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Early-life experience alters response of developing brain to seizures

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Abstract

Prolonged seizures during childhood are associated with behavior problems, memory impairment and school failure. No effective treatment currently exists after seizures to mitigate neuronal injury and long-term neurological sequelae for children with epilepsy. We studied the therapeutic efficacy of early-life environment on seizure-induced behavioral deficits, neuronal injury and the inflammatory reaction using the kainic acid (KA) seizure model. Two rearing conditions, maternal separation for 3-hours daily versus maternal care in an enriched environment, were followed by single housing for the former (Deprived) and group housing in an enriched environment for the latter (Enriched). To examine the influence of differential rearing on the behavioral effects of early-life seizures, KA was injected on P21. On P28, marked reduction in exploratory behavior was noted after seizures only in the Deprived group. To investigate seizure-induced hippocampal injury, a separate group of rats were injected with KA on P35 since consistent seizure-induced neuronal injury is observed only in mature rats. Brains of rats sacrificed on P37 displayed a significant reduction in DNA fragmentation and microglial activation in Enriched compared to Deprived animals. Our results suggest that a nurturing early environment can enhance the ability of the developing brain to recover from seizures and provide a buffer against their damaging effects. While the nurturing environment was neuroprotective, the combination of deprived rearing and the insult of early-life seizures resulted in significant behavioral deficits, an increase in neuronal injury and activation of microglia in young rats.

Keywords

kainic acid; environmental enrichment; status epilepticus; neuroprotection; neuroinflammation; exploratory behavior

1. Introduction

Evidence is compelling that early-life experiences exert a profound and long-lasting influence on the defensive response to threat and stress of many organisms, ranging from plants to mammals (Agrawal, 2001; Fleming et al., 1999; Meaney, 2001). Traumatic early-life

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experiences come in many different forms – from acute episodes, such as a seizure, to more long-term events like neglectful parents. Many studies suggest that early-life social or psychological stress may predispose cognitive dysfunction and depression (Brunson et al., 2005; Fenoglio et al., 2006; Meaney et al., 1988; Sanchez et al., 2001). Experimentally, the quality of maternal care can be altered by housing the dam in a stressful living situation. This, in turn, results in differences in both behavioral and hypothalamic-pituitary-adrenal (HPA) responses to stress in the progeny (Avishai-Eliner et al., 1995; Caldji et al., 2000; Liu et al., 1997). Early-life maternal separation, a well characterized model for early stress (Avishai-Eliner et al., 1995; Colorado et al., 2006; Meaney, 2001), leads to elevated anxiety, suppression of neurogenesis, and diminished plasticity in the hippocampus after exposure to stressors (Huot et al., 2002; Mirescu et al., 2004; Varty et al., 2000). A direct relationship has been observed between good maternal behaviors, such as licking/grooming and arched-back nursing (LG-ABN), and enhanced hippocampal development in offspring; specifically, increased gene expression and cholinergic innervation of the hippocampus and enhanced spatial learning and memory are noted in pups raised by nurturing dams (Fenoglio et al., 2006; Liu et al., 2000).

Environmental enrichment can improve memory and motor skills after brain trauma (Johansson and Ohlsson, 1996; Nudo et al., 2001), reverse age-related decreases in cognition and neuroplasticity (Kempermann et al., 2002), protect against seizure-induced neuronal death (Young et al., 1999) and compensate for seizure-induced visual-spatial memory deficits in young rats (Faverjon et al., 2002). Using the KA-induced status epilepticus (SE) model, we have previously also shown that complex social and sensory-motor stimulations, provided by exposing developing rats to an enriched environment after seizures, improved exploratory behavior and reversed depressive behavior (Koh et al., 2005; Koh et al., 2007).

Here, we extend our study to the neonatal period to investigate the effect of maternal care on seizure-induced behavioral deficit, hippocampal neuronal injury and neuroinflammation. We hypothesize that proper maternal care combined with an enriched environment *before* seizure initiation provides the neuroprotection necessary to shield animals from the detrimental effects of prolonged seizures. We examined (1) the effect of differential maternal care on early life seizure-induced changes in exploratory behavior in a novel environment and (2) the effect of differential rearing on seizure-induced cell death and microglial activation in the hippocampus.

