Increased Seizure Susceptibility and Up-regulation of nNOS Expression in Hippocampus Following Recurrent Early-life Seizures in Rats

INTRODUCTION

The immature neonatal and early-infant brain differs from the mature adult brain in its susceptibility to seizures, seizure characteristics, and responses to antiepileptic drugs (1, 2). While the immature brain is resistant to acute seizure-induced cell loss, there are functional abnormalities following seizures with impairment of visual-spatial memory and reduced seizure threshold. Furthermore, seizures in early-life are associated with an increased likelihood of chronic epilepsy and may themselves contribute to epileptogenesis through adverse effects on brain development (3-5). However, the effects of repeated seizures on the developing brain have long been debated.

Numerous models have been used in the past to determine how early-life seizures exert their effects on the developing brain according to different questions. Nevertheless, the basic questions are often the same as follows: Do seizures cause long-term damage? Do seizures predispose to chronic epilepsy (epileptogenesis) which is long-term spontaneous repetitive seizures? Are these results developmentally regulated? Are the underlying mechanisms of epileptogenesis and brain damage related?

Tetanus toxin, a large protein (MW 150 kDa) produced by Clostridium tetani, is a well-known neurotoxin and potent convulsant and has been used by numerous laboratories to produce seizures in adult animals of several species (6-8). Lee et al. (9) introduced the tetanus-induced seizures models that permit study of the acute and chronic effects of multiple early-life seizures on the developing brain.

Meanwhile, nitric oxide (NO) is produced by nitric oxide synthase (NOS) from L-arginine. Three isofroms of NOS have been identified and are expressed in different tissues. In the brain, a calcium-calmodulin-dependent and constitutive NOS is localized to certain neuronal populations. This so called neuronal isoform of NOS (nNOS) is activated by glutamate via NMDA receptors. NO is thought to be involved in various functions including, vasodilatation, immune function, neurotoxicity, long-term potentiation and long-term depression, memory processes, synaptic plasticity, and the regulation of excitation and firing (10). Also, NO mediates the formation of synaptic connections and regeneration of neurons in the process of brain development (11). The expression of nNOS mRNA is widely distributed in the brain, such
as the hippocampal CA1 and CA3 regions, dentate gyrus, amygdala, cerebral cortex, and cerebellum (12).

This study aimed to determine the effects of repeated seizures on the developing brain by evaluating the long-term changes in seizure susceptibility and nNOS expression late in life following recurrent seizures in early-life induced by a unilateral intrahippocampal injection of tetanus toxin.

**MATERIALS AND METHODS**

**Tetanus toxin injection**

Wistar rat pups (Harlan Sprague Dawley, Indianapolis, IN, USA), 10 days of age, were anesthetized with an intraperitoneal injection of ketamine and xylazine (33 and 1.5 mg/kg, respectively). Tetanus toxin (TNTX, 2.5 or 5 ng) was dissolved in sterile saline (20 or 40 nL) and stereotaxically injected into the right dorsal hippocampus. The tetanus toxin used in this study was a gift of the Massachusetts State Biological Labs (North Worcester, MA, USA). The potency of the toxin was assayed by hindlimb paralysis after injection into the gastrocnemius muscle. The minimal dose (MD_{100}) that produced paralysis in all mice in a group (n=5) was 0.25 ng (9). The surgical procedures and use of tetanus toxin were approved by an Animal Protocol Review Committee, the Infectious Agent and Hazardous Chemical Subcommittee, and the Animal Biosafety Subcommittee of the Baylor College of Medicine (Department of Pediatrics, Division of Neuroscience, Cain laboratory, March 2002-April 2003, IACUC; AN-652). All procedures were in keeping with guidelines established by the National Institutes of Health (Bethesda, MD, USA). To inject tetanus toxin, the pups were placed in an infant rat stereotaxic head holder, a midline incision was made, and a small hole was drilled in the skull. The stereotaxic coordinates for the injection were as follows: anteroposterior, -2.2 mm; mediolateral, 3.0 mm from the bregma; and dorsoventral, -2.95 mm from the dural surface. The toxin was slowly injected at 4 nL/min. After injection, the needle was left in place for 10 min to reduce reflux up the needle track. During injections, the body temperature of the rat pups was maintained by a warmed (electrically regulated) metal plate. Littermates, stereotaxically injected with sterile saline, or untreated rats served as controls (Table 1).

