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Enhanced microglial activation and proinflammatory cytokine upregulation are linked to increased susceptibility to seizures and neurologic injury in a ‘two-hit’ seizure model

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Abstract

Early-life seizures result in increased susceptibility to seizures and greater neurologic injury with a second insult in adulthood. The mechanisms which link seizures in early-life to increased susceptibility to neurologic injury following a ‘second hit’ are not known. We examined the contribution of microglial activation and increased proinflammatory cytokine production to the subsequent increase in susceptibility to neurologic injury using a kainic acid (KA)-induced, established ‘two-hit’ seizure model in rats. Postnatal day (P)15 rats were administered intraperitoneal KA (early-life seizures) or saline, followed on P45 with either a ‘second hit’ of KA, a first exposure to KA (adult seizures), or saline. We measured the levels of proinflammatory cytokines (IL-1 β , TNF- α , and S100B), the chemokine CCL2, microglial activation, seizure susceptibility and neuronal outcomes in adult rats 12 hours and 10 days after the second hit on P45. The ‘two-hit’ group exposed to KA on both P15 and P45 had higher levels of cytokines, greater microglial activation, and increased susceptibility to seizures and neurologic injury compared to the adult seizures group. Treatment after early-life seizures with Minoxidil, a small molecule experimental therapeutic that targets up-regulated proinflammatory cytokine production, attenuated the enhanced microglial and cytokine responses, the increased susceptibility to seizures, and the greater neuronal injury in the ‘two-hit’ group. These results implicate microglial activation as one mechanism by which early-life seizures contribute to increased vulnerability to neurologic insults in adulthood, and indicate the potential longer term benefits of early-life intervention with therapies that target up-regulation of proinflammatory cytokines.

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Keywords

microglia; cytokines; epilepsy; therapeutic; chemokine

1. Introduction

The immature brain is particularly vulnerable to seizures, which may produce permanent changes in synaptic function (Brooks-Kayal, 2005; Cornejo et al., 2007; Sayin et al., 2004; Scantlebury et al., 2007; Zhang et al., 2004). Early-life seizures increase susceptibility to ischemic injury, seizures, and neurologic injury associated with later-life seizures (Dube et al., 2000; Giorgi et al., 2005; Koh et al., 1999; Somera-Molina et al., 2007). A number of lines of evidence implicate inflammation (Galic et al., 2008) and proinflammatory cytokines (Balosso et al., 2008) in the mechanisms by which seizures in the developing brain lead to increased vulnerability to seizures and exacerbation of neurologic injury after a 'second-hit' in adulthood (Haut et al., 2004). Elucidating these mechanisms and identifying therapeutic interventions in childhood may help prevent the long-term neurologic sequelae of such insults, including epilepsy (Lewis, 2005).

Neuroinflammation is the term used to describe the central nervous system (CNS) inflammatory responses produced by activated glia. Glial activation (astrocytes and microglia) occurs as protective or pathologic responses to CNS insults (Perry et al., 2007). Dysregulation of the neurosupportive role of glia can potentially contribute to neurologic disease progression, through an increased production of proinflammatory cytokines such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and S100B (Akiyama et al., 2000; Van Eldik and Wainwright, 2003; Wyss-Coray, 2006).

A recognized response to seizures (Rizzi et al., 2003) and a potential contributor to mechanisms of epileptogenesis is excessive or prolonged glial activation and the associated increase in proinflammatory cytokine production including IL-1 β and TNF- α (Galic et al., 2008; Ravizza et al., 2005; Vezzani and Baram, 2007). Indeed, inflammation in the immature brain leads to long-term increase in seizure susceptibility (Galic et al., 2008).

We previously showed that kainic acid (KA)-induced early-life seizures on postnatal day (P) 15 in the rat produce a rise in proinflammatory cytokines, transient microglial and sustained astrocyte activation, and impaired hippocampal-dependent behavior (Somera-Molina et al., 2007). Treatment with the experimental therapeutic Minoxidil (Mzc) (Hu et al., 2007; Lloyd et al., 2008) after KA-induced seizures on P15 attenuated the acute upregulation of proinflammatory cytokines, long-term astrocyte activation, and subsequent neurobehavioral impairment. However, the cytokine and microglial responses to the second hit and contribution of these responses to the enhanced neurologic injury with the second hit are not known. Therefore, we tested the hypothesis that altering microglial activation and proinflammatory cytokine upregulation induced by early-life seizures can contribute to improved outcomes after a second hit in adulthood. We report here that that suppression of the initial increase in proinflammatory cytokines by treatment with Mzc after early-life seizures prevents the resulting enhanced susceptibility to seizures and greater neuronal injury in adulthood and that an enhanced cytokine and microglial response to the second hit contributes to this susceptibility.

