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Epileptogenesis Is Increased in Rats With Neonatal Isolation and Early-Life Seizure and Ameliorated by MK-801: A Long-Term MRI and Histological Study

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ABSTRACT: Early-life stress has been shown to destabilize the homeostatic synaptic plasticity and compromise the developing brain to the later encountered insults. This study would determine the long-term epileptogenic effect of neonatal isolation (NI) on early-life seizure. There were five groups: normal rearing (NR) rats; NI rats; NR rats suffering from status epilepticus (SE) at P12 (NR-SE); NI-SE rats; NI-SE-MK801 rats. All adult rats were video monitored to detect behavioral seizures, examined with brain magnetic resonance imaging, and assessed for hippocampal NeuN-immunoreactive (NeuN-IR) cells. Behavioral seizures were detected in one of six NR-SE rats, all the NI-SE rats (eight of eight), and none in the NR, NI, or NI-SE-MK801 rats. High hippocampal T2 signal were only found in three of five NR-SE rats, five of six NI-SE rats, and one of five NI-SE-MK801 rats. There was a significant decrease in the number of hippocampal NeuN-IR cells in the NR-SE and NI-SE groups, compared with the NR group, and MK-801 treatment ameliorated the neuronal loss. Our results demonstrated that NI led to an increase in epileptogenesis in rat pups with early-life SE, and treatment with MK-801 could ameliorate brain injuries, indicating a critical role of *N*-methyl-D-aspartic acid receptor in the epileptogenic process. **(*Pediatr Res* 66: 441–447, 2009)**

Seizure is one of the most common pediatric emergencies with the highest incidence in the first year of life. Animal studies have demonstrated that early-life seizures differ essentially from seizures in the adult, including the seizure behaviors, the EEG features, and their consequences. Notwithstanding the higher susceptibility to seizures, the immature brain is less vulnerable to seizure-induced injuries than the mature brain (1–5), and diverse conditions, such as the seizure severity, causes of seizure, or precipitating injuries may affect the long-term neurologic outcome (6–9). In this regard, the circumstances under which a seizure in immature brain can cause permanent brain damage is of great interest (9,10).

In most studies published to date, early-life seizures are induced in the experimental animals under normal rearing (NR) and environmental conditions. For humans, however, most early-life seizures occur in premature and sick neonates (11–13) who are hospitalized and separated from their mothers, and hence, are under stress (14,15). Emerging evidence indicates early-life stress has enduring effects on the neuroendocrine system (16,17) and destabilizes homeostatic synaptic plasticity, particularly on the hippocampal neuroplasticity (18–22). Stress or glucocorticoids (GCs) exposure potentiates the excitotoxic effect of concurrent neurologic insults (23), for example acceleration of kindling epileptogenesis (24), and change of seizure propensity (25). We have earlier demonstrated that rat pups subjected to repetitive neonatal isolation (NI) can exacerbate cognitive deficit and anxiety-like behaviors after recurrent seizures or status epilepticus (SE) (7,9,26). However, whether neonatal stress can precipitate the incidence of epileptogene sis in the context of early-life seizure is currently unknown.

There have been only a few studies applying magnetic resonance imaging (MRI) in the studies of brain injuries after early-life seizures. Dube *et al.* (27) examined the acute temporal profiles of hippocampal T2 signal changes in postnatal (P) 12 rats subjected to febrile seizure. They found that seizure could result in hyperintensity on the T_2-weighted images 24 h and 8 d later, although no accompanying neuronal death was noticed. Roch *et al*. (28) used animal MRI to study the development of chronic epilepsy after lithium−pilocarpine (Li-Pilo)-induced SE in P21 rat. They demonstrated that only rats with acute changes in hippocampal T2 signals or T2 relaxation time developed chronic epilepsy. A recent study shows that in a subpopulation of rats with SE at P12, volume reduction in temporal lobe regions is detected in adulthood by MRI (29). However, the chronic changes of MRI T_2-weighted signal following early-life seizure is still unclear, and also the relation to the development of spontaneous recurrent seizure (SRS), as well as the underlying pathology. Therefore, the goals of this study were 3-fold: 1) the determination of whether NI increases the incidence of epileptogenesis after early-life seizure; 2) the possible role of the *N*-methyl-D-aspartic acid (NMDA) receptor if epileptogenicity is in-

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Abbreviations: GC, glucocorticoids; **Li-Pilo,** lithium-pilocarpine; **NeuN-IR,** NeuN-immunopositive; **NI,** neonatal isolation; **SE,** status epilepticus; **SRS,** spontaneous recurrent seizures

creased; and 3) whether changes on the MRI T2signals reflect neuropathological changes and would be a surrogate marker for chronic epilepsy in rats with a history of early-life seizure.

