 59.7% in DG; control, 100%; *P* < 0.01, Fig. 3F) (Kim *et al.*, 2007b). CBPs such as PV and CB are known as calcium buffers and facilitators of calcium diffusion in the nerve cell. They are widely utilized as excellent markers for specific inhibitory interneurons in the hippocampus (Celio, 1990; Andressen *et al.*, 1993). In addition, enhanced intracellular calcium concentration due to various pathophysiological brain injuries can lead to diverse molecular changes related to neurodegeneration. Thus, declined expression of CBPs during this enhanced process of intracellular calcium levels can cause neuronal loss in brain disorders, including epilepsy (Magloczky *et al.*, 1997; Fritschy *et al.*, 1999; Sutuch *et al.*, 1999). Moreover, studies on epileptogenesis have reported that diverse interneuronal population containing CBPs are reduced in hippocampal complexes of epileptic patients (Magloczky *et al.*, 2000; Gorter *et al.*, 2001). Thus, reduced expression of CBPs is one specific marker for indicating neuronal degenerative phenotype in epileptic hippocampus (Fritschy *et al.*, 1999; Sutuch

Fig. 1 : A representative photomicrograph of rat brain demonstrating loss of necrotic cavity at 1 day following a single episode of cryogenic brain injury in the left ipsilateral hemisphere. Neuronal loss of necrotic cavity was assessed with cresyl violet staining (A-C). A representative cresyl violet stained coronal section shows evidence of morphological changes in the ipsilateral cerebral cortex (C) compared to the contralateral side (B). The open arrow in panel C represents the morphological alteration of cortical neurons at the cortical impact site. Bar = 280 μ m (panels A) and 400 μ m (panels B and D).

Table 1 : Average cell numbers (mean $\pm$ S.E.M per 250 $\times$ 250 $\times$ 3 $^{\circ}$) of PV immunoreactive interneurons in lesioned hippocampus following traumatic brain injury

Traumatic brain injury	Region	Time course	Number of PV-positive interneurons
Lesioned	CA1	Control	11.8 $\pm$ 1.11
		30 min	4.5 $\pm$ 0.72**
		1 day	10.6 $\pm$ 1.39
		3 day	6.1 $\pm$ 1.09**
	CA2-3	Control	20.5 $\pm$ 1.90
		30 min	5.8 $\pm$ 1.27**
		1 day	18.3 $\pm$ 1.36
		3 day	17.9 $\pm$ 1.83
	DG	Control	18.7 $\pm$ 2.37
		30 min	6.1 $\pm$ 1.52**
		1 day	15.7 $\pm$ 2.18
		3 day	16.9 $\pm$ 1.87

*Indicate significant differences from the control group, ** $P < 0.01$

Fig. 2 : PV immunoreactivity in the ipsilateral hippocampus following cryogenic brain lesion. A representative photograph showing the normal hippocampal structure of brain (A). In the control animal, PV immunoreactive neurons are localized in the hilus and the subgranular cell layer of the dentate gyrus (B3). PV immunoreactive neurons are also abundantly observed in CA1 (B1) and CA2-3 region (B2). At 30 min after TBI, themarkedly reduced PV immunoreactive neurons are detected in all hippocampal regions (C1-C3). One day following TBI, although PV immunoreactivity is weakly enhanced, PV immunoreactive neurons are recovered to control levels (D1-D3). PV immunoreactive neurons at 3 day following TBI are unaltered in all region of lesioned hippocampus (E1-E3). Rectangles in panels A indicate the high-magnification of panels B1-B3, C1-C3, D1-D3 and E1-E3. Bars represent 280μm in A and 50μm in B1-B3, C1-C3, D1-D3 and E1-E3. Quantitative analyses of PV immunoreactivities in lesioned hippocampus (F, mean ± S.E.M). Significant differences from control hippocampus, * $P < 0.05$ and ** $P < 0.01$. Densitometric analyses show that PVexpression in lesioned hippocampus after TBI is changed depending on the time course compared to control hippocampus. Average cell numbers of PV immunoreactive interneurons in the lesioned hippocampus following TBI (G, mean ± SD) are shown. Note that down-regulation of PV-positive interneurons in CA1 region at 3 day following TBI seems to be indicative of physical damage. Significant differences from the controls, ** $P < 0.01$.

et al., 1999). Similar to previous investigations, we also observed significantly down-regulated PV and CB immunoreactivity in all hippocampus samples at 30 min following TBI. Such reduced immunohisto chemical expression of both proteins may reveal excessive increment of neuronal hyperexcitability in the hippocampus cause by declined inhibitory neurotransmission through GABAergic inhibitory interneurons. In fact, the diverse neuronal connections projected to hippocampal CA1 region are modulated by inhibitory influence of GABAergic interneurons (Empson and Heinemann, 1995; Fritschy *et al.*, 1999). Furthermore, epileptogenesis in the hippocampus and cerebral

cortex, following TBI is associated with excessive decline of postsynaptic inhibition and inhibitory interneurons (Zhu and Roper, 2000; Andre *et al.*, 2001; Sayin *et al.*, 2003). Excessive impairment of balance between excitatory and inhibitory neurotransmission caused by brain damage following TBI can lead to intense acceleration of neuronal hyperexcitability (Golarai *et al.*, 2001; Graber *et al.*, 2004; Prince *et al.*, 2009). Thus, these processes may play an important role as epileptogenic factors after TBI. Therefore, results of this study suggested that deteriorated balance of neuronal excitation accompanied by inhibitory function of diverse interneurons at hippocampal CA1

