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Characterization of developing rat cortical neurons after epileptiform discharges

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ABSTRACT

The developing brain undergoes major reorganization in response to early environmental changes. The elevated excitation that allows the neonatal brain to develop quickly also makes it highly vulnerable to age-specific seizures that can cause lifelong cognitive and neurological disability. However, it is not yet clear how seizures interfere with the developmental program and how epileptogenesis actualize. Here, by using an *in vitro* model, we report a global abnormal status of cortical cells after epileptiform activity was induced: more NR2B is targeted on the neuronal surface with less NR2A. Dendrotoxicity including dendritic beading, distortion and simplification of dendritic branching patterns were observed. Early-life seizure-like insults also exert effects on the excitatory synaptic size and interactions between PSD-95 and NR2A or NR2B receptor subunits. Our findings support an abnormal development or, worse, cellular degeneration that resembles immature cells, which may enlighten better understanding of the pathological mechanism of early-life seizures and its related injury.

1. Introduction

The developing brain is genetically programmed to adapt its behavior and circuitry to stimulation from the external environment. This heightened flexibility (popularly known as plasticity) during a critical window of early infancy is unmatched at any other time throughout life and is a central tenet of brain development (Kolb and Whishaw, 1998; Johnston et al., 2001; Johnston, 2009). However, the mechanisms (operating essentially in a state of elevated excitation as a baseline) that allow the neonatal brain to develop quickly also make it highly vulnerable to age-specific seizures that can cause lifelong cognitive and neurological disability (Baram, 2003; Johnston, 2004; Haut et al., 2004). Although several lines of research have disclosed numerous biochemical effects of early seizures (Karnam et al., 2009; Lopantsev et al., 2009; Huang and Chang, 2009), it has not been determined how seizures actually interfere with the developmental program and by which process epileptogenesis comes true.

At central excitatory synapses, ionotropic glutamate receptors are organized into multi-protein signaling complexes within the postsynaptic density (PSD). NMDA receptors (N-methyl-D-aspartate receptor, NMDAR) play critical roles in neuronal development, excitotoxicity, and synaptic plasticity (Bliss and Collingridge, 1993; Choi, 1994). Furthermore, the subunit composition and replacement, surface trafficking and lateral mobility, expression, degradation and phosphorylation status are all under tight regulation by developmental experience and synaptic activities (Groc and Choquet, 2006; Wang et al., 2006a,b; Lau and Zukin, 2007; Li and Jimenez, 2008). An obvious feature of the NMDAR worthy notice is its developmental subunit switching, namely, from predominance of NR2B-containing receptors to NR2A-containing receptors (Monyer et al., 1994; Sheng et al., 1994). In addition, a prominent scaffolding protein, postsynaptic density-95 (PSD-95), which couples NMDAR to intracellular proteins and signaling enzymes, may also be implicated in the regulation of the formation, maturation and plasticity of excitatory synapses (Kornau et al., 1995; El-Husseini et al., 2000; Kim and Sheng, 2004; Cui et al., 2007; Bhattacharyya et al., 2009).

Construction of neural circuits is a highly dynamic process consisting of both formation and elimination; thus a much larger number of trial connections are established than are necessary to be maintained in the mature brain (Hua and Smith, 2004). Accordingly, dendritic spines undergo activity-dependent structural remodeling by continually changing their size and shape during early-life development. This has been proposed as a mechanism for structural synaptic plasticity and a cellular basis of learning and memory. In concert with the ongoing spine dynamics, a certain amount of stability of some synapses is also needed to allow continuous, reliable synaptic communication (Matsuzaki et al., 2004; Mysore et al., 2008; Kitanishi et al., 2009; Bhatt et al., 2009). Not surprisingly, this unique sensitivity to local synaptic strength fluctuation and extremely high enrichment of postsynaptic glutamate receptors depict dendrites as an ini-

Abbreviations: PSD, postsynaptic density; NMDAR, N-methyl-D-aspartate receptor; PSD-95, postsynaptic density-95; CD, cortical dysplasia; DIV, days *in vitro*; MGF, magnesium-free; PS, physiological solution.

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tial target of excitotoxic damage during recurrent or prolonged seizures.