2. Results

2.1. Differential quality of maternal care

To quantify the differential quality of maternal care, dams and pups from both environmental conditions were observed for 2 hours each day from P3 to P9 and maternal behaviors were recorded (see Experimental Procedure). Maternal care in a deprived environment, in which pups were separated from their dams for 3 hours daily during the first two weeks of life, was inferior to maternal care in an enriched environment. Dams in the Deprived group spent significantly less time with their pups ($p < 0.05$) and performed significantly less arched-back nursing ($p < 0.02$) than dams in the enriched environment (Fig. 2A & B). This differential quality of maternal care was reflected in both delayed eye opening in the Deprived compared to Enriched group on P15 and a significant difference in weight gain in pups at the time of weaning. Earlier eye opening in animals reared in an enriched environment has been suggested to be a direct result of enhanced levels of good maternal care in the Enriched group (Cancedda et al., 2004). While 80% of pups (43 of 54) in the enriched environment had their eyes open by P15, only 46% of pups (29 of 63) reared in the deprived environment had opened their eyes (Fisher exact test, $p < 0.0003$). At the time of weaning (P21), pups in the Deprived group appeared smaller and weighed significantly less than those in the Enriched ($n=51$, 47.4 ± 0.8 g vs. 50.4 ± 1.0 g; $p < 0.02$) (Fig. 2C).

2.2. Impaired exploratory behavior in deprived animals after early-life seizures

Exploratory behavior was tested in an open field on P28, seven days after injection of either KA or PBS (see Experimental Procedure). Exploration was found to be affected by both rearing environment and experience of seizures. During the five-minute open field trials, rats in the Deprived group who experienced KA-induced seizures (Deprived-KA) often huddled in a corner and rarely approached the center of the arena (Fig. 3A); they only crossed the lines approximately 20 times (21 ± 5), significantly less than all other groups ($n=51$, One-way ANOVA: $p < 0.0005$) (Fig. 3C). Interestingly, neither the Enriched group that experienced seizures (Enriched-KA) nor the Deprived group injected with PBS (Deprived-C) showed impairment in exploratory behavior; there was no significant difference in the number of line crossings among Enriched-KA, Deprived-C and Enriched-C ($p > 0.05$). Despite the difference in open field behavior, both experimental groups in Group A (see Experimental Procedure) experienced statistically similar seizures at P21, which suggests that an enriched environment and maternal care combined could protect against the harmful effects of seizures. There was no difference in severity of seizures ($n=51$, Mann-Whitney U : $p > 0.5$) (Fig. 4A) or in latency to seizures at P21 between the two experimental groups ($n=33$, $p > 0.8$) (Fig. 4B).

2.3. Cell death and microglial activation in the hippocampus

As was also seen in Group A animals at P21, there was no observed difference in seizure intensity ($n=72$, Mann-Whitney U : $p > 0.42$) (Fig. 4C) or latency to seizure ($n=49$, $p > 0.26$) (Fig. 4D) between Enriched and Deprived rats in Group B at P35 (see Experimental Procedure). Even after experiencing seizures of equal latency and severity, hippocampal cell death after a KA seizure was increased in young adult rats that experienced early-life maternal deprivation followed by isolation (Deprived) compared to littermates reared in an enriched environment (Enriched) during the first 5 weeks of life. Seizure-induced DNA fragmentation within the CA3 sub-region of the hippocampus was significantly greater in Deprived compared to the Enriched group ($n=7$, 4.3 ± 1.2 vs. 1.0 ± 0.3 , $p < 0.03$) (Fig. 5C). Similarly, seizure-induced microglial activation in the hippocampus was significantly increased in the Deprived group compared to Enriched ($n=7$, 13.5 ± 0.7 vs. 9.7 ± 0.2 , $p < 0.005$) (Fig. 5F). Only rats with grade IV seizures (see Experimental Procedure) were chosen for immunohistochemistry in order to obtain unbiased results.