MATERIALS AND METHODS

Summary. Only male rat pups were used in this study to investigate whether NI exacerbates brain damage after neonatal SE. Five groups of rats were used: 1) NR group—normal reared rat pups left undisturbed; 2) NI group—rat pups subjected to NI during P2–12; 3) NR-SE group—NR rat pups subjected to Li-Pilo-induced SE at P12; 4) NI-SE group—NI rat pups subjected to SE at P12; and 5) NI-SE-MK801 group—NI-SE rat pups treated with MK-801 (1 mg/kg) intraperitoneally (i.p.) after pilocarpine at P12. To minimize the differences in handling, all rats were weighed and marked in <5 min every day from P0–12. At ~6 mo of age, all rats were video monitored, followed by a brain MRI examination, and then killed for hippocampal neuropathological examinations. Figure 1 provides a graphic summary of the experimental design.

Subjects. Sprague-Dawley (SD) rats, purchased from the National Science Council, were used in this study. Pregnant rats were checked for litters daily at 1000 h. The day of birth was designated P0. Rat pups were weaned at P21. All rats were housed in an animal-care facility at a 1212 h light/dark cycle with light on at 0700 h with access to standard rat chow and water *ad libitum*. All experiments were carried out in accordance with guidelines set by the Animal Care and Use Committee (National Science Council, Taiwan), and all efforts were made to minimize animal suffering during the experiments.

NI procedure. Repeated isolation of neonates from the mother is a well-characterized model of early life stress (30). The rat pups were culled to 10–12 per litter at P1, and the pups were randomly assigned to one of the two treatments: isolated, nonisolated, a between/homogeneous-litter design. The rat pups were isolated from dams for 1 h per day (1000 h to 1100 h) from P2 to P12, modified from our previous study for weighting the effect of NI (9,27). Isolated rat pups were removed from the nest and weighed one at a time. Isolated rat pups were placed individually into a clean plastic cup (9.5 cm diameter × 8 cm deep) in a humidity-controlled (50%) cage with maintained temperature at 30 ± 1°C to maintain the rat pups at a normal body temperature. At the end of the isolation period, pups were returned to the home cage, joining their littermates and dams.

Induction of seizures. Because our previous data showed that SE at P10 did not result in SRS in either NR or NI rats, in this study, a Li-Pilo-induced SE at P12 was used to evaluate the long-term effects on SRS, neuroimage presentations, and hippocampal neuropathological changes. The rat pups of NR-SE, NI-SE, and NI-SE-MK801 groups were treated i.p. with 3 mEq/kg lithium chloride at P11 and then 60 mg/kg pilocarpine at P12. Pilocarpine is a cholinergic agonist that induces seizures with resultant brain damage (31). Pretreatment with lithium potentiates the epileptogenic action of pilocarpine and reduces mortality (31,32). The rat pups of the NR and NI groups were injected i.p. with saline of the same volume per kilogram of the body weight at P11 and P12. MK801-treated animals were injected i.p. with 1 mg/kg MK801, a NMDA receptor antagonist immediately after induction of SE. Lithium chloride, pilocarpine, and MK801 were purchased from Sigma Chemical Co. (St. Louis, MO). All rats were continuously monitored for the behavioral seizures by an experienced observer, and only rats displaying SE (33), including head bobbing, forelimb clonus, and tonic activity, were used.

Direct observation and video monitoring of behavioral seizures. Video monitoring has been demonstrated to detect behavioral seizures characterized by freezing, head nodding, and jaw myoclonus (9). The spontaneous behavioral seizures after pilocarpine-induced SE occurs in cyclicity, peaking every 5–8 d (34), and the seizure phenotype consists of freezing, automatisms, and even jumping (9,10,35,36). Thus, at the age of ~6 mo, the rat's behaviors were monitored using a video camera for 6 h per day for 10 consecutive days, excluding weekends to compare the incidence of behavioral seizures between groups. Monitoring was performed during the daytime (1100 h to 1700 h), and all the tapes were reviewed later for evidence of behavioral seizures. No electrode was inserted to perform a brain MRI examination.