2. Results

2.1. 'Two-hit' seizure model using kainic acid

We used an established (Dube et al., 2000; Giorgi et al., 2005; Koh et al., 1999; Somera-Molina et al., 2007; Weiss et al., 1996) 'two-hit' model of KA-induced early-life seizures in P15 rats combined with a second exposure to KA in adulthood (P45). The experimental paradigm is shown in Fig. 1. Experimental groups (summarized in Table 1) in the 'two-hit' model were: (i) control group (SSS) administered saline on P15, saline 3h and 9h later, and saline on P45; (ii) adult seizure control group (SSK) administered saline on P15, saline 3h and 9h later, and KA on P45; (iii) 'two-hit' control group (KSK) administered KA on P15, saline instead of drug 3h and 9h later, and KA on P45; and (iv) Mzc-treated 'two-hit' group (KMK) administered KA on P15, Mzc (5 mg/kg) at 3h and 9h later, and KA on P45.

The percent survival in the adult seizures (SSK), 'two-hit' (KSK), and 'two-hit' group treated with Mzc (KMK) was significantly reduced compared to the SSS control group (SSK, $p < 0.01$; KSK, $p < 0.05$; KMK, $p < 0.01$ vs. SSS by Log rank test). There were no differences in survival among the three KA-exposed groups. All 9 of the control SSS animals survived, compared to 50% (12/24) in the adult seizures SSK group and 61% (19/31) in the 'two-hit' KSK group. Among the Mzc-treated 'two-hit' KMK group, 58% survived (18/31). There were no differences in weights among groups on P15 or at the time of sacrifice on P45 or P55.

2.2. Acute upregulation of proinflammatory cytokines is enhanced in the 'two-hit' group, and this response is prevented by suppression of cytokine elevation with Mzc after early-life seizures

To determine if early-life seizures altered the proinflammatory cytokine response to a subsequent insult in adulthood, we injected animals with KA on P15, then followed that by a second KA insult ('two-hit') on P45. To determine if suppression of the acute cytokine surge after early-life seizures would affect the proinflammatory cytokine response to a second hit in adulthood, we treated animals with Mzc after seizures on P15 (5 mg/kg at 3h and 9h after KA). Levels of the proinflammatory cytokines IL-1 β , TNF- α , and S100B were measured in homogenates of hippocampal samples taken from animals 12 h after the second insult (Fig. 2).

IL-1 β levels (Fig. 2A) in the 'two-hit' animals (KSK) were significantly higher compared to controls (SSS) or the adult seizures (SSK) group. Mzc treatment after the early-life seizures prevented the enhanced levels of IL-1 β seen in saline-treated 'two-hit' animals (KMK vs KSK). The IL-1 β levels in the Mzc-treated 'two-hit' group (KMK) were not significantly different from the adult seizures (SSK) group.

Results for TNF- α (Fig. 2B) were similar. Animals subjected to a second hit of KA showed a greater increase in TNF- α levels (KSK) compared to control (SSS) and adult seizure (SSK) groups. Treatment with Mzc after early-life (P15) seizures prevented the enhanced increase in TNF- α levels after the second hit on P45 (KMK vs KSK).

The astrocyte-derived cytokine S100B is released by activated astrocytes and plays a pivotal role in the mechanisms of glial activation (Van Eldik and Wainwright, 2003). S100B levels (Fig. 2C) in 'two-hit' animals (KSK) were significantly increased compared both to control (SSS) and adult seizures groups (SSK). Again, suppression of proinflammatory cytokines by Mzc treatment after seizures on P15 (KMK) prevented this enhanced response. While S100B is released by activated astrocytes, there was no increase in S100B in the adult seizure group compared to controls.

2.3. Microglial activation is enhanced following a ‘second hit’ and treatment with Mzc on P15 suppresses this increase

To determine whether the enhanced proinflammatory cytokine response observed in the ‘two-hit’ animals resulted in greater microglial activation, we used immunohistochemical methods to assess microglial responses. We quantified microglial activation in the hippocampus at two time points, 12 h (P45) and 10 d (P55), following KA-induced seizures at P45 by measuring Iba1- and clusterin-immunoreactive cells (Fig. 3).

After a 12 h recovery from the KA treatment on P45, Iba1-immunoreactive microglia in the ‘two-hit’ group (KSK) were increased compared to the control (SSS) and adult seizure (SSK) groups (Fig. 3A). Mzc treatment after early-life seizures (KMK) resulted in significantly fewer Iba1-immunoreactive cells compared to the ‘two-hit’ group (KSK), with values that were similar to the adult seizures group. A similar pattern was seen after a 10 d recovery (P55) from the KA treatment (Fig. 3B, E–H). Iba1 immunostaining in the adult seizures group (SSK) was increased compared to controls, and enhanced even further in the ‘two-hit’ group. Treatment with Mzc on P15 prevented the enhanced microglial activation in the ‘two-hit’ animals.

