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Full-length Article

# Selective activation of cannabinoid receptor-2 reduces neuroinflammation after traumatic brain injury via alternative macrophage polarization

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ABSTRACT

Inflammation is an important mediator of secondary neurological injury after traumatic brain injury (TBI). Endocannabinoids, endogenously produced arachidonate based lipids, have recently emerged as powerful anti-inflammatory compounds, yet the molecular and cellular mechanisms underlying these effects are poorly defined. Endocannabinoids are physiological ligands for two known cannabinoid receptors, CB1R and CB2R. In the present study, we hypothesized that selective activation of CB2R attenuates neuroinflammation and reduces neurovascular injury after TBI. Using a murine controlled cortical impact (CCI) model of TBI, we observed a dramatic upregulation of CB2R within infiltrating myeloid cells beginning at 72 h. Administration of the selective CB2R agonist, GP1a (1–5 mg/kg), attenuated pro-inflammatory M1 macrophage polarization, increased anti-inflammatory M2 polarization, reduced edema development, enhanced cerebral blood flow, and improved neurobehavioral outcomes after TBI. In contrast, the CB2R antagonist, AM630, worsened outcomes. Taken together, our findings support the development of selective CB2R agonists as a therapeutic strategy to improve TBI outcomes while avoiding the psychoactive effects of CB1R activation.

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## 1. Introduction

Traumatic brain injury (TBI) is a major cause of injury-related deaths and long-term disability. Cerebral edema, a life-threatening complication that presents in the hours and days after TBI, contributes to elevated intracranial pressure (ICP) (Catala-Temprano et al., 2007; Levin et al., 1991; Saul and Ducker, 1982), cerebral hypoperfusion (Catala-Temprano et al., 2007), inadequate tissue oxygenation (Narotam et al., 2009), brain herniation (Katayama et al., 1990; Lenzlinger et al., 2001; Sarabia et al., 1988), and a poor clinical patient prognosis (Eisenberg et al., 1990; Levin et al., 1991; Narayan et al., 2002). Neither neurosurgical nor medical approaches adequately reduce edema formation or improve cerebral perfusion (Bloch and Manley, 2007; Sahuquillo and Arikan, 2006). Given the high incidence rate of TBI, novel approaches are needed to ameliorate both the acute and long-term pathological outcomes following TBI.

The initial impact produces immediate mechanical damage and loss of tissue due to necrosis. This is followed by a coordinated set of immune responses that contribute towards both tissue repair and secondary neurological injury. Along these lines, we and others found a temporal and functional association between acute neuronal necrosis and the development of innate immune activation after both pre-clinical and clinical TBI (Braun et al., 2017; Czigner et al., 2007; Kawamata and Katayama, 2006; Laird et al., 2014). Macrophages are professional phagocytes that aid in clearance of

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cellular debris and tissue repair, yet the sustained release of pro-inflammatory mediators from infiltrating macrophages may exacerbate neuronal death, increase neurovascular injury, and contribute to long-term loss of white matter (Bartnik-Olson et al., 2014; Shi et al., 2015; Su and Bell, 2016). Although the molecular mechanisms underlying these opposing roles remain poorly defined, macrophages polarize along a continuum from a classical pro-inflammatory (M1) state to an alternative anti-inflammatory (M2) state. Notably, expression of both M1- and M2-like phenotypic markers are observed early after TBI, with the transient expression of M2 phenotypic markers yielding to a predominantly M1 phenotype that has been associated with the release of pro-inflammatory cytokines, edema development, the development of long-term adaptive immune responses, and neurodegeneration (Braun et al., 2017; Kim et al., 2016; Kumar et al., 2016). Thus, the development of therapeutic approaches to reduce pro-inflammatory M1 polarization may improve TBI outcomes.

Endocannabinoids, such as anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), are endogenously produced, arachidonate based lipids that serve as physiological ligands for the cannabinoid receptors, CB1R and CB2R (Ashton and Moore, 2011; Buch, 2013; Munro et al., 1993). CB1R, which was originally detected in neurons, mediates the psychoactive effects of marijuana (Ashton and Moore, 2011; Buch, 2013; Ramirez et al., 2012). Conversely, CB2R is predominantly expressed on immune and endothelial cells and does not generate psychoactive activity (Anday and Mercier, 2005; Ramirez et al., 2012; Rom et al., 2013). Of note, inhibition of either CB1R or CB2R reversed the neuroprotective effects of minocycline (Lopez-Rodriguez et al., 2015) whereas inhibition of fatty acid amide hydrolase (FAAH), which boosts anandamide levels, increased anti-inflammatory microglia/macrophage activation and reduced neurodegeneration after TBI (Tchantchou et al., 2014). Thus, mechanistically defining the anti-inflammatory and neuroprotective roles of cannabinoid receptors may advance the therapeutic development to improve TBI outcomes.