**Behavioral monitoring of seizures in infant rats**

The frequency of behavioral seizures was monitored 1 hr each day for 10 consecutive days after tetanus toxin injections. The types and duration of seizures were noted. Wild running seizures were most easily identified. Rats that had a total of 2 or more wild running seizures during the 10 monitoring sessions were selected for performing western blots, seizure susceptibility tests, and video-EEG monitoring. Other than seizures, no other behavioral signs of neurological abnormalities were observed in the tetanus toxin-treated rats.

**EEG electrode implantation**

At 50-60 days-of-age, EEG electrodes were implanted in 5 toxin-treated animals, 2 saline-injected animals, and 3 untreated controls rats. Chlorided silver wires (exposed tips of 0.005 inches in diameter, Teflon coated silver wires, A-M Systems, Inc., Carlsborg, WA, USA) were stereotaxically implanted bilaterally in each hippocampus and over each somatosensory cortex (surface electrodes). A reference electrode was placed over the right occipital cortex. These electrodes were held in place with dental acrylic. A ground electrode was placed in the right occipital muscle group. Dental cement was used to secure the electrode wires in place and to form a protective cap around a plug to which the electrodes were connected.

**Video EEG monitoring**

During the week following EEG electrode implantation, a 20 channel Nihon Kohden EEG machine with an attached video camera was used to monitor each animal twice for at least 2 hr during each recording session. Recordings were made at a paper speed of 30 mm/sec and a sensitivity of 30-75 μV/mm. A referential montage was used in which the left and right cortical and hippocampal electrodes were referred to the medial occipital cortex.

**Western blotting**

On postnatal day 50, rats were anesthetized with isoflurane vapors and the brains were quickly removed and submerged in ice-cold ACSF (sucrose 110 mM, NaCl 60 mM, KCl 3 mM, NaH2PO4 1.25 mM, NaHCO3 28 mM, CaCl2: 906 D.-K. Kim

**Table 1. Summary of experiments**

| Animal group | Behavioral monitoring of seizures (n) | Early seizure susceptibility, PND 22 (n) | Western blot, PND 45 (n) | Late seizure susceptibility, PND 50 (n) | Video-EEG monitoring, PND 50-60 (n) |
|--------------|--------------------------------------|---------------------------------------|--------------------------|----------------------------------------|----------------------------------|
| TNTX         | 39                                   | 10                                    | 16                       | 8                                      | 5                                |
| SC           | 33                                   | 10                                    | 12                       | 9                                      | 2                                |
| NC           | 32                                   | 10                                    | 12                       | 9                                      | 3                                |

TNTX, tetanus toxin treated; SC, saline injected; NC, normal control; PND, postnatal day.
0.5 mM, MgCl\(_2\) 7 mM, glucose 5 mM, and ascorbate 0.6 mM) that was bubbled with 95% O\(_2\)/5% CO\(_2\). Both hippocampi were then dissected free of all adhering tissue. After removing the most septal and temporal poles, the right hippocampus was sliced into transverse sections, approximately 3,000 μm in thickness. On an ice-cold plate and under direct visual control, areas CA1, CA3, and the dentate gyrus were then dissected from each slice using a shard of a razor blade. All microdissected samples were placed on dry ice and stored at -80°C.

The samples were homogenized by sonication in homogenizing buffer containing a protease inhibitor cocktail tablet (Complete, Mini, Roche, Germany). The homogenized samples were placed immediately on ice, and the protein content was assayed by the Bradford method. Each sample was separated on 10% polyacrylamide electrophoresis gels (Novex, Invitrogen, Carlsbad, CA, USA) and electrophoresed to PVDF membranes (Immobilon P, Millipore, Bedford, MA, USA).

After blocking with 5% nonfat dry milk and T-TBS (0.05% Tween) for at least 1 hr at room temperature, and then incubated overnight with a primary antibody, rabbit-nNOS, 1:1,000 (Sigma-Aldrich, St. Louis, MO, USA) at 4°C. After incubation, the PVDF membrane was washed and then incubated with a secondary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG, 1:1,000, Sigma-Aldrich). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Uppsala, Sweden). The optical densities of the immunoreactive bands were quantified using the Scion Image software (NIH Image) from film exposures in the linear range for each antibody. To ensure equal protein loading among wells, membranes were stripped and re-probed with mouse anti-β-actin 1:1,000 (Sigma-Aldrich). Measures of optical densities for TNTX-treated and saline-injected controls were normalized to the optical densities of immunoreactive bands from untreated control rats on the same gel.