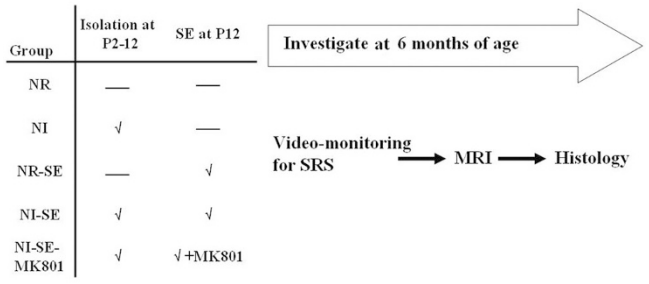

Figure 1. Schematic diagram of the study design.

Brain MRI protocols. At the age of ~6 mo, rats are anesthetized with ketamine (50 mg/kg) and xylazine (23 mg/kg). The MRI was performed on a clinical 3.0-T scanner (GE Signa, Excite HD, Milwaukee, WI) with a surface coil Mayo Clinic BC-10 3.0-T coil (Mayo Foundation for Medical Education and Research). The brain was scanned from the brain stem to the olfactory bulb with fast spin-echo T2-weighted imaging pulse sequences (TR/TE, 3800/85). Field of view was 40 mm, pixel matrix was 256 × 256, and 1-mm slice thickness, no gap, and two excitations were used. The T2 signal intensities were compared with normal control rats, and all the signal intensities were measured by ROI by using PACS imaging display software (Centricity RA1000, GE Healthcare, Taipei, Taiwan). All the MRI scans were read by a radiologist without the knowledge of the group.

Neuropathology examination. After completion of brain MRI examination, the rats were killed and brains were removed, postfixed in 10% formaldehyde for 7 h at 4°C, and then stored in a 30% sucrose solution. The brains were sectioned in the coronal plane (20 μm) with a freezing microtome and stored at −20°C until processed. NeuN (Chemicon, Temecula, CA, monoclonal 1;200), a neuron-specific nuclear marker was used for immunohistochemical detection of surviving pyramidal neurons in the hippocampus, as well as counterstaining with hematoxylin-eosin in the same sections for detection of neurons that underwent delayed neuronal death. The sections were selected from the septal hippocampus, a region between 2.8 mm and 3.8 mm posterior to the bregma (37).

Cell counts were performed under 400× magnification (40× objective, 10× ocular). A counting box 100 μm × 100 μm was placed over the region of interest (ROI). The number of NeuN-immunopositive neurons (NeuN-IR) with >50% perikarya surface within the box was measured separately in three randomly selected regions of the hippocampal CA1 and CA3 subfields in each hippocampal slice in a blinded manner. An averaged value from three ROIs in each subfield of CA1 or CA3 was obtained in each slice. For each group, the analysis was performed on eight slices for each region in four to six brains per group ($n = 4$ for the NR and NI groups; $n = 5$ for the NR-SE and NI-SE-MK801 groups; $n = 6$ for NI-SE group). Data were given as number of NeuN-IR neurons within a 100 μm × 100 μm area in each specific brain region (mean ± SEM).

Statistical analysis. Changes of body weight over the time were compared by analysis of variance (ANOVA) with repeated measures. *t* test was used to compare acute seizure duration between NR-SE and NI-SE groups. Hippocampal NeuN-IR cells between groups were analyzed using the ANOVA with *post hoc* Bonferroni test. Nonparametric tests were used to compare the mortality, the high signal findings on T_2-weighted MRI images between groups, and the correlation between MRI findings and the rates of SRS. Correlations of the number of NeuN-IR cells in CA1 and CA3 subfields and T2 signal was analyzed using point-biserial correlation coefficient. Significance was defined as $p < 0.05$ for all tests.

RESULTS

Behavioral effects in the acute stage. There were no differences in body weights between any groups compared either

Table 1. *Summary of the seizure duration and the mortality at acute stage and long-term morbidity of spontaneous recurrent seizures*

	Acute stage		Long term
Group	Seizure duration, min	Mortality, %	Chronic epilepsy
NR	—	0	0
NI	—	0	0
NR-SE	420.86 ± 28.96	14.26	1/6 (17%)
NI-SE	511.00 ± 19.61*	11.11	8/8 (100%)
NI-SE-MK801	Immobile, asleep	16.67	0

*$p < 0.05$ comparing with NR-SE group.