Similar changes were seen with another microglial marker, clusterin. The pattern of clusterin changes (Fig. 3C, D, I–L) in the ‘two-hit’ model and the prevention of the enhanced response in the Mzc-treated ‘two-hit’ group were similar to that observed for Iba1 immunoreactivity. For example, at 12 h following KA-induced seizures at P45, clusterin immunoreactivity in the hippocampus was significantly increased in adult seizures (SSK) compared to controls (SSS), the % clusterin-positive cells in the ‘two-hit’ group (KSK) was further increased, and Mzc treatment (KMK) suppressed the enhanced clusterin levels back to those comparable to the adult seizures group (Fig. 3C).

At P55 (Fig. 3D, I–L), we found a similar pattern of changes in clusterin immunoreactivity to that seen at P45. Clusterin immunoreactive cells remained significantly increased after 10 days recovery from KA insult (P55) and, similar to the response observed at 12 h recovery, the increase in clusterin in the ‘two-hit’ group was greater compared to the adult seizure group. Again, Mzc treatment on P15 suppressed the enhanced increase in clusterin found in the ‘two-hit’ animals.

2.4 The chemokine CCL2 is increased following KA-induced seizures, and this response is enhanced in the ‘two-hit’ animals

Chemokines serve to recruit activated microglia in the CNS (Streit et al., 2005). To determine whether the increase in microglial activation in the ‘two-hit’ group was associated with increased chemokine signaling, we measured changes in the chemokine CCL2 (Ghoshal et al., 2007), in hippocampal homogenates (Fig. 4) after seizures on P45 (data expressed as % control $\pm$ SEM). CCL2 levels were significantly increased in the ‘two-hit’ group (KSK) compared to controls (SSS), the adult seizures group (SSK), and the Mzc-treated group (KMK). Again, treatment with Mzc on P15 prevented the enhanced response in the ‘two-hit’ animals.

2.5. Mzc treatment after early-life seizures prevents the increased neuronal injury, susceptibility to seizures, and neurobehavioral impairment induced by a second hit in adulthood

Neuronal injury produced by a second hit of KA-induced seizures in adulthood is increased in animals that have previously experienced early-life seizures (Koh et al., 1999; Somera-Molina et al., 2007; Weiss et al., 1996). We next determined whether suppression of the acute proinflammatory cytokine increase after early-life seizures would prevent the increase in susceptibility to neurologic injury produced by the second hit. We treated animals with Mzc after early-life seizures and measured neuronal injury after the second hit with KA on P45 (Fig.

5). Injured neurons in all hippocampal subfields were detected using the fluorescent dye FJB both at 12 h (data not shown) and 10 days recovery after KA injection on P45 (example shown in Fig. 5A). No neuronal injury was present in controls (SSS) at either time point.

Consistent with previous studies (Koh et al., 1999; Somera-Molina et al., 2007; Weiss et al., 1996), injury in the 'two-hit' (KSK) group was increased compared to the adult seizures group (SSK), and Mzc treatment on P15 prevented this enhanced neuronal injury to a second hit in adulthood (Fig. 5B). Specifically, neuronal injury was significantly increased in the adult seizures group compared to the control group (SSK vs SSS, $p < 0.01$), and neuronal injury in the 'two-hit' group was increased compared to controls at both time points (P45 KSK, $p < 0.01$ vs. P45 SSS; P55 KSK, $p < 0.001$ vs. P55 SSS). Neuronal injury in the Mzc-treated group (KMK) was reduced significantly compared to the 'two-hit' animals both at 12 h ($p < 0.05$ vs. KSK) and 10 day recovery ($p < 0.001$ vs. KSK).

There were no significant differences in susceptibility to injury among hippocampal subfields. The majority of FJB-positive cells in the KA-exposed groups were present in the CA1, CA2, and CA3 subfields of the hippocampus, DG, piriform, and entorhinal cortex. In the P45 animals, the majority of FJB-positive cells were found in the DG and CA3 of the hippocampus as well as the cortex. In P55 animals, the CA1, CA2, and DG also showed significant increases in labeled cells compared to controls. FJB-positive cells were also observed in the entorhinal and frontal cortex, lateral septal nuclei, piriform cortex, and perirhinal cortex, as described previously (Somera-Molina et al., 2007).

As a second measure of neuronal injury, we selected representative P55 hippocampal sections for each experimental group (SSS, SSK, KSK, and KMK; $n = 4$ per group) and performed immunostaining for NeuN (Fig. 5C). Qualitative assessment of neuronal loss by reduced NeuN immunoreactivity was consistent with the results using FJB staining. Neuronal loss was more prominent in the 'two-hit' group compared to adult seizures group, and the neuronal loss was prevented by Mzc treatment after early-life seizures.