In this study, we tested the hypothesis that administration of the selective CB2R agonist, GP1a [N-(piperidin-1-yl)-1-(2,4-dichloro phenyl)-1,4-dihydro-6-methylindeno[1,2-c]pyrazole-3-carboxamide], induces M2 macrophage polarization and improves TBI outcomes. We also explored whether administration of the selective CB2R antagonist, AM630 [6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone] would exacerbate neurovascular injury after TBI.

## 2. Materials and methods

### 2.1. Controlled cortical impact

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and was approved by the Committee on Animal Use for Research and Education at Augusta University. C57BL/6 mice were used for bone marrow chimera and adoptive transfer studies, while CD1 mice were used for all other experiments. Briefly, male C57BL/6 mice or CD-1 mice (12–16 weeks old; Charles River Laboratories) were housed under a 12-h light/12-h dark cycle at 23 ± 1 °C. Food and water were provided *ad libitum*. Mice were anesthetized with 2% isoflurane and subjected to either sham injury or controlled cortical impact, as detailed by our laboratory (Braun et al., 2017; Kimbler et al., 2012; Laird et al., 2010). Mice were placed in a stereotaxic frame and a circular craniotomy was made in the right parietal bone midway between the lambda and the bregma by carefully outlining a 2.0 mm radius bone flap centered at stereotaxic coordinates-AP = −2 mm from bregma; ML = +2.0 mm from mid line, leaving the dura intact. Mice were impacted at 3 m/s with 85 ms dwell time and 3 mm depression using a 3 mm diameter convex tip (PinPoint PCI3000 Precision Cortical Impactor, Hatteras Instruments, Cary, NC). Bone wax was used to seal the craniotomy, the incision was surgically stapled, and mice were placed in a clean warm cage until recovered. Sham-operated mice underwent the identical surgical procedures, but were not impacted. Body temperature was maintained at 37 °C using a small animal temperature controller throughout all procedures (Kopf Instruments, Tujunga, CA, USA). For treatments, placebo (PBS), CB2R selective agonist GP1a (Tocris Bioscience, Bristol, UK) (1–5 mg/kg b.wt.) or the CB2R antagonist AM630 (Tocris Bioscience, Bristol, UK) (5 mg/kg b.wt.) were intraperitoneally administered at 10 min post-TBI.

### 2.2. Behavioral tests and neurological scoring

#### 2.2.1. Habituation

For all tasks, mice were habituated for thirty minutes in the behavior room prior to training/testing. All tasks were performed under identical lighting conditions at the same time each day. All behavioral chambers were cleaned with 70% ethanol cleaning solution before and after use.

#### 2.2.2. Assessment of motor coordination

Motor coordination was assessed using an accelerating rotarod task. Mice were trained three days prior to injury and re-assessed at day 3 after TBI. Mice were placed on the rotarod moving at a constant speed of 4 rpm for thirty seconds followed by an acceleration to 30 rpm over the course of 4 min. The length of time each animal maintained balance while walking on top of the drum was recorded. Trials were ended when the animal either fell off the rod or clung to the rod for one complete rotation (Cernak et al., 2014). Motor coordination was further determined by measuring the time required to traverse a stationary 1 m narrow beam (6 mm width) (Luong et al., 2011). Each mouse was tested three-times and the average was recorded. All behavioral analyses were done by investigators blinded to the experimental groups.

#### 2.2.3. Open field test

Mice were placed in a 40 cm × 40 cm × 40 cm box for 10 min and activity was digitally recorded. Latency to enter the center zone and time spent in the center zone was recorded and analyzed using Ethovision XT video tracking software (Noldus Information Technology, Asheville, NC). Results were presented as mean ± SEM.

### 2.3. Tissue collection

At 72 h post-TBI, mice were euthanized with 5% isoflurane. Blood was collected by cardiac puncture and placed into ice-cold heparinized tubes. Mice were next perfused transcardially with 30 mL of ice-cold phosphate buffered saline and brains were carefully removed. A 3-mm coronal brain section centered on the contusion was prepared using an acrylic brain matrix. Ipsilateral (injured) cerebral cortex from each brain was collected for different analyses, as detailed below. For histological and immunohistochemical analysis, brains were perfused with ice-cold phosphate buffered saline followed by 4% paraformaldehyde.

### 2.4. Immunostaining and quantification

Five μm thick coronal sections were obtained from paraffin embedded tissue blocks and deparaffinized with xylene and alcohol gradients. Following an antigen retrieval step, deparaffinized sections were incubated with rabbit anti-CB2R antibody (1:200; Bioss#bs-2377R) overnight at 4 °C followed by incubation with a

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