3. Discussion

We found that the combination of a deprived rearing environment and the insult of early-life seizures resulted in significant behavioral deficits in young rats. Significant seizure-induced impairment in exploratory behavior was observed only in animals that experienced poor maternal care and isolation. Adequate maternal care provided in an enriched environment appears to protect young animals from the detrimental effect of seizures on their anxiety-like behavior. Further support of the neuroprotective potential of maternal care and environmental enrichment before brain injury is the observation that seizure-induced cell death and brain inflammation was ameliorated in animals in the Enriched group.

Decreased exploratory behavior in Deprived rats after seizures in the present study may imply heightened fearfulness and an exaggerated stress response to a novel environment. Early-life experience has been shown to influence the expression of fearfulness and HPA responses to stress (Caldji et al., 1998; Liu et al., 1997; Sanchez et al., 2001). Maternal separation of rat pups leads to enhanced cellular responses to psychological stressors (Colorado et al., 2006; Huot et al., 2002; Varty et al., 2000). Studies have shown that better maternal care during infancy reduces the levels of pituitary adrenocorticotrophic hormone (ACTH) and adrenal corticosterone to stress in adulthood (Liu et al., 1997). Likewise, the offspring of mothers who exhibit high levels of LG-ABN show significantly reduced behavioral fearfulness later in life

compared to pups that experience unusually low LG-ABN levels (Caldji et al., 1998). These observations indicate that poor maternal care and isolation render developing animals more vulnerable to stress in later life. Protection from this vulnerability is afforded by proper maternal care and an enriched environment, and demonstrates the therapeutic power of environment to influence behavioral outcomes following early-life seizures.

The hippocampus is especially susceptible to damage due to stress (Bremner et al., 2000; Fenoglio et al., 2006; Sapolsky, 2002) and to seizures (Nadler et al., 1981). CA1 and CA3 pyramidal neurons are two highly sensitive neuronal populations to seizure-induced damage, sustaining injury prior to other brain sites (Dragunow et al., 1995; Sperk, 1994). In our study, stressors in the form of maternal deprivation and subsequent isolation increased susceptibility of animals to seizure-induced microglial activation and hippocampal neuronal death. Stress and exposure to glucocorticoids early in life have been associated with hippocampal atrophy and impairments in learning and memory (Huot et al., 2002). The deprived environment could potentially subject the developing animals to non-physical chronic stress, resulting in a hyperactivated HPA-axis, elevated glucocorticoid secretion, heightened immune cell response, and an increased proinflammatory response to acute seizures in young adults, as has been reported in response to chronic unpredictable stress (de Pablo et al., 2006) and chronic high stress levels of corticosterone (Dinkel et al., 2003; Sorrells and Sapolsky, 2007).

The influence of rearing environment appears to be mediated through tissue-specific effects on gene expression (Fischer et al., 2007). Genes involved in synaptic plasticity and memory consolidation such as *Arc*, *Homer1a*, and *Egr1* as well as serotonin receptors (5HTR) (Koh et al., 2005; Koh et al., 2007) were decreased after early-life seizures, but increased after rearing in an enriched environment. Further supporting the gene-by-environment interaction, a recent epidemiological study showed that a polymorphism in the serotonin transporter gene (5HTT) modulates the relationship between childhood maltreatment and adulthood depression. Individuals with a less efficient (short) allele were two times more likely than those with the long allele to develop major depression when faced with similar traumatic life events (Caspi et al., 2003).

Placement of animals in an enriched environment following seizure-induction has previously been shown to enhance neurogenesis in the hippocampal granule cell layer (GCL), as measured by a greater number of BrdU-labeled neurons in animals that experienced enriched versus control environments (Faverjon et al., 2002; Koh et al., 2005; Young et al., 1999). However, post-SE enrichment did not reverse seizure-induced cell loss or synaptic reorganization (Faverjon et al., 2002; Rutten et al., 2002). In contrast, environmental enrichment before KA-seizures has been shown to promote survival of dentate granule cells, decrease spontaneous apoptosis, stimulate adult neurogenesis and increase GCL volume, supported by increased subgranule cell expression (Young et al., 1999). Young et al. report that enriched rats that experienced SE showed significantly less cell death in the CA3 region than controls, suggesting that environmental enrichment before brain insult can produce resistance to excitotoxic injury. In the current study, differences in both maternal care and environment that occur *prior* to seizure induction also have a significant histological effect. Seizure-induced cell death and microglial activation in the hippocampus were reduced in animals reared with good maternal care in an enriched environment compared to deprived littermates. These results demonstrate that a more complex environment during neonatal life may induce changes that facilitate neuronal survival and recovery during postnatal life, and may even provide a buffer against neuronal injury – thus providing a scientific basis to support environmental enrichment therapies.