To determine whether the acute microglial activation and cytokine upregulation response after early-life seizures contribute to the increased susceptibility to seizures with a second hit in adulthood, we treated rats with Mzc after exposure to KA on P15 and quantified seizure severity and latency after the second hit of KA on P45. We found that seizure severity in the 'two-hit' group was attenuated by Mzc administration at P15 (Fig. 5D). Specifically, in the adult seizures (SSK) group, 28 animals exhibited grade IV seizures, identical to the results in the 'two-hit' KSK group. However, significantly fewer animals (20/36, 55.6%, chi-square $p < 0.0001$) exhibited grade IV seizures in the Mzc-treated 'two-hit' KMK group compared to the 'two-hit' KSK group, despite receiving Mzc 30 days prior to the second administration of KA.

To quantify further the change in susceptibility to seizures, we measured latency to onset of first seizure (defined as onset of forelimb clonus) after KA administration on P45. Latency was significantly reduced in the 'two-hit' KSK group compared to the adult seizures SSK group (Fig. 5E). In contrast, latency times in the Mzc-treated KMK group were significantly longer than those of the 'two hit' KSK group and were not different from the adult seizures (SSK) group (Fig. 5E).

Our previous data (Somera-Molina et al., 2007) showed that suppression of the acute proinflammatory cytokine increase with Mzc treatment following early-life seizures on P15 prevented neurobehavioral impairment. To determine whether Mzc treatment also modulated the behavioral sequelae of a second hit on P45, we measured Y-maze performance (Fig. 5F) from P46 until P55. We found that animals in the adult seizures group (SSK) and the 'two-hit' group (KSK) were impaired compared to controls, but we could not detect with these methods any difference between the adult seizures (SSK) and two hit groups (KSK). However, animals

treated with Mzc on P15 (KMK) showed improved performance on the Y-maze compared to both the SSK and KSK groups and were similar to controls.

3. Discussion

There are two major new findings of this study. First, in response to a second KA ‘hit’ in adulthood, there is an enhancement of both the upregulation of proinflammatory cytokines, microglial activation, and expression of the chemokine CCL2 in adult animals who had previously experienced early-life seizures. Consistent with the exaggerated proinflammatory cytokine and microglial activation responses after the second hit, these animals also show greater susceptibility to seizures and greater neuronal injury. Second, administration of Mzc to suppress of the upregulation of proinflammatory cytokines produced by early-life seizures prevents the exaggerated cytokine and microglial responses to the second KA hit in adulthood. Importantly, regulating the cytokine response to early-life seizures also prevents the enhanced neuronal injury, behavioral impairment, and increased susceptibility to seizures associated with the second KA insult. These results implicate microglial activation in the mechanisms by which early-life seizures lead to increased susceptibility to seizures and enhanced neurologic injury with a second hit in adulthood.

Excessive microglial activation and the associated overproduction of proinflammatory cytokines have been suggested to play a pivotal role in mechanisms of epileptogenesis and post-seizure injury in both experimental animal models and human epilepsy (Galic et al., 2008; Ravizza et al., 2005; Ravizza et al., 2008; Rizzi et al., 2003; Vezzani and Granata, 2005; Vezzani and Baram, 2007). Consistent with this suggestion, the data reported here for KA-induced injury indicate that suppression of the acute proinflammatory cytokine surge induced by early-life injury can influence the outcomes caused by a second neurologic insult in adulthood. The cellular and molecular mechanisms that can bring about increased susceptibility to adulthood injury or its prevention by early-life therapeutic intervention are not known.

The increase in microglial responses in the ‘two-hit’ animals is consistent with the capacity of microglia to switch between anti- and proinflammatory phenotypes in response to a new insult (Perry et al., 2007). A protective function for microglia in neurodegenerative disease has been proposed (El_Khoury et al., 2007), but activated microglia may also directly promote neuronal injury (Knoch et al., 2008).

One possible explanation for the mechanisms involved in the results reported here is the idea that a primary insult can sensitize microglia to react with a potentiated proinflammatory phenotype to a subsequent stimulus (Block et al., 2007; Lee et al., 2002). Microglial ‘priming,’ manifest as an enhanced production of proinflammatory cytokines caused by a new neurologic insult during ongoing cerebral pathology, occurs in a mouse model of prion disease (Perry et al., 2003). The results reported here, including the effect of Mzc intervention after early-life injury on adulthood injury outcomes, are consistent with this concept in which the primary insult predisposes to a more robust microglial response to injury in adulthood. The lack of effect of Mzc treatment on the high mortality in two-hit group most likely reflects the high dose of KA used in this model as mortality is also high in the adult seizure group.