NR, normal-rearing rats; NI, rats treated with neonatal isolation; NR-SE, normal-rearing rats suffering status epilepticus; NI-SE, isolated rats suffering status epilepticus; NI-SE-MK801, NI-SE rats receiving MK-801 treatment.

at P2, P21, or P30. The Li-Pilo-treated rat pups uniformly had behavioral changes consisting of immobility, piloerection, and scratching within 4–5 min, which evolved into SE during the following 15–20 min. Rat pups in the NR-SE and NI-SE groups had similar degrees of seizure severity, characterized by head bobbing, extension of the tail and hindlimbs, and forelimb clonus without falling. The seizure duration was longer in the NI-SE group (511.00 ± 19.61 min), compared with the NR-SE group (420.86 ± 28.96 min) ($t_{(14)} = 2.667$, $p < 0.05$). In the NI-SE-MK801 group, rats became floppy, immobile, and drowsy. Five of the six rat pups in the NI-SE-MK801 group survived on through to the next day and were used for subsequent studies. The mortality was similar for all groups during seizure induction or follow-up (Table 1).

Direct observation and video monitoring of behavioral seizures as evidence of chronic epilepsy. Observed over a consecutive 10-d period, behavioral seizure phenotype consisted of freezing, automatisms, such as chewing or facial clonus, and jumping was directly observed in one of six NR-SE rats (17%) and eight rats in the NI-SE group (eight of eight; 100%). Behavioral seizure was not detected in any rat in the other three groups. The incidence of chronic epilepsy was substantially higher in the NI-SE group than in the NR-SE group.

Hippocampal MRI findings. The MRI examinations were performed after the completion of video monitoring. All rats in the NR group ($n = 5$) and the NI group ($n = 5$) had unremarkable brain MRI (Fig. 2*A* and *B*). In the NR-SE group, the one rat with behavioral seizure died during MRI examination, three of the remaining five rats had increased T_2 weighted in the hippocampus (Fig. 3). Hippocampal high T_2-weighted signal was found in five of the six rats in the NI-SE group (Fig. 4) but only in one of the five NI-SE-MK801 rats (Fig. 5). There was a lower rate of hippocampal high T2

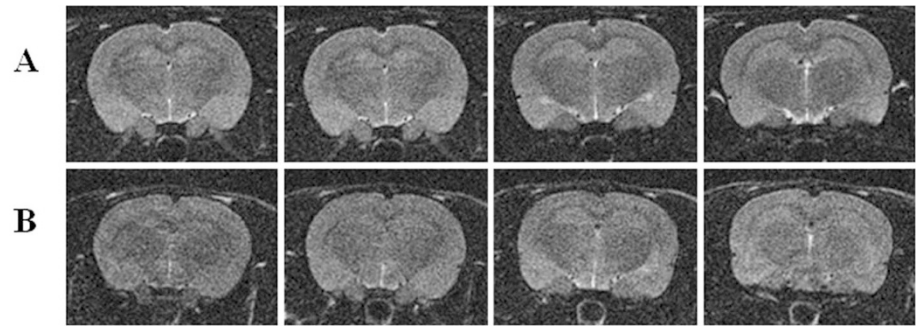

Figure 2. Coronal T_2-weighted MRI images from the NR (*A*) and NI (*B*) rats in adulthood.