Children with epilepsy are often feared, shunned and isolated (Sillanpaa et al., 1998). Isolation alone is known to diminish spatial learning, suppress hippocampal neurogenesis, increase

anxiety-like behavior and render the brain hypersensitive to corticosterone while blunting the stress response in adulthood (Huot et al., 2002; Huot et al., 2001; Mirescu et al., 2004). Recovery from seizures can be hampered by lack of social support, such that the additive effects of seizure-induced brain injury and social isolation may result in a compounding vicious cycle. Despite an increasing awareness of the social and neuropsychiatric problems associated with epilepsy, therapeutic intervention specifically addressing these issues is extremely rare. Many anticonvulsants commonly used in children with epilepsy are known to slow mental processes and even impede neurogenesis (Farwell et al., 1990; Mikati et al., 1994). Hippocrates, the father of medicine, offered another view of therapy for epilepsy – that epilepsy was just another natural disease and could be treated through natural methods. Our finding of the environmental influence on the detrimental effects of prolonged seizures in early-life has important implications regarding therapy for childhood epilepsy. Intense educational and social intervention may be viewed as a therapeutic modality that could compensate for the vulnerability of children with epilepsy and afford an improved long-term psychosocial outcome.

4. Experimental Procedure

4.1. Animals

A total of 10 Long Evans timed pregnant mother rats (E14) were used. Each rat was randomly assigned either to a standard vivarium cage (24cm × 45cm × 31cm) or to an enriched environment consisting of a larger cage made of two clear plastic boxes (40cm × 58cm × 38cm) connected by two clear plastic tunnels and equipped with a running wheel and various movable objects, such as wooden chew toys, balls, bells and a plastic igloo. The enlarged space and objects allowed for complex social interaction and opportunities for voluntary physical exercise.

The day of birth was designated postnatal day 0 (P0). On P2, pups from all litters were combined together in one cage and randomly redistributed (cross-fostered) into litters of equal-sex ratio, culled to 10 pups per litter, and assigned to one of two rearing conditions: maternal deprivation (Deprived) or environmental enrichment (Enriched) (Fig. 1). For 3 hours daily from P2 to P14, Deprived pups were placed out of sight of the dam in a bedding-lined cage in an incubator held constant at 34–36 °C to maintain body warmth. At the end of the separation period, pups were returned to the nest and reunited with the dam. Maternal deprivations were carried out between 12PM–3PM each day. Pups in the Enriched group were left undisturbed in the enriched environment. Regular cage cleaning was suspended in all cages to avoid disturbing the nests until P14.

From P3–9, dams and pups from both environmental conditions were observed 2 hours per day, prior to maternal deprivation. Dams were observed during the light phase, from 10AM–12PM consistently. For each session, the behavior of each dam was scored every 2 minutes and the amount of time the dam spent with the pups and maternal behaviors such as sleeping, licking, and arched back nursing were quantified. Four variables were measured to determine the quality of maternal care: (1) time spent with pups and (2) arched-back nursing were used to measure maternal behavior, while (3) eye opening at P15 and (4) the weight of pups at the time of weaning were indicators of the effects of maternal care on the offspring.