Human and animal data (Heida and Pittman, 2005; Ravizza et al., 2005; Ravizza et al., 2008) identify a pivotal role for IL-1 β and persistent brain inflammation triggered by status epilepticus in the mechanisms of epileptogenesis. Long-term increase in seizure susceptibility produced by inflammation in the immature brain may also be produced by TNF- α dependent mechanisms, independent of IL-1 β (Galic et al., 2008). Our data also support a model in which early-life seizures lead to long-term astrocyte activation as we have previously described

(Somera-Molina et al., 2007) and priming of microglia. As a result, a ‘second-hit’ in adulthood produces greater cytokine release, microglial activation and recruitment, leading to increased susceptibility to seizures, enhanced neuronal injury and neurobehavioral impairment. These data indicate a role for microglia in amplifying the neurologic sequelae of the ‘second hit’ (Block et al., 2007).

The chemokine CCL2 mediates neuroinflammation by activating microglia (Ghoshal et al., 2007) and is increased by excitotoxic injury (Galasso et al., 2000). The enhanced increase in CCL2 in the ‘two-hit’ animals and the suppression of upregulation in animals treated with Mzc suggest that the recruitment of microglia is also enhanced by early-life seizures.

Other mechanisms may also contribute to the long-term neurologic consequences of early life seizures. Seizures may alter neuronal function and hippocampal circuitry without causing neuronal injury, through loss of dendritic spines (Jiang et al., 1998; Swann et al., 2000). These mechanisms may involve either decrease or increase in actin polymerization (Kim and Lisman, 1999; Ouyang et al., 2005) through calcineurin-dependent regulation of the actin cytoskeleton (Zeng et al., 2007). In the immature brain, seizures also produce functional changes in glutamate receptors (Crino et al., 2002; Zhang et al., 2004), cyclic nucleotide-gated ion channels (Brewster et al., 2002), impaired induction of long-term potentiation and hippocampal dependent behaviors (Lynch et al., 2000) (Huang et al., 1999).

Our data support a role for activated glia responses in the mechanisms by which early-life seizures produce greater susceptibility to a second neurologic insult. The improved outcomes with Mzc administration in multiple acute or chronic injury models where proinflammatory cytokine upregulation contributes to neurologic injury (Hu et al., 2007; Somera-Molina et al., 2007; Karpus et al., 2008; Lloyd et al., 2008) suggest that disease-specific interventions may be more effective if combined with therapies that modulate glial responses. These results are additional evidence that glial activation may be a common pathophysiologic mechanism and therapeutic target in diverse forms of neurologic injury (Akiyama et al., 2000; Craft et al., 2005; Emsley et al., 2005; Hu et al., 2005; Perry et al., 2007). Therapies, which selectively target glial activation following acute brain injury in childhood, may serve to prevent neurologic disorders in adulthood. These findings raise the possibility that interventions after early-life seizures with therapies that modulate the acute microglial activation and proinflammatory cytokine response may reduce the long-term neurologic sequelae and increased vulnerability to seizures in adulthood.

4. Experimental Procedures

4.1. Animal use

A total of 126 P15 male Sprague-Dawley rats (Charles River Laboratories, Cambridge, MA) were randomly divided into experimental groups as summarized in Table 1. Values for the number of animals used prior to treatment are given in Table 1 and the ‘n’ for each outcome measure in the surviving animals given in the results. At P21, animals were weaned and randomly distributed to four animals per cage. Animals were monitored throughout the experiments and adequate measures taken to minimize pain and discomfort. The Institutional Animal Care and Use Committee of Children’s Memorial Research Center, Chicago, Illinois approved all procedures, which conformed to the guidelines established by the NIH Guide for the Care and Use of Laboratory Animals.

4.2. ‘Two-hit’ model of KA-induced seizures and administration of Mzc

In these studies we used an established (Dube et al., 2000; Giorgi et al., 2005; Koh et al., 1999) ‘two-hit’ model of KA-induced early-life seizures combined with a second exposure to

KA in adulthood. Briefly, P15 rats were administered by intraperitoneal (i.p.) injection either 5 mg/kg KA (Sigma-Aldrich, St. Louis, MO) or an equivalent volume of saline (Sal) vehicle as previously described (Somera-Molina et al., 2007). To determine whether suppression of the acute increase in proinflammatory cytokines after KA exposure on P15 would prevent the increase in susceptibility to neurological injury after a 'second-hit' with KA on P45, we used Mzc, a small molecule, selective inhibitor of proinflammatory cytokine production by activated glia (Hu et al., 2007; Somera-Molina et al., 2007). The dose of Mzc (5 mg/kg dissolved in Sal) and timing of administration (i.p. injection at 3 h and 9 h following KA injection on P15) were identical to that used in our previous study (Somera-Molina et al., 2007). Control animals were injected i.p. with an equal volume of Sal. Surviving animals were allowed to recover for 30 days to P45. On P45, rats were administered KA (15 mg/kg i.p.) as previously described for the 'two-hit' model of KA-induced neurological injury (Koh et al., 1999; Somera-Molina et al., 2007). Experimental groups (see Table 1) in the 'two-hit' paradigm were: (i) control group (SSS) administered saline on P15, saline 3h and 9h later, and saline on P45; (ii) adult seizure control group (SSK) administered saline on P15, saline 3h and 9h later, and KA on P45; (iii) 'two-hit' therapeutic intervention control group (KSK) administered KA on P15, saline instead of drug 3h and 9h later, and KA on P45; and (iv) Mzc-treated 'two-hit' group (KMK) administered KA on P15, Mzc (5 mg/kg) at 3h and 9h later, and KA on P45.

