Brief Communication

Change of presynaptic vesicle cycling in the hippocampus after status convulsion

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Infants and children are at a higher risk for seizures compared with adults. Increasing experimental animal data strongly suggest young animals are less vulnerable than mature animals to cell loss in the hippocampus with more neurogenesis after status convulsion (SC). Early-life seizures cause permanent functional alterations in neuronal networks and render the brain susceptible to later epilepsy and cognitive deficits. Such seizures may intervene with developmental programs, and lead to inadequate construction of cortical networks rather than induction of neuronal cell loss. The developing brain has a greater capacity in long-term synaptic plasticity than the adult brain, but the molecular mechanism is not well understood. Long-term synaptic plasticity involves both functional and morphological changes of synapses. In the CNS, postsynaptic silent synapses have been identified as showing only N-Methyl-D-aspartate (NMDA), but not alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors, and activation of these postsynaptically silent synapses has been demonstrated to contribute significantly to long-term synaptic plasticity. Presynaptic silent synapses have also been reported, which are possibly caused by a very low probability of synaptic vesicle release or the small quanta. The influence of SC on activation of presynaptic silent synapses and their contribution to long-term synaptic plasticity is widely unknown. The current study aims to observe the dynamic changes and age-related differences of vesicle pool organization at hippocampal synapses by monitoring vesicle recycling, and to explore the influences of SC on hippocampal synaptic transmission in immature and adult rats through a presynaptic mechanism.

This study was carried out at the Children's Hospital Affiliated to Chongqing University of Medical Sciences, Chongqing, China between October 2010 and March 2011. Twenty-five healthy adult (postnatal day 60 [P60], weight 250g-350g) and 25 young (postnatal day 21 [P21], weight 60g-100g) Wistar rats (ARs and IRs) were provided by the Animal Central of Chongqing University of Medical Sciences, Chongqing, China. The gender was random. Experimental Animal Care Guidelines, which were in compliance with the National Animal Ethical Policies, were followed. The SC animal model evoked by lithium and pilocarpine (Sigma, Saint Louis, MO, USA) was adopted. Seizures were induced in IRs and ARs individually by intraperitoneal injection of lithium (3 mEq/kg), and pilocarpine (40 mg/kg) 18-20 hours later. According to the Simialowski 6 classes' evaluation,[1] rats with class IV or V, and one hour duration seizures were included in the study. Fifteen minutes after seizure onset, the rats were injected intraperitoneally with atropine one mg/kg (He Feng Tragacanth Company, Shanghai, China). Seizures were terminated with chloral hydrate (He Feng Tragacanth Company Shanghai, China) injected intraperitoneally after one hours duration of SC. Rats were sacrificed at 4 time points (3 hours, one day, 3 days, and 7 days) after one hour of SC termination (n=5). In addition, normal controls were set up (n=5).

The brain tissue of decapitated rats was examined as followed. The rats were anesthetized with chloral hydrate (400mg/kg) prior to decapitation. The brains were removed and cooled off in an ice-cold solution for 30 seconds. The solution contained: 140 mM choline (CL), 2.5 mM potassium chloride (KCl), 0.5 mM calcium chloride ($CaCl_2$), 7 mM magnesium chloride ($MgCl_2$), 25 mM sodium bicarbonate ($NaHCO_3$), 1.25 mM sodium phosphate (NaH_2PO_4), 1.3 mM ascorbic acid, and 7 mM dextrose. Hippocampal slices (300-400 μm thick) were cut with a vibratome (NVSLM1 from World Precision Instruments, Sarasota, FLA, USA) in the same ice-cold solution, and they were subsequently stored in artificial cerebrospinal fluid (ACSF) containing: 126 mM sodium chloride (NaCl), 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose; saturated with 95% O_2 and 5% CO_2, pH 7.4. Hippocampal slices were labeled with FM 1-43 by perfusing the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 uM, Sigma, Saint Louis, MO, USA) for 10 minutes to reduce spontaneous activity, followed by CNQX + FM 1-43 (5 μM; Biotium, Hayward, CA, USA) + APV (NMDA receptor antagonist, DL-2-Amino-5-phosphonovaleric acid, Sigma, Saint Louis, MO, USA) for 5 minutes, and then CNQX + FM 1-43 + APV in high K^+ (45 mM) for 15 minutes to stimulate uptake of FM 1-43 via endocytosis of vesicles. After loading, high K^+ and FM 1-43 were washed out in ACSF containing ADVASEP-7 (200 μmol/L; Biotium, Hayward, CA, USA) for 5 minutes x 3 prior to imaging. The ADVASEP-7 was used to reduce background fluorescence by removing FM1-43 staining nonspecifically. Images were taken before and after cells were destained in high KCl solution for 30 seconds.[2] All