On P21, pups in Group A (Fig. 1) were weaned from their dams and prolonged seizures were induced by injection of KA, while control littermates received phosphate buffered saline (PBS). The animals were also weighed on 21, one week following the last day of maternal deprivation, in order to eliminate the possibility of growth retardation being the result of food deprivation or hypothermia. Three hours after injection, the animals previously in the maternal separation rearing condition were placed singly in a standard vivarium cage (Fig. 1, isolated), while the

rats previously in the enriched environment were returned to the same cage (Fig. 1, enriched). To study the influence of differential rearing on exploratory behavior in early-life after seizures, Group A rats remained in these housing conditions until they were tested in the open field test on P28. Since seizure-induced DNA fragmentation is inconsistent and variable at P21 and undetectable 7–10 days after KA at P21 (Koh et al., 2005), environmental influence on seizure-induced neuronal injury and microglial activation was investigated after KA injection later in life. All rats in Group B (Fig. 1) were allowed to mature until P35, at which time seizures were induced. On P37, rats in Group B were perfused and their brains were harvested. Fig. 1 summarizes the experimental design.

4.2. KA induced seizures

Seizures were induced by intraperitoneal injection of KA (10mg/kg); control littermates received PBS. Animals were observed over a 3-hour period. Seizure severity and latency to the first sign of seizure were recorded. A seizure severity grade was assigned based on the maximal response achieved on a scale from 0 to V as follows: 0 - no response; I - wet dog shake (WDS) and/or behavioral arrest; II - WDS, staring, pawing, and clonic jerks; III - WDS, staring, pawing, clonic jerks, rearing and falling; IV - continuous grade III seizures for longer than 30 minutes (status epilepticus (SE)); V - death. Animals with clonic jerks (grade II) or prolonged clonic seizures (grade III or IV) were included in the portion of the study regarding effect of maternal care and enrichment on early life seizure-induced changes in exploratory behavior in a novel environment. Only rats with grade IV seizures were selected to quantify the effects of differential rearing on neuronal injury and inflammation in the hippocampus.

4.3. Open field test

Explorative behavior in an open field, an accepted measure of fear and emotional reactivity, was quantified to determine the effect of differential rearing environments on seizure-induced behavioral deficits. On P28, seven days after KA or saline injection, rats were tested in an open field. The arena consisted of a 152.5cm × 152.5 cm square, made up of twenty-five 30.5cm × 30.5cm connected individual vinyl tiles marked off with lines and housed in a semi-secluded, brightly lit room. Each individual animal was placed in the center square of the arena at the start of the trial and was allowed to explore undisturbed for five minutes. The total number of lines crossed was counted and their path traced on a paper grid by hand.

4.4. Quantification of cell death and microglial activation

Only rats that experienced grade IV seizures were chosen for immunohistochemistry (IHC) in order to standardize results. From 11 animals that experienced grade IV seizures, 7 (3 Deprived, 4 Enriched) were selected for statistical analysis. P37 rats were deeply anesthetized and perfused transcardially with PBS, then fixed with cold 4% paraformaldehyde/0.1M sodium phosphate buffer. Brains were harvested and 50 µm horizontal sections were cut on a freezing microtome. The tissue was processed as previously described (Koh et al., 1999) for *in situ* end labeling (ISEL) nick translation to detect DNA fragmentation, a robust positive marker of cell injury. Adjacent brain sections were processed for IHC using Allograft Inflammatory Factor-1 (Aif-1, Wako Chemicals, Richmond, VA, USA) to visualize activated microglia. A total of 84 hippocampal sections (12 hippocampi per brain × 7) were quantified. Images were captured digitally at 20× magnification, converted to gray scale, and areas of positively stained immunoreactive cells directly adjacent to the fimbria were highlighted at a threshold held constant for all specimens and quantified using an image analysis system, MetaMorph (v. 6.1, Universal Imaging Corp., Downingtown, PA), as previously described (Koh et al., 2005). An average percent area above the threshold was calculated per animal from both right and left hippocampi. Consistency in location was maintained by using three horizontal sections located at ~6.6, 6.8 and 7.1 mm ventral to the bregma. The anterior commissure was used as a specific

landmark to match sections across experiments. A comparison was then made between animals from different rearing environments.