To determine the effects of early-life seizures on acute changes in proinflammatory cytokines, glial activation, and neuronal injury induced by KA on P45, some animals were sacrificed on P45, 12 h after the second administration of KA. To determine long-term changes in glial activation and neuronal injury, some animals were allowed to recover for an additional 10 days before sacrifice on P55.

4.3. Quantification of seizure latency and severity

Following administration of KA or Sal at P45, all animals were monitored continuously for three hours. Observers were blinded to animal treatment groups by assigning numeric labels to all animals. Seizure susceptibility was quantified by determining the latency to onset of first seizure (defined as onset of forelimb clonus) after KA administration (SSK, $n = 28$; KSK, $n = 28$; KMK, $n = 36$). Seizure severity was quantified using published criteria (Somera-Molina et al., 2007). The following grading scheme was followed: Grade 0, no response; Grade I, wet dog shakes and/or behavioral arrest; Grade II, wet dog shakes, pawing, clonic jerks; Grade III, wet dog shakes, staring, clonic jerks, rearing and falling; Grade IV, continuous Grade III seizures for more than 30 min. Only animals with seizure grade of IV at P15 received a second injection of KA at P45 to ensure an equal baseline of injury for all animals. This was necessary for accurate comparison of seizure latency and seizure severity among groups at P45.

4.4. Hippocampal-linked task behavioral testing

The Y-maze test of spontaneous alternation was used to evaluate hippocampal-dependent spatial learning after KA insult at P45, as previously described (Somera-Molina et al., 2007; Weiss et al., 1998). A blinded observer performed Y-maze testing on alternate days from P46 until P55 (SSS, $n = 9$; SSK, $n = 12$; KSK, $n = 14$; KMK, $n = 16$), and the percent alternation over the duration of testing was calculated for each animal.

4.5. Brain tissue preparation

Animals were anesthetized with 4% isoflurane and transcardially perfused with PBS (pH 7.4). Brains were excised, and left and right hemispheres were separated. The hippocampus was isolated from the right hemisphere and flash frozen in liquid nitrogen. The left hemisphere was fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4 overnight at 4°C, paraffin embedded, and sectioned for immunohistochemistry.

4.6. Measurement of proinflammatory cytokines and the chemokine CCL2

Frozen hippocampus was thawed, homogenized in a buffer containing protease inhibitor cocktail, and centrifuged as described (Somera-Molina et al., 2007). Supernatant was collected and total protein concentration was measured using commercially available reagents (BCA, Pierce, Rockford, IL). Levels of IL-1 β , TNF- α and CCL2 ($n = 4$ –11 per group) were measured in hippocampal supernatants by sandwich immunoassay methods using commercially available electrochemiluminescent detection system, plates, and reagents (Meso-Scale Discovery (MSD), Gaithersburg, Maryland). S100B levels were measured by ELISA as previously described (Somera-Molina et al., 2007). For each assay, samples were analyzed in duplicates and compared with known concentrations of protein standard. Plates were analyzed using the SECTOR Imager 2400 for MSD plates and Victor²TM (PerkinElmer, Waltham, MA) for S100B.

4.7. Immunocytochemistry for microglial activation and neuronal injury

Immunohistochemical detection of hippocampal markers of microglial activation (Iba1 and clusterin; $n = 5$ –15 per group) and neuronal number (NeuN, $n=4$ per group) was performed on 5 μ m paraffin-embedded axial sections using Vectastain Elite ABC immunodetection kits and diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame CA). The following primary antibodies were used: Iba1 (1:400, goat polyclonal, Abcam, Cambridge, MA); β -clusterin (1:100, goat polyclonal, Santa Cruz); and NeuN (1:50, mouse monoclonal, Chemicon). Control sections were incubated in normal serum or PBS in place of primary antibody. For clusterin, control peptides were used instead of the primary antibodies to determine specific immunoreactivity. Sections were incubated for one hour at 38°C with the appropriate biotinylated secondary antibody (Vector). Sections were incubated with ABC complex (Vector) and developed with DAB substrate (brown). Sections were counterstained with hematoxylin (blue) for contrast.