Seizure susceptibility

The volatile convulsant, flurothyl (bis-2,2,2-trifluoroethyl ether, Sigma-Aldrich) was used to induce seizures in order to evaluate differences in seizure susceptibility between the treatment groups. Ten TNTX-treated, 10 saline-injected and 10 control rats were tested either at 20 or 50 days-of-age. Rats were placed individually in an airtight plexiglass chamber (11"×6"×6"). Liquid flurothyl was pumped (Harvard Apparatus, Holliston, MA, USA) at a constant rate of 50 μL/min from a syringe and dripped onto filter paper on a raised platform in the center of the chamber. The chamber was flushed with room air and cleaned between trials at 10 min intervals. All rats were exposed to flurothyl until tonic extension of both the forelimbs and hindlimbs were observed. Rats were then quickly removed from the chamber and allowed to recover in room air. After recovery, they were returned to their home cage. Seizure susceptibility was expressed as the latency between the first drop of liquid flurothyl and the first myoclonic jerk and was measured independently by two observers.

Statistics

All data were presented as the mean ± SEM. ANOVA for comparison of two or more independent means was used in comparing nNOS protein expression, and seizure susceptibility in tetanus toxin treated, saline injected, and control rats.

**RESULTS**

Tetanus toxin-induced seizures in infancy

As has been reported previously (13), after a TNTX injection, behavioral seizures were first observed in rat pups 24-48 hr later. The frequency of seizures peaked during the monitoring sessions on the second day after the injection (Fig. 1). At that time, the rats often displayed repetitive behavioral seizures which included a constellation of the following behaviors: wild running, clonic face and limb movements, wet dog shakes, and head nodding. The wild running seizures were accompanied by vocalizations, jumping, and infrequent tonic-clonic seizures. A flurry of wet dog shakes were noted to precede or follow a wild running episode. Because wild running episodes were the easiest to identify, wild running seizures were counted during daily 1 hr observation periods. All TNTX-treated rats displayed wild running seizures and they were never observed in saline-injected or untreated litter-mate controls. Following the peak frequency on post-injection day 2, seizures declined in number over the next 5 days (Fig. 1). Seizures were not observed 7 days after the injections. On average, rats had 8.4 ± 2.7 seizures during the daily 1 hr monitoring sessions. The duration of seizures varied widely but most (66%) were less than 1 min and the remainder no more than 2-3 min long.

![Fig. 1. Behavioral monitoring of seizures in infant rats. Average seizures during the 10 hr of observation (one hour/1 day for 10 days); 8.4 ± 2.73, Seizure frequency peaks; within 24-48 hr. WRS, wild running seizure; TNTX, tetanus toxin.](image-url)
Long-term effects of early-life seizures: Video EEG findings

Two previous studies have examined the long term effects of TNTX-induced recurrent early-life seizures (13, 14). In the first study, rat pups experienced severe seizure which as adults resulted in nearly 90% of the animals displaying frequent interictal spikes and 40% of the animals having electrographic seizures. However, in the second study, the rat pups had milder seizures and as adults only 50% of the animals displayed interictal spikes and none had seizures. In the experiments reported here EEG recordings were undertaken in order to resolve these seemingly divergent results. The recordings from 3 normal control rats (Fig. 2A) and 2 saline injected rats did not display epileptiform activity. Video EEG recordings from 5 rats that had experienced recurrent seizures early in life all displayed frequent interictal spikes (Fig. 2B, C) in the hippocampus and/or neocortex but only 1 had electrographic seizures (Fig. 2B). This rat had brief electrographic events that had no behavioral accompaniments.

