The endocannabinoid 2-AG protects the blood–brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines

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Endocannabinoids are involved in neuroprotection through numerous biochemical pathways. We have shown that the endocannabinoid 2-arachidonyl glycerol (2-AG) is released in mouse brain after closed head injury (CHI), and treatment with exogenous 2-AG exerts neuroprotection via the central cannabinoid receptor CB2. This process involves inhibition of inflammatory signals that are mediated by activation of the transcription factor NF-kB. The present study was designed to examine the effect of 2-AG on the blood–brain barrier (BBB) and the possible inhibition of the early expression of proinflammatory cytokines, which are implicated in BBB disruption. We found that 2-AG decreased BBB permeability and inhibited the acute expression of the main proinflammatory cytokines: TNF-α, IL-1β and IL-6. It also augmented the levels of endogenous antioxidants. We suggest that 2-AG exerts neuroprotection in part by inhibition of the early (1–4 h) inflammatory response and augmentation of the brain reducing power.

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Introduction

The major endocannabinoid 2-arachidonyl glycerol (2-AG) has been identified both in the central nervous system and in the periphery (Mechoulam et al., 1995; Sugiura et al., 1995). Stressful stimuli such as brain trauma in mice (Panikashvili et al., 2001), picrotoxin administration into rat brain (Sugiura et al., 2000) or reserpine treatment of mice, which models for Parkinson disease (Di Marzo et al., 2000) as well as stimulation of prefrontal cortex in rats (Melis et al., 2004) or short-term fasting in mice (Hanus et al., 2003; Kirkham et al., 2002) enhance brain 2-AG levels. Elevated levels of 2-AG and anandamide were found in the spinal cord of mice in a model of amyotrophic lateral sclerosis (Witting et al., 2004).

Various exogenous and endogenous cannabinoids are neuroprotective in in vivo models of closed head injury (Panikashvili et al., 2001, 2005), ischemia (Nagayama et al., 1999) and excitotoxicity (van der Stelt et al., 2001a,b). These effects may derive from the ability of cannabinoids to act through various biochemical mechanisms (for review, see Mechoulam et al., 2002) including inhibition of glutamate release or direct blockade of NMDA receptors, inhibition of intracellular calcium mobilization through inhibition of calcium channels, acting as reactive oxygen species (ROS) scavengers (McCarron et al., 2003), inhibition of proinflammatory cytokines, inhibition of NF-κB activation (Juttler et al., 2004; Panikashvili et al., 2005). Some of the neuroprotective cannabimoids mechanisms are not CB1 receptor mediated (Juttler et al., 2004), including blockade of NMDA receptors (Feigenbaum et al., 1989), antioxidant properties (Hampson et al., 1998; Chen and Buck, 2000) and inhibition of calcium accumulation (Nadler et al., 1995).

The endocannabinoids act via multiple receptors, of which the CB1 receptors are mostly abundant in the CNS (Matsuda et al., 1993) and the CB2 receptors are mostly present in peripheral immune cells (Pertwee, 1997). In a model of cerebral ischemia, CB1 receptors knockout mouse showed increased infarct volume (Parmentier-Batteur et al., 2002), and CB1 receptor agonists protected rat hippocampal neurons from excitotoxicity (Shen et al., 1996). Recently, we showed that CB1 knockout mice display slower functional recovery after closed head injury (CHI) and do not respond to treatment with 2-AG (Panikashvili et al., 2005). These findings agree with the report of Marsicano et al. (2003) who demonstrated that, in mutant mice that lack expression of the CB1 receptor in principal forebrain neurons, but not in adjacent inhibitory interneurons, the excitotoxin kainic acid (KA) induced

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excessive seizures in vivo. They showed that the endogenous cannabinoid system thus provides on-demand protection against acute brain excitotoxicity which is CB1 mediated.

