Experimental Neurology 290 (2017) 85–94

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research paper

Rapid neuroinflammatory response localized to injured neurons after diffuse traumatic brain injury in swine

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ARTICLE INFO

Article history:
Received 1 November 2016
Received in revised form 23 December 2016
Accepted 6 January 2017
Available online 9 January 2017

Keywords:
Neuroinflammation
Microglia reactivity
Diffuse traumatic brain injury
Permeabilized neurons
Concussion

ABSTRACT

Despite increasing appreciation of the critical role that neuroinflammatory pathways play in brain injury and neurodegeneration, little is known about acute microglial reactivity following diffuse traumatic brain injury (TBI) — the most common clinical presentation that includes all concussions. Therefore, we investigated acute microglial reactivity using a porcine model of closed-head rotational velocity/acceleration-induced TBI that closely mimics the biomechanical etiology of inertial TBI in humans. We observed rapid microglial reactivity within 15 min of both mild and severe TBI. Strikingly, microglial activation was restrained to regions proximal to individual injured neurons – as denoted by trauma-induced plasma membrane disruption – which served as epicenters of acute reactivity. Single-cell quantitative analysis showed that in areas free of traumatically permeabilized neurons, microglial density and morphology were similar between sham or following mild or severe TBI. However, microglia density increased and morphology shifted to become more reactive in proximity to injured neurons. Microglial reactivity around injured neurons was exacerbated following repetitive TBI, suggesting further amplification of acute neuroinflammatory responses. These results indicate that neuronal trauma rapidly activates microglia in a highly localized manner, and suggest that activated microglia may rapidly influence neuronal stability and/or pathophysiology after diffuse TBI.

1. Introduction

Traumatic brain injury (TBI) is a major health and socioeconomic problem, with over 1.7 million individuals afflicted each year and over 5 million exhibiting chronic neurological deficits in the U.S. alone (Faul et al., 2010; Hyder et al., 2007; Langlois et al., 2006). Even so-called "mild" TBI – otherwise known as concussion – may lead to cognitive disruptions immediately post-injury as well as persistent neurological deficits likely resulting from disruption of neuronal circuitry (De Kruijk et al., 2001; De Monte et al., 2006; Leininger et al., 1990; Wolf and Koch, 2016). However, it is not well understood how microglia, the inflammatory modulators of the CNS, contribute to acute neuronal health, homeostasis, and/or pathophysiology after TBI. Previously, murine CNS injury models indicated that microglia react to laser ablation within minutes of the initial insult (Davalos et al., 2005; Dibaj et al., 2010). It remains unclear if a similar phenomenon occurs following closed-head (i.e., non-penetrating) TBI generally resulting from falls, collisions and/or blunt impacts (Coronado et al., 2012; Faul et al., 2010; Langlois et al., 2006). Indeed, the vast majority of clinical TBIs are closed-head injuries caused by a sudden jolt or blow to the head, resulting in rapid rotational velocity/acceleration (inertial loading) and diffuse strain fields throughout the brain (Adams et al., 1989; Ommaya and Gennarelli, 1974; Povlishock, 1992; Smith and Meaney, 2000). These TBIs are difficult to model because there is no injury epicenter as is the case with predominantly focal TBIs – generally the dominant component of preclinical models using rodents – and the resulting

Abbreviations: TBI, traumatic brain injury; LY, Lucifer yellow; Iba1, ionized calcium-binding adapter molecule 1; GFAP, glial fibrillary acidic protein.

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http://dx.doi.org/10.1016/j.expneurol.2017.01.004
0014-4886/

neuropathology is often extremely subtle. Moreover, large brain mass and gyrencephalic architecture are key features in replicating the biomechanics and pathophysiological progression of closed-head TBI.

Therefore, we investigated acute microglial reactivity immediately following inertial TBI using an established porcine model with biomechanical fidelity to closed-head TBI in humans (Cullen et al., 2016; Meaney et al., 1995; Smith et al., 1997). Since diffuse TBIs by definition lack a focal injury epicenter, we explored microglial activation in the context of neuronal trauma, specifically breaches in plasma membrane integrity (Cullen et al., 2011; Geddes et al., 2003; LaPlaca et al., 2009; Singleton and Povlishock, 2004). Such plasma membrane disruptions are a hallmark consequence of supra-threshold loading during TBI, and may alter cell homeostasis due to loss of ionic gradients, osmotic imbalance, and calcium dysregulation (LaPlaca et al., 1997; Stone et al., 2004; Weber et al., 1999). These acute membrane breaches are generally transient, as initially compromised cells survive the insult but may be dysfunctional or undergo delayed cell death (Cullen et al., 2011; Farkas et al., 2006; Singleton and Povlishock, 2004; Whalen et al., 2008). In addition, microglia have been shown to be reactive at intermediate and chronic (e.g., days to years) time points after TBI (Johnson et al., 2013; Kelley et al., 2007; Lafrenaye et al., 2015), and glial activation is likely mediated by warning factors released from damaged neurons (Davalos et al., 2005; Lee, 2013). Thus we hypothesized that microglia would be most reactive, as measured by distribution and morphology, in regions exhibiting acute neuronal damage and correlating with injury severity and number of injuries. We tested this hypothesis by subjecting swine to single or repetitive closed-head rotational TBI at levels previously established to result in an injury phenotype consistent with clinical definitions of "mild" or "severe" TBI in humans (Browne et al., 2011; Cullen et al., 2016; Smith et al., 2000). In order to assess trauma-induced alterations in membrane permeability, the cell-impermeant dye Lucifer yellow (LY) was delivered into the ventricles to diffuse throughout the interstitial tissue prior to injury. LY would only gain intracellular access if the plasma membrane became compromised during or immediately following injury, similar in principle to other fluorescent permeability tracers used in the neurotrauma field (Cullen et al., 2011; Geddes et al., 2003; Harris, 2015; LaPlaca et al., 2009; Simon et al., 2009; Singleton and Povlishock, 2004; Stone et al., 2004). Following closed-head rotational TBI, microglial cell characteristics were quantified as a function of distance from injured neurons. This model presents a unique potential for analyzing acute microglia reactively following closed-head diffuse TBI, and our findings suggest that rapid microglia activation depends on proximity to traumatically permeabilized neurons.