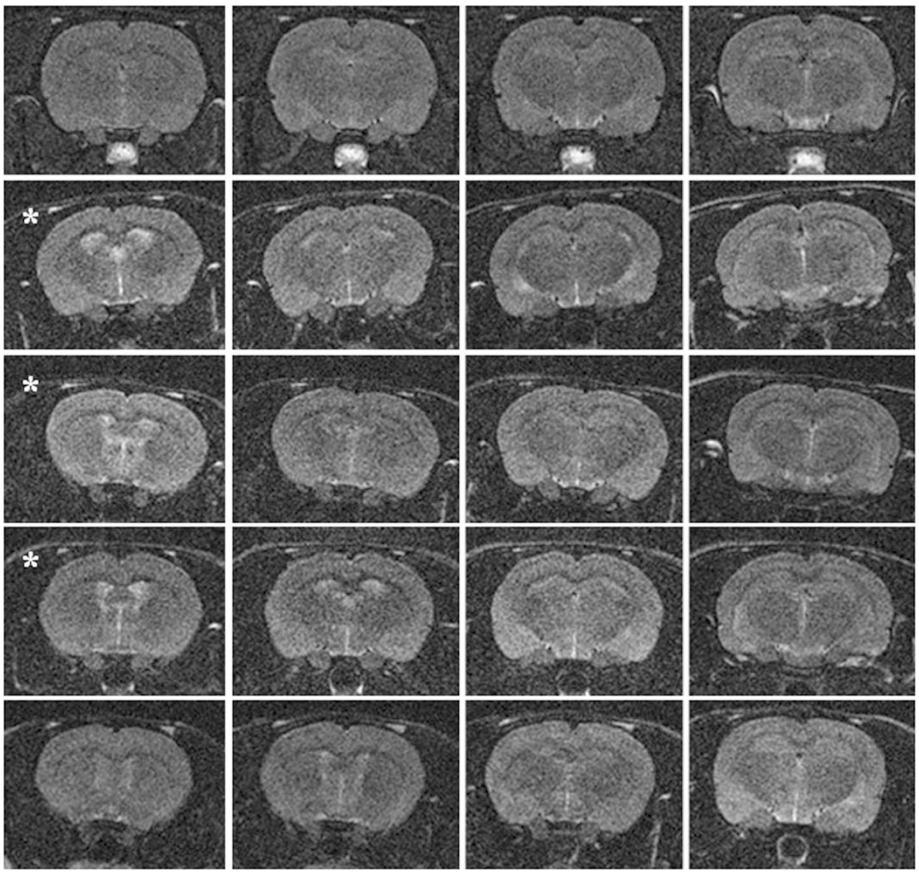

Figure 3. Coronal T_2-weighted MRI images from the NR-SE rats in adulthood. Increased T2 signals were seen in three of five of NR-SE rats. *Rats presenting increased T2 signals in the hippocampus.

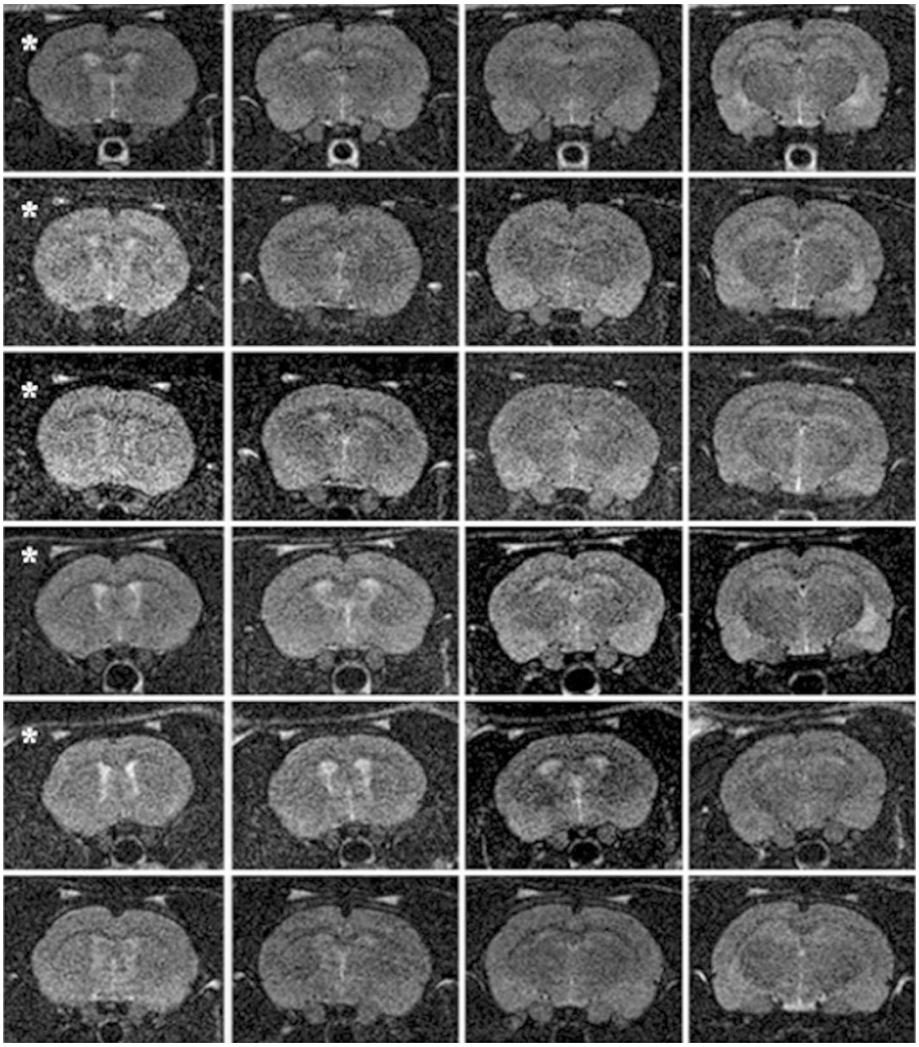

Figure 4. Coronal T_2-weighted MRI images from the NI-SE rats in adulthood. Increased T2 signals were seen in five of six of NI-SE rats. *Rats presenting increased T2 signals in the hippocampus.