Recent studies of cortical dysplasia (CD), the most frequent pathology found in pediatric epilepsy surgery patients, suggest that there are prenatal neurons with immature cellular and synaptic properties retained in pediatric CD tissues (Cepeda et al., 2006). Following this dysmature cerebral developmental hypothesis, several authors have described animal model studies in the last few years indicating that neuronal hyperexcitability may interfere with the normal maturation of glutamatergic synapses and lead to impairment of the gross structure of the brain (Swann et al., 2007; Karnam et al., 2009; Lopantsev et al., 2009). However, these articles only reported discontinuous snapshots of the whole scenario by detecting some molecular markers for glutamatergic synapses, while the particular transformation process and underlying mechanisms are still large missing pieces of the puzzle. Here, we report a comprehensive long-lasting abnormal status of cultured rat cortical neurons after an early-life single event of epileptiform activity. We propose that early-life epileptiform activity may hinder normal development of neocortical neurons and retain them at a much more naïve state with disturbed NMDAR subunit expression and replacement, less organized dendrites, larger stable spines with less plasticity and dysregulated protein interaction.

2. Materials and methods

2.1. Primary cortical neuronal culture and discharges inducement

Primary cortical neuron cultures were prepared from 18-day-old Wistar rat embryos (Peking University Health Science Center Experimental Animal Department) as described (Meberg and Miller, 2003; Jiang et al., 2007). All experimental procedures were in accordance with the Guide for Care and Use of Laboratory Animals, which was approved by the Medicine Animal Studies Committee of Peking University. Dissociated neurons were suspended in plating media (minimum essential medium containing 10% fetal bovine serum, 5% heat-inactivated horse serum, 2 mM glutamine, 0.2 μM cysteine, 100 IU/ml penicillin and 100 mg/ml streptomycin), transferred to poly-L-lysine-coated 25 cm^2 flasks (at a density of $(5.0–5.5) \times 10^5$ cells) or coverslips (at a density of $(0.8–1.0) \times 10^5$ cells) in 12-well culture plates and incubated at 37 °C. After 4–6 h, all the plating medium was removed and replaced with maintenance medium (Neurobasal medium supplemented with 2% B-27 and 0.5 mM L-glutamine). Medium replacement was performed every 3–4 days, and cells were used at days *in vitro* (DIV) 7–21.

To induce epileptiform activity, the maintenance medium was replaced with magnesium-free (MGF) physiological solution, pH 7.3, containing (in mM): 145 NaCl, 2.5 KCl, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 1 $CaCl_2$, 10 glucose, and 0.001 glycine. Two kinds of control cultures were developed in parallel: one was cells maintained in Neurobasal/B27 medium (control) and the other was cells treated with physiological solution (PS, pH 7.3) including 1 mM $MgCl_2$. Corresponding medium replacement was simultaneously performed at DIV 6 and maintained under identical incubation conditions for 3 h (Cao et al., 2003). After treatment, all neurons were transferred to the regular Neurobasal/B27 medium and returned to the original incubator.

2.2. Subcellular fractionation and immunoblotting

Biochemical fractionation was performed with standard protocols with minor modifications (Blackstone et al., 1992; Dunah and Standaert, 2001). Briefly, primary cultured neurons were washed with ice-cold phosphate-buffered saline (PBS), and scraped from the flasks. Cells collected by centrifugation (1,000 × *g* for 10 min at 4 °C) were homogenized in ice-cold homogenization buffer (HB, 10 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA and 320 mM sucrose, pH 7.4) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and a complete protease inhibitor cocktail (4 mM aprotinin, 0.1 mM leupeptin, 0.05 mM pepstatin, 0.25 mM bestatin, 5 mM AEBSF and 0.075 mM E-64, KangChen Bio-tech, KC-440, China.). After sonication on ice for 1 min, the homogenization was centrifuged at 1,000 × *g* to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 13,000 × *g* to obtain a crude synaptosomal fraction (P2) which was subsequently lysed hypo-osmotically with lysis buffer (LB, HB omitting sucrose). Concurrently, the supernatant (S2) above P2 was used for immunoblot as total cytosolic fractions, which contained the cytosolic fraction and a light membrane/microsome-enriched fraction. After each centrifugation, the resulting pellet was rinsed briefly with ice-cold homogenization buffer before subsequent fractionations to avoid possible crossover contamination.

The indicated amount of protein was loaded and separated using 8.0% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were subjected to subunit-specific antibodies overnight at 4 °C. To further normalize the equal loading of neuronal protein extracts, the membranes were also probed with β-tubulin III. After washing for 40 min with three changes of PBS containing Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Zhongshan Biochem Com, Beijing, China) for 1 h at room temperature (RT). Following another four washes with PBS containing Tween-20, bands were visualized on film by enhanced chemiluminescence (Pierce Biotechnology, Inc., USA) and quantified via computer-assisted densitometry (Bio Image Analysis System, USA). The net intensities of the bands are expressed as fold increases or decreases over the respective control values.