2. Materials and methods

2.1. Animal care and anesthesia

All procedures and protocols were approved by the Animal Care and Use Committee of the University of Pennsylvania. All animal care complied with the Guide for the Care and Use of Laboratory Animals (Clark et al., 1997) and followed the ARRIVE Guidelines. Female Yorkshire swine ($n = 19$) with an average weight of 22.5 kg were utilized in this study. No special diets were provided as the animals were housed indoors with feed and water, *ad libitum*. The housing facility was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Prior to the injury procedure, animals were fasted for 18–20 h with water remaining *ad libitum*. After induction with a cocktail of ketamine (20–30 mg/kg; Hospira, 0409-2051-05) and midazolam (0.4–0.6 mg/kg; Hospira, 0409–2596-05), anesthesia was provided with 5% isoflurane (Piramal, 66794-013-25) via a snout mask and glycopyrrolate (0.01 mg/kg; West-Ward Pharmaceutical Corp., 0143-9682-25) was given subcutaneously. The animal was then intubated, and isoflurane was connected at maintenance levels (1.5–2.0%). Eye lubricant was used to minimize drying. Physiological monitoring allowed titration of anesthesia so that SpO_2, heart rate, and respirations were within acceptable ranges (SpO_2 between 97 and 100%, heart rate between 110 and 130 beats per minute, and respirations between 9 and 12 breaths per minute).

2.2. Delivery of Lucifer yellow via intracerebroventicular injections

LY (Invitrogen, L453, Carlsbad, CA) was delivered into the lateral ventricles of all animals before the rotational/sham injury to demarcate cells permeabilized during the rotational injury. This aldehyde-fixable, normally cell-impermeable dye was delivered via a Hamilton syringe (Reno, NV). Animals were placed in a stereotactic head frame. The scalp was shaved and swabbed with betadine before a 4 cm incision was made. The scalp was reflected from the skull in order to visualize Bregma. Two 5 mm craniectomies were made at the following stereotactic co-ordinates: 1.0 mm posterior to Bregma, ±6.0 mm lateral. A small incision in the dura was made with a 19-gauge needle. For each site in serial fashion, a sterile Hamilton syringe was lowered 18.0 mm from the surface of the dura mater to access the ventricles. Tissue was left to stabilize for 2 min before delivering 500 μL of LY (0.4 mg/kg in sterile saline) over a period of 10 min on each side using an UltraMicroPump III (World Precision Instruments, Sarasota, FL). The needle was slowly withdrawn at a rate of 2 mm/min, and the burr holes were sealed with bone wax. The incision site was sutured with 0-0 sutures, and the area was swabbed with betadine. A surgical plane of anesthesia was maintained through the injury/sham procedure and sacrifice. The injury occurred 2 h after the start of the first injection, which was optimized to allow even LY distribution throughout the interstitial tissue.

2.3. Porcine closed-head rotational/inertial TBI

Closed-head rotational TBI was induced under anesthesia using the HYGE pneumatic actuator, a device capable of producing pure impulsive non-impact head rotation with a controlled relationship between maximum rotational acceleration and injury severity, as previously described (Browne et al., 2011; Cullen et al., 2016; Smith et al., 1997, 2000). The well-characterized model rapidly accelerates the head and induces inertial forces representative of human TBI from falls, collisions, or blunt impacts. Angular velocity was recorded using a magneto-hydrodynamic sensor (Applied Technology Associates, Albuquerque, NM) connected to a National Instruments DAQ, controlled by LabVIEW. Briefly, the animal was randomly assigned to an injury group and the animal's head was secured to a padded bite plate under anesthesia, and mounted to the HYGE device. In pigs, sagittal plane head accelerations (transverse to the brainstem) cause an enhancement of strain in the brainstem region compared to coronal plane accelerations (circumferential to the brainstem), therefore reduced levels of angular velocity/acceleration are required to elicit loss of consciousness and coma (Browne et al., 2011; Cullen et al., 2016; Meaney et al., 1995; Smith et al., 1997, 2000). Therefore, to induce a "mild" injury phenotype, single rapid head rotation was performed in the coronal plane (peak angular velocities of 193–299 rad/s; $n = 7$), whereas to induce a moderate-to-severe injury phenotype rotation occurred in the sagittal plane (peak angular velocities: 80–139 rad/s; $n = 5$). In prior studies, it was empirically determined that head rotation at these levels of angular velocity/acceleration in the coronal plane did not induce measurable loss of consciousness or hematoma. However, head rotation at these levels of angular velocity/acceleration in the sagittal plane induced prolonged loss of consciousness and often significant hematoma (Cullen et al., 2016). In addition to the single injuries, we performed repetitive injuries on a small cohort of animals in both the sagittal and coronal planes. Repetitive head rotation was either performed on the same day (separated by 15 min) or separated by 7 days; these animals only received LY intracerebroventicular injection on the day of their second injury