signal in the NI-SE-MK801 rats than in the NI-SE rats ($p < 0.05$, Mann-Whitney Test). Although there was a trend toward a positive correlation between the hippocampal high T2 signal and SRS phenomena, the trend did not reach statistical significance ($p = 0.06$, Fisher exact test).

Morphologic studies in the hippocampus. There were significant differences in the average numbers of NeuN-staining cells within the counted areas of the CA1 ($F_{(4,19)} = 21.075$, $p < 0.05$) and CA3 ($F_{(4,19)} = 16.020$, $p < 0.05$) subfields among the five experimental groups (Figs. 6 and 7). *Post hoc* test showed significant decrease of cell counts in both the NR-SE and NI-SE groups, compared with the NR group. However, the NI-SE-MK801 group showed less neuronal cell loss compared with the NI-SE group. Among rats suffering SE in NR-SE, NI-SE, NI-SE-MK01 groups, only the number of NeuN-IR in CA1 had a negative correlation to high T2 signal (correlation coefficient: -0.688, $p < 0.05$).

DISCUSSION

The current study presents evidence that NI potentiates early-life seizure-induced epileptogenesis and brain damages. In addition, immediate injection of MK-801 after SE induction can ameliorate brain injuries, including incidence of chronic epilepsy, decrease in hippocampal NeuN-IR cells, and rate of high hippocampal T2 signal, indicating a critical role of NMDA receptor in the epileptogenic process.

There is an age-dependent increase of epileptogenesis after SE in immature rats. Li-Pilo-induced SE at P10 rat does not lead to the development of SRS in adulthood (35,38). However, 25, 50, and 92% of rats developed SRS after Li-Pilo-induced SE at P12, P25, and P28, respectively (35,39). Similarly, 14 and 29% of rats exhibited SRS after kainic acid-induced SE at P20 and P30, respectively (5). Apparently, the incidence of chronic epilepsy after SE is both age- and model-dependent (40–42). In our study, using video monitoring, P12 SE resulted SRS in 17% of NR rats but more in the NI rats (100%).

How NI increases the vulnerability of immature brain to seizure insults and epileptogenesis is a matter of interest of our study. Stress and/or exposure of GCs are known to potentiate the effects of concurrent neurologic insults in the hippocampus (23). Excessive GCs increase extracellular glutamate levels (43,44), calcium conductance, either voltage or ligand gated (43), alter expressions of NMDA receptor subunits (45), and reduces the uptake of glutamate by glia (46). Indeed, one previous study has shown that GCs exacerbate NMDA receptor-mediated toxicity (47). Thus, it is reasonable to speculate that repeated