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the solutions were saturated with 95% O_2 and 5% CO_2. The image after FM dye destaining was subtracted from the initial image, and only those terminals containing activity-dependent releasable FM dye (about 90% of total staining) were analyzed. Images of FM1-43 were acquired with a confocal microscope (Leica TCS SP2, Solms, Germany). Analysis of the imaging data was performed by using the Leica TCS SP2 image software. Three brain slices were taken from each animal, and from each slice we randomly choose 5 high power fields (scale 75 μm) in the CA1 region. The image was used to calculate the brightness value of FM1-43 for each pixel in the field of view.

All data were presented as mean±standard error of mean (SEM). Statistical analysis was performed with one-way analysis of variance test with Bonferroni's corrections and 2-tailed independent-samples t-test, using the Statistical Package for Social Sciences version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Two-sided *p*-values of less than 0.05 were regarded as statistically significant.

Class IV or V seizures occurred in all the experimental rats after the intraperitoneal injection of lithium and pilocarpine. They were sacrificed at 4 time points (3 hours, one day, 3 days, and 7 days) after one hour of SC termination (n=5). The fluorescent probe FM1-43 was used for imaging vesicle recycling in hippocampal slices. The positive staining of FM1-43 was located mainly in the dendrites and axons of neurons, and took on red fluorescence particles. The function of synaptic vesicle recycling was measured by the semi-quantitative analysis of FM1-43 fluorescence intensity (**Table 1**). The fluorescent intensity of FM1-43 in the CA1 region of IRs and ARs were inhibited after SC termination. The expression of FM1-43 dropped sharply 3 hours after SC in the AR group ($p<0.01$), with minimum levels at this point, and then rose steadily. These decreases lasted for at least 7 days ($p<0.01$). The pattern of FM1-43 expression in the IR group was similar to that in the AR groups after SC. It declined abruptly to below control levels at 3 hours ($p<0.01$), reaching the minimum level at this point, and then rose steadily. The decrease process continued until at least 7 days ($p<0.01$). In normal IRs, the fluorescent intensity of FM1-43 was lower than that in normal ARs. The age-dependent difference was persistent for at least 7 days after SC ($p<0.01$).

The fluorescent styryl dye, FM1-43, is commonly used to monitor synaptic transmission. In our study, vesicle cycling in live hippocampus slices was directly examined by using confocal imaging with FM 1-43. We can analyze the synaptic function by calculating activity-dependent staining and destaining of synaptic boutons during vesicular cycling. At early developmental stages, the number of silent synapses is quite high in different brain areas including the hippocampus, and decreases during development. Our results demonstrated that in normal IRs, the fluorescent intensity of FM1-43 was lower than that in normal ARs. It indicated that, in the developmental hippocampus, a proportion of glutamatergic synaptic connections were presynaptically rather than postsynaptically silent. With the maturation of the CNS, synaptic vesicle recycling was enhanced and synaptic function was increased. The developmental hippocampus had stronger synaptic plasticity. Alternatively, the difference between the 2 age groups may be the result of the lower synaptic density in the younger animals.

