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Minocycline attenuates microglia activation and blocks the long-term epileptogenic effects of early-life seizures

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ABSTRACT

Innate immunity mediated by microglia appears to play a crucial role in initiating and propagating seizure-induced inflammatory responses. To address the role of activated microglia in the pathogenesis of childhood epilepsy, we first examined the time course of microglia activation following kainic acid-induced status epilepticus (KA-SE) in Cx3cr1$^{GFP/+}$ transgenic mice whose microglia are fluorescently labeled. We then determined whether this seizure-induced microglia activation primes the central immune response to overreact and to increase the susceptibility to a second seizure later in life. We used an inhibitor of microglia activation, minocycline, to block the seizure-induced inflammation to determine whether innate immunity plays a causal role in mediating the long-term epileptogenic effects of early-life seizure. First status epilepticus was induced at postnatal day (P) 25 and a second status at P39. KA-SE at P25 caused nearly a two-fold increase in microglia activation within 24 h. Significant seizure-induced activation persisted for 7 days and returned to baseline by 14 days. P39 animals with prior exposure to KA-SE not only responded with greater microglial activation in response to "second hit" of KA, but shorter latency to express seizures. Inhibition of seizure-induced inflammation by 7 day minocycline post-treatment abrogated both the exaggerated microglia activation and the increased susceptibility to the second seizure later in life. The priming effect of early-life seizures is accompanied by modified and rapidly reactivated microglia. Our results suggest that anti-inflammatory therapy after SE may be useful to block the epileptogenic process and mitigate the long-term damaging effects of early-life seizures.

Introduction

There is growing recognition that neuroinflammation mediates many aspects of disease for chronic neurodegenerative disorders as well as for acute brain injury, autistic spectrum disorder and epilepsy (Choi and Koh, 2008; Vargas et al., 2005; Vezzani et al., 1999). Cytokines, free radicals, and excitatory neurotransmitters released from activated microglia provide the commonality between neuroinflammatory processes in many neurological conditions including multiple sclerosis, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Balistreri et al., 2007; Conductier, 2010; McCoy and Tansey, 2008). Chronic microglial activation is an important component of these diseases and likely contributes to neuronal dysfunction, injury, loss, and hence to progression of neurological conditions. Dysregulated microglial activity may be responsible for detrimental rather than reparative central responses in various CNS diseases.

The role of brain inflammation in childhood epilepsy has long been implicated in pediatric infectious or autoimmune diseases that are often accompanied by recurrent seizures. In addition, pro- and anti-inflammatory molecules are synthesized during seizures in the brain at the sites of seizure initiation and propagation (Murashima et al., 2008). Extensive microglia activation occurs in the brain parenchyma of individuals with chronic intractable epilepsy and in animal models of seizures (Avignone et al., 2008; Beach et al., 1995; Choi et al., 2009; Drage et al., 2002; Fabene et al., 2010; Somera-Molina et al., 2009; Yang et al., 2010). Microglia, the resident macrophage in the brain parenchyma, constitute the first line of defense against pathological changes within the central nervous system (CNS) microenvironment. CNS injury triggers rapid activation of microglia with concomitant changes in distribution, morphology, immunophenotype, and metabolism. Swelling of the microglial cell body, a thickening of the proximal processes, and a reduction in the number and complexity of distal processes are key morphological signs of this activation (Buttini et al., 1996; Kloss et al., 2001). The extent to which activated microglia are involved in CNS homeostasis or diseases is a delicate balance. On

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the one hand, activation of the innate immune system controls infections, removes debris and promotes repair. On the other hand, these otherwise normal glial functions can sometimes result in a more severe and chronic neuroinflammatory cycle that actually promotes the shift from a physiological to a pathological inflammatory response.

We have previously developed a "two-hit" seizure model demonstrating that an early-life seizure permanently decreases seizure threshold and increases the susceptibility to seizure-induced cell death in adulthood (Koh et al., 1999; Somera-Molina et al., 2007). Seizures in immature animals cause subtle functional changes and alter the response to seizures later in life (Dube et al., 2000; Holmes et al., 1998; Koh et al., 2004; Sankar et al., 1998). To elucidate the role of activated microglia "primed" by early-life convulsions in later seizure susceptibility, we determined whether heightened seizure susceptibility is accompanied by exaggerated microglia activation in animals with prior exposure to early-life SE in our "two-hit" rodent seizure model. We hypothesize that the inflammatory reaction provoked by early-life seizures primes the developing brain so that microglia are modified, leading to rapid reactivation by a second seizure in adulthood. We first visualized and quantified the time course of microglia activation after kainic acid (KA)-induced status epilepticus (SE) in juvenile Cx3cr1$^{GFP/+}$ transgenic mice whose parenchymal microglia are fluorescently labeled. To test the functional significance of marked activation of microglia caused by an early-life prolonged seizure, we determined whether inhibition of inflammation by minocycline, a known inhibitor of microglia activation (Henry et al., 2008; Pabreja et al., 2011), abrogates the exaggerated microglia activation and thus susceptibility to second seizure later in life.