Fig. 3 : CB immunoreactivity in the ipsilateral hippocampus following TBI. A representative photograph shows the normal expression of CB-immunoreactivities in the hippocampus after TBI (A). In the control animal, CB immunoreactivity is mainly detected in the granular cell layer in the dentate gyrus (B3). In the CA1 region (B1), CB immunoreactivity is observed both in pyramidal cells and in interneurons. CB expression in the CA2-3 regions is detected in the mossy fibers as well as interneurons (B2). At 30 min after TBI, CB immunoreactivity is altered in all lesioned hippocampus compared to control levels (C1-C3). Although CB immunoreactivity in CA2-3 region at one day following TBI is enhanced over control levels, CB-positive interneurons are recovered to control levels (D1-D3). Three day following TBI, CB immunoreactive interneurons are unaltered in all region of lesioned hippocampus whereas its immunodensities in CA1 and CA2-3 are up-regulated compared to control levels (E1-E3). Rectangles in panels A indicate the high-magnification of panels B1-B3, C1-C3, D1-D3 and E1-E3. Bar represent 400 μm in A and 50 μm in B1-B3, C1-C3, D1-D3 and E1-E3. Quantitative analyses of CB immunoreactivities in lesioned hippocampus (F, mean ± S.E.M) are shown. Significant differences from control hippocampus, * $P < 0.05$ and ** $P < 0.01$. Densitometric analyses show that CB expression in lesioned hippocampus following TBI are changed compared to control hippocampus, similar to immunohistochemical data. Average cell numbers of CB immunoreactive interneurons in lesioned hippocampus following TBI (G, mean ± SD) are shown. Note that CB-positive neurons in the CA1-3 region at 30 min following TBI are down-regulated. Significant differences from controls, * $P < 0.05$ and ** $P < 0.01$.

region is caused by reduced PV and CB expression at this early time point after TBI, thus contributing to the development and spread of epileptogenic phenotype, following brain trauma.

On the other hand, the immunohistochemical distribution of PV-positive interneurons following TBI was recovered to control levels in all hippocampus regions (TBI 1 day, 10.6% in CA1, 18.3% in CA2-3, 15.7% in DG; Fig. 2D1-D3, G and Table.1). PV immunoreactivity was maintained until 3 days after TBI, although its expression in CA1 decreased similar to that at 30 min

after TBI (TBI 3 day, 6.1% in CA1, 17.9% in CA2-3, 16.9% in DG; Fig. 2E1-E3, G and Table.1). Quantitative data revealed some enhancement of PV immunodensity at 1 day which maintained at 3 days, similar to control levels (TBI 1 day, 89.7% in CA1, 92.7% in CA2-3, 93.8% in DG; TBI 3 days, 91.2% in CA1, 87.4% in CA2-3, 86.1% in DG; $P < 0.05$, Fig. 2F) (Kim *et al.*, 2007b). Moreover, the expression of CB-positive interneurons at 1 day was recovered in all hippocampal regions to levels similar to control (TBI 1 day, 44.1% in CA1, 24.8% in CA2-3, 101.3% in DG; Figs. 3D1-D3, G and Table.2). Its distribution was maintained until 3

Table 2 : Average cell numbers (mean $\pm$ S.E.M per $250 \times 250 \times 3^3$) of CB immunoreactive interneurons in lesioned hippocampus following traumatic brain injury

Traumatic brain injury	Region	Time course	Number of CB-positive interneurons
Lesioned	CA1	Control	42.7 $\pm$ 3.21
		30 min	15.8 $\pm$ 2.78**
		1 day	44.1 $\pm$ 3.85
		3 day	47.2 $\pm$ 4.92
	CA2-3	Control	21.7 $\pm$ 2.89
		30 min	7.8 $\pm$ 3.79*
		1 day	24.8 $\pm$ 3.41
		3 day	21.1 $\pm$ 4.24
	DG	Control	98.7 $\pm$ 6.07
		30 min	67.2 $\pm$ 5.76**
		1 day	101.3 $\pm$ 7.92
		3 day	99.7 $\pm$ 4.45

Footnote: Asterisks indicated significant differences from the control group, * $P < 0.05$, ** $P < 0.01$

days following TBI (TBI 3 days, 47.2% in CA1, 21.1% in CA2-3, 99.7% in DG; Figs. 3E1-E3, G and Table.2). However, densitometric analysis of CB immunoreactivity showed enhanced expression in CA1 and CA2-3 at both time points, although its immunodensity was rehabilitated in the dentate gyrus, similar to control level after TBI (TBI 1day, 102.1% in CA1, 126.7% in CA2-3, 94.6% in DG; TBI 3 days, 125.8% in CA1, 114.3% in CA2-3, 98.4% in DG; $P < 0.05$ and 0.01 , Fig. 3F) (Kim *et al.*, 2007b). Previously, some investigators have demonstrated that the remaining interneurons in the epileptic hippocampus are exposed to hyperexcitation as compensational response to decreased GABAergic inhibition (Beau and Alger, 1998; Isokawa, 1998; Cossart *et al.*, 2001). In addition, excessive abnormal response, including axonal sprouting in the injured specific brain region can appear at early time period until 3 days after TBI and such response can remain till one week (Yang *et al.*, 1996; King *et al.*, 2001). Thus, brain tissues injured by trauma should be treated within 3 days after TBI because delayed treatment can lead to deteriorating epileptogenesis in the brain (Graber and Prince, 2006). Moreover, interictal epileptiform activity in the damaged brain tissue is significantly enhanced at this early time points, hence revealing the occurrence of abnormal neural activity (Jacobs *et al.*, 2001). Thus, the results of this study demonstrates that the re-enhanced immunohisto-chemical expression of PV and CB might be compensatory response to protect hippocampal neurons against diverse pathophysiological influences during epileptogenic processes after brain trauma. Therefore, inhibitory and excitatory neurotransmission ineffectiveness might occur at initial time after traumatic brain injury, leading to the development of epilepsy after TBI.

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