The recent research has shown that SC not only induced hippocampal neuronal death and neural stem cell proliferation, but also induced synaptogenesis. Synaptogenesis is the formation of synapses, including the synaptic reorganization and the conversion of silent synapses. The SC could induce hippocampal mossy fiber sprouting (MFS) and synaptic reorganization. The currently dominant theory illustrates that MFS and synaptic reorganization could establish aberrant synapses on granule cells, which may contribute to the physiopathology of the dentate gyrus in epileptic animals. However, we do not know what roles SC plays in conversion of silent synapses.

Could seizure induce conversion of silent synapses and establish normal synaptic transmission? Shen et al[3] indicated that either theta burst stimulation (TBS) or electric field stimulation (EFS) could induce conversion

Table 1 - The average fluorescence intensity of FM1-43 at different time points after one hour status convulsion (SC) termination in immature and adult rat hippocampal CA1 regions.

Time-points	Immature group	Adult group	t	*P*-value
Normal control group	136±10	152±13	8.52	0.000
3 hours after SC	101±8*	122±7*	17.178	0.000
1 day after SC	113±7*	127±10*	10.199	0.000
3 days after SC	123±8*	138±17*	6.818	0.000
7 days after SC	127±11*	145±12*	9.641	0.000

*Compared with normal control group $p<0.01$

of silent synapses into functional synapses. Yao[4] revealed that many presynaptic boutons in immature neurons were functionally silent at resting conditions, but could be converted into active ones after repetitive neuronal stimulation. The current studies only confirmed that the induction of seizure activity in cultured neurons could activate silent synapses, but no in vivo experiment proved whether seizure could affect conversion of silent synapses. We demonstrated that the change trends of the fluorescent intensity of FM1-43 in the IRs and ARs were similar. The fluorescent intensity in the IRs and ARs hippocampal CA1 regions dropped sharply ($p<0.01$) at 3 hours after SC termination, and then rose steadily. The decrease process continued until at least 7 days. Contrary to in vitro experiments, severe seizure could inhibit vesicular release and uptake in hippocampus CA1 in vivo, suggesting that the silent synapses could be generated by a presynaptic mechanism after SC. It was not clear what caused the difference from in vitro to in vivo experiments. We demonstrated that severe seizure induced neuronal apoptosis in the hippocampus. Alternatively, it was possible that the reduced staining after SC was related to neuronal degeneration.

What was the significance of silent synapses in the hippocampus? Conversion of silent synapses into functional synapses was not only an important developmental stage in the CNS, but also the key to formation and maintenance of long-term potentiation (LTP). In the hippocampal CA1 region of young animals, conversion of silent synapses to functional synapses occurred rapidly after pairing post-synaptic depolarization with one Hz pre-synaptic stimulation, a protocol that also induced LTP.[5] Silent synapse formation was associated specifically with the expression of long-term depression.[6] The bidirectional plasticity may be the adaptive change in neural circuits that occurred as a basis for new memory storage; however, it may also cause an abnormal synaptic response and lead to brain dysfunction. We confirmed that the fluorescent intensity of FM1-43 in hippocampal CA1 of IRs was always lower than in that of ARs before or after SC. There were probably more silent synapses in IRs after SC. The decrease in vesicle recycling or the more silent synapses formation could not establish normal synaptic connections in the brain, which may be the cause of higher risk for seizures in the immature brain.

In conclusion, severe seizure could inhibit vesicular release and uptake in the hippocampal CA1. The silent synapses could be generated by a presynaptic mechanism after SC. The synaptic activity in the hippocampal CA1 of IRs was always lower than in that of ARs before or after SC. There were probably more silent synapses in IRs. The inhibition of synaptic function in IRs and ARs was persistent at least 7 days after SC ($p<0.01$). This suggested that hippocampal neural synaptic plasticity, which was induced by SC had a regulation time-window. We should perform an in-depth systematic study to describe the role of silent synapses in seizure brain damage, then make an appropriate regulation (whether improve or inhibit the formation of silent synapses) in the time-window and stimulate endogenous repair of brain damage.

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