4.5. Statistics

One-way analysis of variance (ANOVA) with a post hoc t-test and Tukey corrections (GraphPad Prism v. 4.0, GraphPad Software Inc., San Diego, CA) was used to compare differences in maternal behavior (time spent with pups and arched back nursing), quality of maternal care (weight on P21) and exploratory behavior among different experimental groups. The Fisher exact test was used to compare the differences in proportion of animals with eyes open at P15. Mann-Whitney *U*-test was employed to compare seizure severity between experimental groups, and Student's *t*-test was used to compare the latency to grade II seizure, fractional area of DNA fragmentation and microglial activation. Values are expressed as mean $\pm$ standard error of the mean (SEM). Significance was defined as $p < 0.05$ for all tests.

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Fig. 1. Experimental Design

E14 timed pregnant dams were placed either in a standard cage or enriched environment. On P2, the pups were culled and assigned to one of two rearing conditions: maternal separation (Deprived) or maternal care (Enriched). From P2–14, Deprived pups were separated from their dams for 3 hours daily while Enriched pups were left undisturbed. **A.** On P21, status epilepticus was induced by injection of KA in 6 pups in each litter, while control littermates received phosphate buffered saline (PBS). Three hours after injection, the animals previously in the maternal separation rearing condition were placed singly in standard vivarium cages (isolated), while the rats previously under the maternal care in the enriched environment were returned to the same cage (enriched). Rats remained in these housing conditions until they were tested in the open field test on P28. **B.** A separate group of rats were allowed to mature and all rats were injected with KA on P35 and perfused on P37.

Fig. 2. Differential maternal care and weight gain in pups

A. During the 2 hour observation period from P3–P9, dams in the Enriched group spent significantly more time with their pups than dams in the Deprived group (total $n=7$ litters, $p < 0.05$). **B.** Time spent arched-back nursing was also significantly different between Deprived versus Enriched group (total $n=7$ litters, $p < 0.02$). **C.** On the day of weaning (p21), pups reared in the enriched environment weighed significantly more than pups in the Deprived group ($n=51$, 47.4 ± 0.8 g vs. 50.4 ± 1.0 g; $p < 0.02$).

Fig. 3. Exploratory behavior in a novel environment: Effects of KA seizures and differential rearing conditions

A. Path of exploration taken by a maternal deprivation-isolated rat during the 5-minute trial was severely limited one week after KA-induced seizures (Deprived-KA). **B.** A rat in the maternal care-enriched environment that experienced seizures on P21 explored the open field similarly to control littermates (Enriched-KA). **C.** Rats in the Deprived group exhibited impaired exploratory behavior compared to all other groups ($n=51$, One-way ANOVA: $p<0.0005$) indicating the unique vulnerability of this group to seizure-induced behavioral deficits. Neither the Enriched group that experienced seizures (Enriched-KA) nor the Deprived group injected with PBS (Deprived-C) showed impairment in exploration of a novel environment.

Fig. 4. Severity and latency to seizure at P21, P35

A & B. No significant difference was observed in severity of seizure ($n=51$, Mann-Whitney U : $p>0.5$) or in latency to seizure at P21 ($n=33$, $p>0.8$) between Enriched and Deprived groups.

C & D. There was also no difference found in seizure severity ($n=72$, Mann-Whitney U : $p>0.42$) or latency to seizure ($n=49$, $p>0.26$) in animals at P35.

Fig. 5. KA-seizure induced DNA fragmentation and microglia activation in the hippocampus
A & B. ISEL of fragmented DNA in the CA3 region of the hippocampus. Marked hippocampal neuronal injury is observed in Deprived animals (A) 48 hours after KA-induced seizure at P35, while minimal cell death occurred in Enriched animals (B). **C.** Quantification of DNA fragmentation shows significantly increased cell death within CA3 of the hippocampus in Deprived compared to Enriched rats ($n=7$, 4.3 ± 1.2 vs. 1.0 ± 0.3 , $p < 0.03$). **D & E.** Aif-1 immunohistochemistry in the CA3 region of the hippocampus. There are numerous large, activated microglia diffusely distributed in the Deprived group (D) after KA-induced seizure at P35, whereas activated microglia are less abundant in the Enriched group (E). **F.** Quantification of activated microglia in CA3 shows significantly greater neuroinflammation in Deprived rats compared to Enriched animals after KA-induced seizures. ($n=7$, 13.5 ± 0.7 vs. 9.7 ± 0.2 , $p < 0.005$).