Immunofluorescent detection of neuronal injury was measured in 5 μ m paraffin-embedded axial sections using Fluoro-Jade B (FJB) (Chemicon International, Temecula, CA) as described (Schmued and Hopkins, 2000; Somera-Molina et al., 2007). The nuclear dye 4,6-diamidino-2-phenylindole (DAPI, Sigma) was used to counterstain and to identify cell nuclei. Two tissue sections representing the hippocampus from P45 12 h recovery animals (SSS, $n = 6$; SSK, $n = 6$; KSK, $n = 9$; KMK, $n = 9$) as well as P55 animals (SSS, $n = 6$; SSK, $n = 12$; KSK, $n = 9$; KMK, $n = 15$) were analyzed and quantified by blinded observers.

4.8. Image acquisition and quantification of immunoreactive cells

Sections were examined under brightfield microscopy (Nikon Eclipse E800) by two blinded observers. For Iba1 and clusterin, the CA1, CA2, CA3, dentate gyrus (DG), and polymorph dentate gyrus (PoDG) were photographed at 10x magnification, and images were converted to grayscale for quantification of immunoreactive cell density. Digitized images were analyzed using commercially available software (Metamorph, Universal Imaging Corporation, Sunnyvale, CA) as previously described (Somera-Molina et al. 2007). Immunoreactive cells were identified by thresholding for dark objects and the percentage of staining intensity in each hippocampal regions was quantified. The total hippocampal immunoreactivity as well as hippocampal region-specific immunoreactivity were obtained for each sample (Somera-Molina et al., 2007).

4.9. Statistical analysis

Values are expressed as mean $\pm$ SEM for each group. Test for normality was performed for each data set. For comparisons of three or more groups, One-way analysis of variance (ANOVA) was performed, followed by Tukey's Multiple Comparison Test. Two groups were

compared using Student's t-test. To determine statistical differences in seizure severity, chi-square analysis was performed. Kaplan-Meier Survival Analysis and log rank test were performed to analyze differences in mortality between groups. Significance was defined as $p < 0.05$. Prism 4.0 (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses.

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Abbreviations

Mzc	Minozac
KA	Kainic acid
FJB	FluoroJade-B
IL-1β	Interleukin-1 β
TNF-α	Tumor necrosis factor- α
C1	clusterin

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Figure 1. Schematic of experimental design

Timeline of kainic acid (KA) injection, Minozac (Mzc) treatment, and Y-maze testing are shown. Following intraperitoneal (i.p.) injection of KA or saline (Sal) on postnatal day (P)15, rats were injected i.p. with Mzc (5 mg/kg) or Sal at 3 h and 9 h. Animals were allowed to recover for 30 days to P45. On P45, animals were administered KA or Sal. Y-maze testing was performed on alternating days from P46–P55. Animals were sacrificed at 30 d (P45 12 h) and 40 d (P55). Experimental groups at P45 and P55 include control (SSS), adult seizures (SSK), 'two-hit' (KSK), and Mzc-treated 'two-hit' (KMK).

Figure 2. Enhanced upregulation of proinflammatory cytokines in the ‘two-hit’ group, and prevention of this response by treatment with Mzc after early-life seizures

Effect of KA-induced early-life seizures on levels of the proinflammatory cytokines IL-1 β (A), TNF- α (B), and S100B (C) in hippocampal homogenates after a second hit of KA on P45 and 12 h recovery. The cytokine levels were significantly increased in the ‘two-hit’ group (KSK) compared to controls (SSS) and adult seizures (SSK). Mzc treatment (KMK) on P15 prevented the enhanced increase in cytokine levels at P45. **, $p < 0.01$; ***, $p < 0.001$ vs. SSS.

Figure 3. Microglial activation is enhanced following a ‘second hit’ of seizures in adulthood, and this exaggerated response is prevented by administration of Mzc after early-life seizures

Quantification of microglial markers, Iba1 (A,B) and clusterin (C,D), in combined regions (CA1, CA2, CA3, DG, PoDG) of the hippocampus at 12 h (P45) and 10 d (P55) recovery following seizures at P45. (A and B) Increased numbers of Iba1-immunoreactive cells indicate increased microglial activation in adult seizures (SSK) and ‘two-hit’ (KSK) groups compared to controls (SSS) after exposure to KA on P45 at both time points. Prior treatment with Mzc on P15 (KMK) suppressed the enhanced increase in microglial activation in the ‘two-hit’ group at 12 h (A) and 10 d (B) recovery. (C and D) Quantification of clusterin-immunoreactive cells at the same time points. Clusterin-immunoreactive cells in controls, adult seizures, ‘two-hit’, and Mzc-treated groups show a pattern similar to Iba1 immunoreactivity. Mzc treatment suppressed the enhanced increase in clusterin immunoreactivity in the ‘two-hit’ group at 12 h (C) and 10 d (D). Representative photomicrographs of Iba1 (E–H) and clusterin (I–L) immunoreactive cells in the CA1 region of the hippocampus are shown. All photomicrographs are of P55 animals obtained at 10 d recovery after KA exposure on P45. Positively stained cells are brown. Sections were counterstained with hematoxylin (blue) for contrast. Scale bar, 100 μ m. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. SSS.