Seizure susceptibility after early-life seizures

Since rats did not display spontaneous behavioral seizures, experiments were undertaken to determine if the rats were markedly different in their susceptibility to seizures induced by the volatile convulsant, flurothyl. As has been reported previously (15), following exposure to flurothyl neonatal rats initially became quite agitated with head bobbing or turning from side to side. This was followed by attempts at running, squealing and loss of posture. The rats would then invariably develop tonic posturing with both the forelimb and hindlimbs stiffly extended. Mild cyanosis, urinary and fecal incontinence, and salivation were frequently noted. Rats were removed from the chamber as soon as the tonic phase began and allowed to recover in room air. Typically the rats returned to baseline behavior within 10-15 min. Given that rats treated in early-life with TNTX had frequent interictal spikes. However, as shown in Fig. 3B while 50 day-old rats that had experienced early-life seizures were more susceptible to seizures, this difference was quite modest. The time to seizure onset, as measured by the first appearance of myoclonus was decreased by 15% (TNTX: 138.63 ± 11.43 sec, saline control: 162.44 ± 21.01, control: 163.56 ± 26.97). By comparison, 22 day-old rats, which were also treated as infants with TNTX, were markedly more seizure prone (Fig. 3B). Time to seizure onset was decreased by at least 50% (TNTX: 80.38 ± 21.19 sec, saline control: 174.63 ± 56.22, normal control: 157.75 ± 14.88). Thus, the rats appeared to progress from a period of overt spontaneous seizures (postnatal days 12-17), through a period of marked seizure susceptibility (day 22) to one of lessened seizure susceptibility (day 50).

Up-regulation of nNOS expression late in life

Considering a period of marked seizure susceptibility (day 22) to one of lessened seizure susceptibility (day 50) during development in the TNTX treated rats, it seemed reasonable
to propose that during development there is a consequence of reestablishment of neuronal and network excitability. In an attempt to explore the reason for the decline in seizure susceptibility, immunoblotting for nNOS which was known to be related was undertaken.

The results are shown in Fig. 4. On P50, hippocampal slices were microdissected into CA1 and, CA3 subfields and the dentate gyrus, and assayed separately. The results were revealed that area CA1 displayed a 300% increase in nNOS expression (TNTX: 78.7 ± 3.77, saline control: 26.2 ± 3.20), and CA3 displayed an 164% increase (TNTX: 79 ± 3.80, saline control: 48.2 ± 3.97). However, there was statistically no change in nNOS expression level in the dentate gyrus (TNTX: 57 ± 3.01, saline control: 47 ± 2.98).

**DISCUSSION**

Several animal models have been used in the past to study the effects of early-life seizures on brain development. Animal models using systemic and intracerebral injections of convulsants such as pilocarpine and kainic acid produce prolonged seizures in young rats (16, 17), which would be not a reasonable model for a study of recurrent seizures in early-life. Meanwhile, the kindling model has been used successfully in rat pups (18-21). However, even though these animals have a lowered seizure threshold in adulthood, kindling in neonates has not been shown to produce spontaneous seizures or an overt epileptic condition later in life.

Tetanus toxin-induced seizure model has been used by numerous laboratories to produce seizures in adult animals of several species (6-8). Tetanus toxin acts by enzymatically inactivating the synaptic vesicle docking protein, synaptobrevin, thus, preventing neurotransmitter release. Studies have shown that tetanus toxin blocks the release of both excitatory and inhibitory neurotransmitters (22). However, the toxin has also been shown to preferentially block the release of inhibitory neurotransmitters, primarily GABA and glycine (23). Jefferys (24) have shown that an intrahippocampal injection in adult rats results in epileptiform activity in both the injected and contralateral hippocampus for up to 6-8 weeks following injection. Whether similar mechanisms underlie epileptiform activity in adult rats that were treated as infants with tetanus toxin is unknown. Lee et al. (9) introduced the tetanus-induced seizures models that permit study of the acute and chronic effects of multiple early-life seizures on the developing brain. It has been shown the clinical situation where an infant or young child has many repeated seizures over days and weeks. Therefore, the tetanus toxin model was certainly though to be a valid tool to examine both the features of early-life seizures originating in the developing nervous system and the consequences such seizures impact on brain maturation. Thus, author selected this tetanus toxin induced seizure model to create the ideal environment for studying the long term effects of repetitive early-life seizures.

Preliminary observations on this study performed in vivo suggest that tetanus toxin injection into the hippocampus of infant rats can result in abnormal hippocampal epileptiform activity in adulthood. Thus far, author has recorded interictal discharges in rats at 50-60 days of age. Results from the experiments reported herein demonstrate that microinjection of tetanus toxin into infant rat brain may be used to study elec-
trographic seizure activity in the developing nervous system as well as the effects of early-life seizures on brain development.