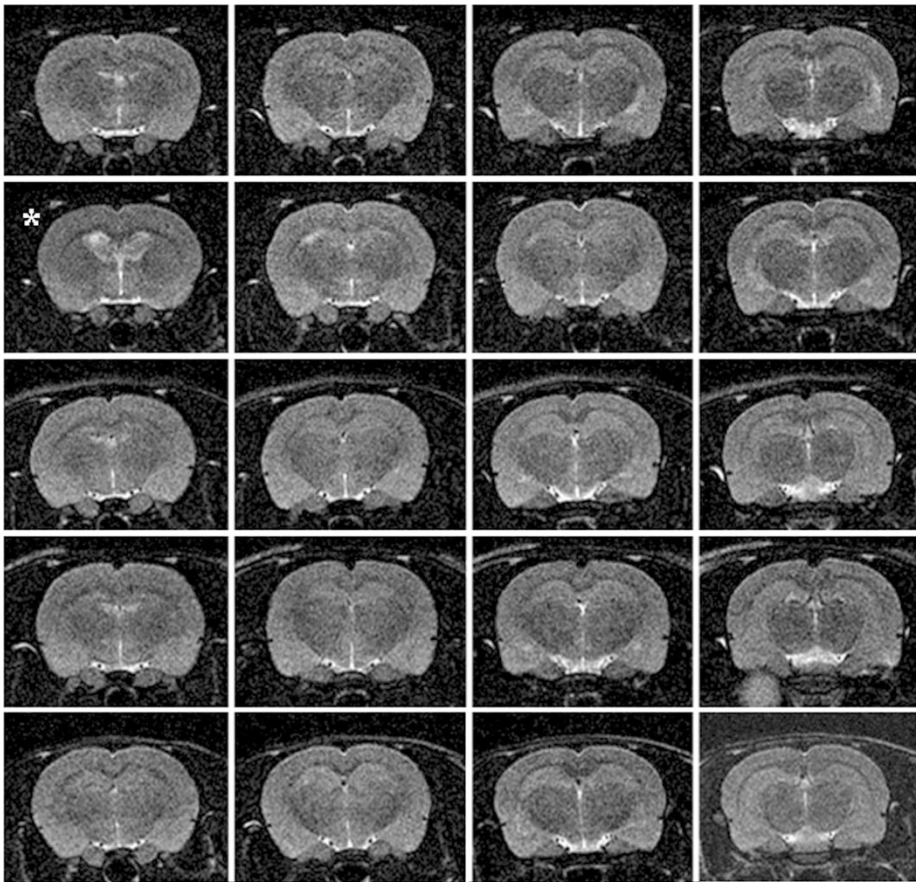

Figure 5. Coronal T_2-weighted MRI images from the NI-SE-MK801 rats in adulthood. Increased T2 signals were seen in one of five of NI-SE-MK801 rats. *Rats presenting increased T2 signals in the hippocampus.

NI can change the balance of excitatory and inhibitory synaptic connectivity in the hippocampus, a critical region for epileptogenesis, and exacerbate brain injury after early-life seizure. Further, a recent study demonstrated that repeated parental separation resulted in elevated excitatory spine density (12) and alteration of the numbers of hippocampal GABAergic neuronal subpopulations, reflecting reduced inhibitory activity in the CA1 region (14). In this study, we found that treatment with MK-801 immediately after SE induction prevented the development of chronic epilepsy, ameliorated hippocampal neuronal loss, and decreased the rate of chronic hippocampal T_2-weighted MRI abnormality in isolated rat pups subjected to early-life seizures. Although MK801 shows an anticonvulsant effect in several models of epilepsy, data from a variety of models suggest that MK801 treatment can either in long-term (48) or acutely increase neuronal excitability and seizure propensity, possibly related to an up-regulation of NMDA receptors (49–51).

Increased T_2-weighted MRI signal implies that tissue-free water concentration is increased that might be caused by neuronal loss, brain edema, or gliosis (52,53). Roch *et al.* (28) studied the chronic MRI finding in adult rat with Li-Pilo-induced SE and found that hippocampal T_2-weighted signal gradually intensified with the progression of epilepsy and correlated well with neuronal loss and gliosis in the hippocampus. Nairismagi *et al.* (29) studied P12 rat pups with Li-Pilo-induced SE and detected chronic volume reduction in hippocampus, amygdala, or perirhinal cortex. In this study, high T2 signal in the hippocampus was found in 60% of NR-SE group rats, in 83% of NI-SE group rats, whereas less in NI-SE-MK801 group rats (20%) as in adulthood. In addition, there was a negative correlation between the hippocampal high T2 signal and the number of NeuN-IR in CA1. However, no statistical association between the hippocampal high T2 signal and SRS rate was seen, despite a trend toward a positive correlation. For the relatively small number of subjects in this study, further work is necessary before any definitive conclusions can be made.

Hitherto, research on the mechanisms leading to increased epileptogenesis has focused on functional and structural alterations in the hippocampus. Although the NI-SE group rats had significantly higher incidence of chronic epilepsy than the NR-SE group, there was no significant difference of hippocampal neuronal loss between these two groups. The reasonable speculation is that other mechanisms accompanying hippocampal neuronal loss account for the difference of epileptogenicity. A recent study by Galanopoulou (54) showed that maternally separated male and female rat pups exhibited a hyperpolarizing shift of GABA postsynaptic potential, and its effect was analogous to three episodes of kainic acid-induced SE in male pups. Although our stress model is different from that of Galanopoulou's model, we postulate that NI might have effects on GABA receptors and increase epileptogenicity on concomitant neonatal seizure. Other possible mechanisms involved in epileptogenesis after early-life seizure such as neurotransmitter receptors, gene expression programs, growth factors, cytokines, neurogenesis, and glia pathology could be the subject of further research (55,56).