2.3. Immunocytochemistry and quantification

To characterize neuronal development and maturation, we detected alterations in dendritic morphology, as well as density and size changes of excitatory synapses in permeabilized control or treated neurons at DIV 7 and DIV 17. Neuronal processes were detected by MAP2 staining, while excitatory synapses were displayed by postsynaptic marker PSD-95 labeling. Cells were fixed with 4% paraformaldehyde/PBS for 15 min at RT. After permeabilization using 0.2% Triton X-100 for 15 min, the cells were incubated with 5% BSA in goat serum for 1 h at RT. Immunostaining was performed with corresponding antibodies overnight at 4 °C before staining with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (A11008, Molecular Probes, Eugene, OR, USA) and/or TRITC-conjugated goat anti-mouse IgG polyclonal secondary antibody (ZF-0313, Zhongshan Biochem Com, Beijing, China). Cell number and distributions were revealed by staining with nuclear marker Hoechst 33342 (Gift from Dr. Q.F. Fan, Division of Nephrology, Department of Pediatrics, Peking University First Hospital) for 5 min at RT.

Confocal time-lapse microscopy was performed on a Leica confocal microscope (TCS-SP5, Germany) with a 60× oil immersion objective. Images were acquired in the linear range with constant settings and analyzed using Image-Pro Plus software version 6.0 (MediaCybernetics, Bethesda, MD, USA). All analyses were performed blind to the stimulation history of the culture. Immunoreactive puncta were defined as discrete regions along the dendrite with fluorescence intensity twice the background. For quantification, 13–18 neurons from two to three different batches of cultures and experiments for each condition were randomly chosen on the basis of healthy morphology. Negative controls, in which one or both primary antibodies were omitted and treated only with the secondary antibodies, were run for each condition to exclude false positive secondary antibody binding. The *n* value refers to the number of cells analyzed.

2.4. Co-immunoprecipitation

Co-immunoprecipitation was performed under non-denaturing conditions. For preparation of membrane protein samples, we first washed neurons twice with ice-cold PBS, then added ice-cold modified RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate) supplemented with PMSF (1 mM) and a complete protease inhibitor cocktail (4 mM aprotinin, 0.1 mM leupeptin, 0.05 mM pepstatin, 0.25 mM bestatin, 5 mM AEBSF and 0.075 mM E-64, KangChen Bio-tech, KC-440, China) immediately before harvesting by scraping the cells off the flask. Subsequently, the cell suspension was transferred into a centrifuge tube and gently rocked at 4 °C for 15 min. After centrifuging the lysate at 14,000 × *g* for 15 min, the supernatant was immediately transferred into a fresh centrifuge tube and the pellet was discarded. Before determining protein concentration, a portion of the cell lysate was diluted at least 1:10 to avoid interference of the detergents in the lysis buffer with the Coomassie-based reagent. At the same time, the remaining samples were aliquotted and stored at −80 °C for further use.

To prepare protein-A sepharose (Zymed, San Francisco, USA), the beads were washed twice with PBS and resuspended in PBS to obtain a 50% slurry. The membrane proteins (400 μg, 1 μg/μl) were pre-cleared by adding 100 μl of protein-A bead slurry and incubating at 4 °C for 30 min. The protein-A beads were removed by centrifugation at 2,500 × *g* for 5 min at 4 °C. The supernatant was transferred to a new centrifuge tube and gently mixed with 10 μg of PSD-95 antibody. The protein/antibody mixture was incubated overnight at 4 °C, after which the immunocomplexes were captured by adding 100 μl protein-A bead slurry and gently rocking for 2 h at RT. The sepharose beads were collected by pulse centrifugation and washed 3 times with 200 μl of ice-cold PBS. The beads were resuspended in 60 μl of 2× sample buffer and mixed gently. The proteins were eluted from the beads and denatured by boiling for 5 min and then resolved by SDS-PAGE. After transferring the proteins on the gels to membranes, they were probed with antibodies against NR2A or NR2B subunit and PSD-95.

2.5. Antibodies

Rabbit polyclonal antibody against NR2A was obtained from Abcam plc (Cambridge, UK). Rabbit polyclonal antibody against NR2B and mouse monoclonal antibody against NR1 were obtained from Upstate Cell Signaling Solutions (Lake Placid, NY, USA). Rabbit polyclonal antibody against MAP2 (Microtubule-associated protein 2), and mouse monoclonal antibody against β-tubulin III and PSD-95 were obtained from Chemicon International, Inc. (Temecula, CA, USA).