Materials and methods

Animals

We used Cx3cr1$^{GFP/+}$ transgenic mice in which the fractalkine chemokine receptor has been replaced by a green fluorescent protein (GFP) reporter gene by targeted deletion via homologous recombination in embryonic stem cells (Davalos et al., 2005; Jung et al., 2000). Mice were group housed in polypropylene cages and maintained at 21 °C with ad libitum access to water and rodent chow. All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Children's Memorial Research Center Institutional Animal Care and Use Committee.

Kainic acid-induced seizures

It has long been shown that systemic injection of KA in rodents induces prolonged seizures (Ben-Ari, 1985). These seizures originate in the CA3 region of the hippocampus and can spread to other limbic structures and can be followed by neuronal loss in selected regions of the brain reminiscent of brain damage seen in patients with temporal lobe epilepsy. Here, seizures were induced by intraperitoneal (*i.p.*) injection of KA as previously described (Hu et al., 1998). In brief, postnatal day (P) 25 and 39 mice received an *i.p.* injection of either sterile phosphate buffered saline (PBS) or KA (20 mg/kg). 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—behavioral arrest; II—staring, pawing, and head bobbing; III—clonic jerks, rearing and falling; IV—continuous grade III seizures for longer than 30 min (status epilepticus); V—death. Only mice with status epilepticus (Grade IV) were included in the study. Latency to Grade III was calculated and compared between groups; Grade 0–II seizures were excluded from latency calculation.

Experimental protocol

To study the time course of seizure-induced microglia activation, P25 Cx3cr1$^{GFP/+}$ mice were sacrificed 1, 7 or 14 days after administration of PBS or KA. To determine the effects of early-life seizures on seizure susceptibility and microglial activation in response to the later-life "second hit" KA, a separate group of mice were injected with PBS or KA on P25 and followed by an injection of KA on P39. On P40, 24 h after KA injections, mice were perfused transcardially.

To establish whether the tetracycline derivative, minocycline, inhibits acute seizure-induced microglia activation, P25 mice received an *i.p.* injection of PBS or minocycline (20 mg/kg) 3 h after KA-SE induction and for the following six consecutive days. Minocycline (Sigma, St. Louis, MO) was dissolved in sterile water and sonicated to ensure complete solubilization. On the 7th day, 24 hours (h) after the last dose, mice were sacrificed and area of green fluorescent microglia in the hippocampus was quantified and compared between PBS and minocycline treated animals.

To determine the efficacy of minocycline in blocking the long-term effect of early-life seizures on later-life seizure susceptibility and subsequent microglia activation, a separate study was conducted where P25 mice received a KA injection followed by PBS or minocycline treatment for 7 days as detailed above. Following an additional 7 days, P39 mice received an injection of KA and sacrificed 24 h later on P40.

Quantification of microglial cell activation

Mice were deeply anesthetized and perfused transcardially with PBS followed by ice-cold 4% performaldehyde/0.1 M sodium phosphate buffer. Brains were harvested, post-fixed with 4% performaldehyde/30% sucrose solution over night, and mounted on a freezing microtome. 40 μm horizontal sections were cut, and every 6th section collected and mounted on slides for microscopic examination. At least 6 hippocampal sections per brain from at least 4 animals ($n=4$–10) per group were selected for quantification. The anterior commissure was used as a specific landmark to match sections across experiments. Images were captured digitally at 20× magnification, converted to gray scale, and areas of positively labeled green fluorescent cells were highlighted within the hilus of the hippocampus for initial time course experiments (Fig. 1) to allow consistent comparison between controls and KA animals over time. For all subsequent experiments (Figs. 2–4), area directly adjacent to the fimbria (near CA3 hippocampal subregion), where microglia activation was maximal after KA-SE, was captured for quantification. Sum total spot count—defined as cell counts/0.27 mm^2 unit area reflecting density of microglia—was quantified manually by including brightly fluorescent microglia with visible processes and distinct soma of at least 5 μm diameter while excluding cells out of focus with indistinct borders. Sum total spot count was quantified within the hilus where microglia were uniformly dispersed (Figs. 1D, H and L). Because spot counts of individual cells within the aggregates in CA3 subregion could not be determined reliably, quantification of microglia activation was limited to % area fluorescence in Figs. 2C, 3D and 4D. To control for intra/inter observer reliability, the quantification threshold was held constant for all specimens within each experimental group and quantified using ImageJ (1.43u, Public Domain, NIH) by a single observer (A.B.) who was blinded to the treatment groups.

Statistical analysis

Student's *t*-test (GraphPad Prism v. 4.0, GraphPad Software Inc., San Diego, CA) was used to compare the latency to seizure onset and microglial activation between SS and SK. One-way analysis of variance (ANOVA) with a post-hoc *t*-test and Tukey corrections was used to compare differences in microglia activation and seizure