Although behavioral seizures can be quite stereotyped in many rat pups, the severity of the response can vary from no overt clinical manifestation to as many as 10 seizures per hour during the first few days after injection. This study has shown that routine EEG/video recordings were critical in our interpretation of the behavioral responses to tetanus toxin injection. While some of the responses attributed to the generation of seizures, such as wild running, are clearly abnormal, other responses, such as wet dog shakes, can be observed in control rat pups. Further, it is important to emphasize that behavioral scoring underestimates the total number of seizures that occur electrographically. It is not uncommon to record prolonged focal or generalized electrographic seizures with no apparent behavioral correlates (i.e., subclinical seizures). Thus, to obtain an accurate record of seizure frequency, EEG/video recordings are necessary. Although the neocortex appeared to be involved in the generation of some electrographic seizures in rat pups, recordings from the hippocampus indicated that this area contributes significantly to seizure generation. Indeed, the behavioral correlates of seizures reported herein are very similar, if not identical, to those reported previously for amygdala kindling in 2-week-old rats (25). Specifically, wild running seizures, which were commonly observed in our studies, have been reported as stage 6 seizures in kindled rat pups. Thus, it appears that discharges in temporal lobe structures, such as the hippocampus, contribute to behavioral seizures in the tetanus toxin model.

The volatile convulsant, flurothyl was used to induce seizures in order to evaluate differences in seizure susceptibility between treatment groups at 20 or 50 days-of-age. Twenty-two day-old rats, which were also treated as infants with TNTX, were markedly more seizure prone (Fig. 3A). The time to seizure onset was decreased. Therefore, rats appeared to progress from a period of overt spontaneous seizures (postnatal days 12-17), through a period of marked seizure susceptibility (day 22, Fig. 3A) to one of lessened seizure susceptibility (day 50, Fig. 3B). These changes in seizure susceptibility in adult rats suggested that there may be neuronal hyperexcitability induced by recurring seizures in infant rats and neuronal plasticity during brain development. This study suggested that these changes in seizure susceptibility could be explained as the effects of seizures on the connectivity and circuitry of the developing brain. In recent studies, there were valid results that can support our explanation. Swann et al. (26) reported spine loss and other persistent alterations of hippocampal pyramidal cell dendrites at CA3 in a tetanus toxin induced seizure model in infant rats. They introduced alterations in the distribution of recurrent excitatory synapses on dendrites could lead to promote the reverberation of recurrent excitation in networks of mutually excitatory pyramidal cells.

To explain the decreased seizure susceptibility in the adult rats which experienced recurring seizures in early-life, the expression of nNOS level was evaluated in the hippocampal subfield, CA1, CA3, and dentate gyrus. Distinctively, the significant up-regulation of nNOS was revealed in the CA1 and CA3 subfields of the hippocampus. However, there was statistically no change in nNOS expression level in the dentate gyrus (Fig. 4). These results resemble a previous study of NOS mRNA expression by Elmer et al. (27). The reason for the meaningful regional difference in nNOS expression may be related to a seizure generation mechanism or distribution of the NMDA receptor which activates NOS via glutamate. Thus, repetitive seizures on developing rat brain caused changes in nNOS expression level. Furthermore, there were differences in nNOS expression especially according to the hippocampal subfield. Which is interesting, the nNOS is even considered to be related to neuronal development in gestational stage (28, 29). Recently, Sunico et al. (30) suggested that NO mediates the formation of synaptic connections and regeneration of neurons in the process of brain development. This characteristics of nNOS expression on brain development suggests that nNOS contributes to neuron maturation as well as vulnerability in each period and region, and NO may play an important role in the basic development of human brain functions.

In conclusion, repetitive seizures in early-life eventually cause long term effects, which represents as decrease in seizure susceptibility on the mature brain of late-life stage. Furthermore, the hippocampus of the mature brain has an increase in nNOS expression. Considering these results, it appears that nNOS causes a seizure prone effect on the neuronal network and neuronal plasticity. Therefore it is suggested that nNOS plays an important role in both down regulating the seizure threshold and causing epileptogenesis.

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