Figure 4. The chemokine CCL2 is upregulated following KA-induced seizures and this response is enhanced following a ‘second hit’ of seizures in adulthood

Levels of the chemokine, CCL2 were measured in hippocampal homogenates 12 hours after exposure to KA or Sal on P45. CCL2 levels were increased by KA (SSK), and this increase was greater in the ‘two-hit’ (KSK) group. Prior treatment with Mzc on P15 (KMK) prevented this enhanced response. **, $p < 0.01$ vs. SSS.

Figure 5. Mzc treatment after early-life seizures prevents the increased neuronal injury, susceptibility to seizures, and neurobehavioral impairment induced by a second hit in adulthood (A) Representative photomicrographs of injured neurons (green) identified by FluoroJade-B (FJB), and DAPI (blue) fluorescent staining in the CA1 subfield of the hippocampus in a ‘two-hit’ animal (KSK) on P55 show the extent of neuronal injury in the ‘two-hit’ group and attenuation of this injury by Mzc treatment (KMK) on P15. (B) Quantification of FJB-fluorescent cells in the hippocampus of P55 animals. Neuronal injury is increased in the ‘two-hit’ (KSK) group compared to adult seizures (SSK), and this enhanced injury is prevented in the two-hit group (KMK) treated with Mzc on P15. (C) Qualitative assessment of neuronal nuclei (NeuN) staining shows increased neuronal loss in adult seizures and ‘two-hit’ groups,

with greater neuronal loss evident in 'two-hit' animals. Minimal or no neuronal loss was observed in controls and Mzc-treated animals. All photomicrographs are of P55 animals obtained at 10 d recovery after KA exposure on P45. Scale bar, 100 μ m. (D) Reduced seizure severity in the Mzc-treated group (KMK) compared to the adult seizure (SSK) and the two-hit (KSK) groups. (E) Reduced seizure latency in the 'two-hit' (KSK) group after a second hit of KA on P45 compared to values for adult seizures (SSK). Prior treatment with Mzc on P15 in the 'two-hit' group (KMK) prevented this increased susceptibility, and was not different from the adult seizure group. (F) Hippocampal-dependent behavior measured by the Y-maze test from P46 to P55. Both the SSK and KSK groups showed behavioral impairment (reduced % alternation) compared to Sal-treated controls (SSS). Mzc treatment (KMK) after early-life seizures prevented this behavioral impairment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. SSS.

TABLE 1
Summary of Experimental Groups and Outcome Measures

Group (n)	P15 0 h	Treatments P15 3h & 9h	P45	Recovery from P45 Treatment	Age at Sacrifice	Behavior	Endpoints	
							ELISA	Immunocytochemistry
SSS (7)	Sal	Sal	Sal	12 h	P45	Latency;	Cytokines;	Iba1; Cl; NeuN; FJB
SSK (6)	Sal	Sal	KA	12 h	P45	Severity Latency;	CCL2 Cytokines;	Iba1; Cl; NeuN; FJB
KSK (9)	KA	Sal	KA	12 h	P45	Severity Latency;	CCL2 Cytokines;	Iba1; Cl; NeuN; FJB
KMK (9)	KA	Mzc	Sal	12 h	P45	Severity Latency;	CCL2 Cytokines	Iba1; Cl; NeuN; FJB
SSS (9)	Sal	Sal	Sal	10 d	P55	Severity; Y-maze Latency; Severity;	Iba1; Cl; NeuN; FJB	
SSK (24)	Sal	Sal	KA	10 d	P55	Severity; Y-maze Latency; Severity;	Iba1; Cl; NeuN; FJB	
KSK (31)	KA	Sal	KA	10 d	P55	Severity; Y-maze Latency; Severity;	Iba1; Cl; NeuN; FJB	
KMK (31)	KA	Mzc	KA	10 d	P55	Severity; Y-maze	Iba1; Cl; NeuN; FJB	
Total (126)								

P, postnatal day; Sal, Saline; KA, Kainic acid; Mzc, Minozac; Latency, time to seizure onset; Severity, seizure severity following KA injection; Cytokines (IL-1 β , TNF- α , and S100B); Cl, clusterin; FJB, Fluoro-Jade B; (n), sample size prior to treatment.