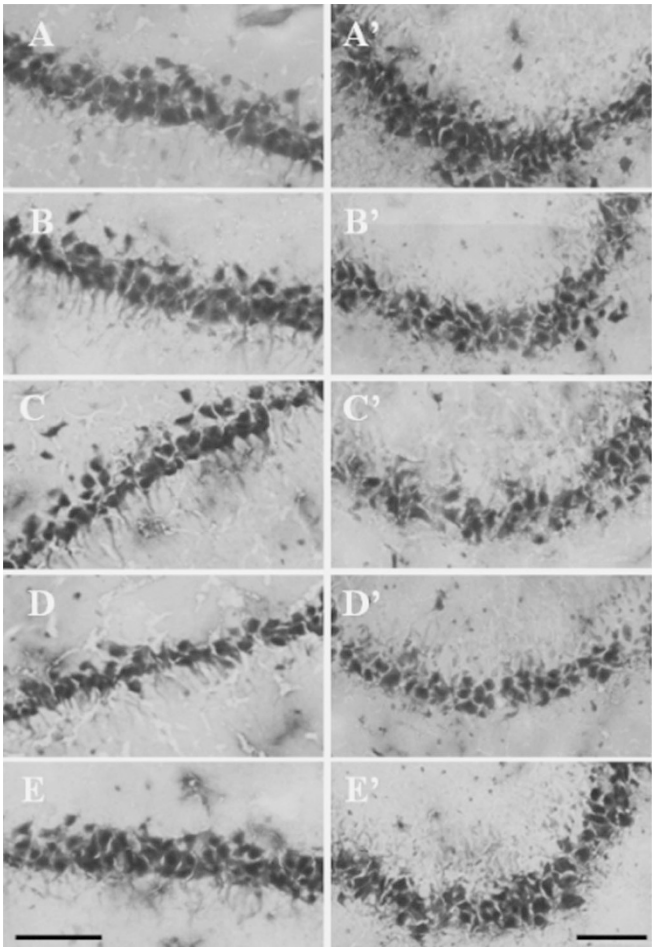

Figure 6. Representative photomicrographs of NeuN-immunoreactive (NeuN-IR) cells in the CA1 (*A–E*) and CA3 (*A'–E'*) subfields of the hippocampus from the NR (*A*, *A'*), NI (*B*, *B'*), NR-SE (*C*, *C'*), NI-SE (*D*, *D'*), and NI-SE-MK801 (*E*, *E'*) rats. Decrease in NeuN-IR cells was present in CA1 and CA3 subfields in NR-SE and NI-SE rats. Scale bar = 100 μm.

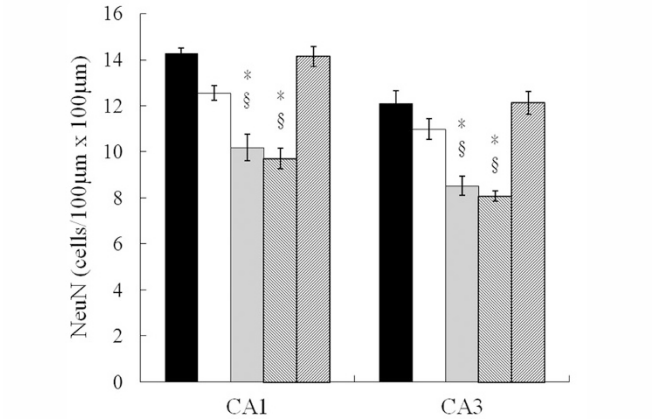

Figure 7. Comparing NeuN-immunoreactive (NeuN-IR) cells in the CA1 and CA3 subfields of the hippocampus between groups. Statistical decrease was seen in NR-SE and NI-SE rats, and NI-SE-MK801 rats showed fewer hippocampal neuronal loss. Results are presented as mean ± SEM. *$p < 0.05$, compared with the NR rats, §$p < 0.05$, compared with the NI-SE-MK801 rats. ■NR NI ■NI-SE ▨NI-SE ▨NI-SE-MK801

In clinical practice, the neonates with seizures are usually treated in neonatal intensive care unit, a stressful environment for neonates. Our data indicate that adequate treatment of neonatal seizure may need to include medications to control seizure, as well as environmental maneuvers to reduce concomitant stress.

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