CNS inflammation is an intrinsic part of many neurodegenerative disorders including traumatic brain injury (TBI) (Dusart and Schwab, 1994; Shohami et al., 1999). Inflammation is mediated by proinflammatory cytokines (TNF-α, IL-1β, IL-6), reactive oxygen species (ROS) and prostaglandins and might promote further brain damage or augment cytotoxicity. These factors are mainly produced by reactive microglia (Liu and Hong, 2003) which migrate towards the site of injury and affect neurons which in turn become necrotic or apoptotic.

One of the early harmful events of TBI is the disruption of the BBB allowing infiltration of peripheral macrophages and leukocytes into cerebral tissue and aggravation of the inflammatory response. Reactive microglia produce proinflammatory cytokines such as TNF-α and IL-6, which in turn, together with NO, contribute toward the impairment of the BBB function (Walter and Stella, 2004; Lu et al., 2001). TNF-α converting enzyme (TACE) is a membrane protein that belongs to the ADAM (a disintegrin and a metalloprotease) family. It cleaves various membrane proteins, including the transmembrane precursor form of TNF-α. Little is known about the physiological role of TACE, yet, some neurotoxic and neuroprotective functions have been reported recently (Moro et al., 2003). Reactive oxygen species (ROS) were also shown to play a role in altering blood–brain barrier (BBB) permeability and formation of brain edema induced by trauma. Antioxidants (e.g., nitroxides) were reported to protect the BBB (Beit-Yannai et al., 1996), and the findings of McCarron et al. (2003) provide evidence of 2-AG antioxidant activity. In addition, 2-AG was shown in in vitro studies to act as chemo-attractant of microglial cells (Walter et al., 2003).

We previously reported that the endocannabinoid 2-AG is massively accumulated in mouse brain after CHI (Panikashvili et al., 2001). It may represent an attempt of the endocannabinoid system to recruit microglia under neuroinflammatory conditions. We also showed that the endocannabinoid 2-AG inhibits nuclear translocation of one of the hallmarks of the inflammatory pathway—nuclear factor kappaB (NF-κB), 24 h following CHI in mice (Panikashvili et al., 2005). We therefore designed the current study to investigate the effects of 2-AG on BBB integrity after CHI and on the expression profile of the proinflammatory cytokines and the enzymes TACE and matrix metalloproteinase (MMP-9) over the initial 2–4 h after the injury, the time preceding the maximal disruption of the BBB. We also investigated the effect of 2-AG on total antioxidant capacity of the injured brain, as we have demonstrated that high antioxidant capacity is associated with better clinical recovery (Shohami et al., 1997b).

**Methods**

**Animals**

The study was performed according to the guidelines of the Institutional Animal Care Committee. Adult (8–9 weeks of age) male Hebrew University strain mice (Sabra) weighing 38–45 g were used.

**Trauma model**

Mice were subjected to CHI under ether anesthesia using a weight-drop device as described elsewhere (Chen et al., 1996; Yatsiv et al., 2002). To assess the functional impairment after trauma, a scoring system (neurological severity score, NSS) was used based on the ability of mice to perform ten different tasks that evaluate their motor ability, balancing and alertness (Beni-Adani et al., 2001). One point is awarded for failure to perform a task, such that healthy mouse scores zero, and severely injured 8–9. This scoring system was applied to ensure that mice of both treatment groups (drug and vehicle) suffered of similar degree of damage.

**Evaluation of blood–brain barrier (BBB) integrity**

The integrity of the BBB was tested using the Evans Blue (EB) dye extravasation method. The total dye uptake by the cerebral hemispheres was measured 4 h after CHI (time of maximal disruption of BBB, Chen et al., 1996). EB (2% w/v in normal saline) was injected into the tail vein 3 h after CHI, and 1 h later, animals were anesthetized with an intraperitoneal injection of 100 mg/kg body weight ketamine hydrochloride and were transcardially perfused with normal saline to clear blood vessels of the dye. The brains were then removed, the hemispheres separated, EB was extracted, and its fluorescence was measured using a Perkin-Elmer LS-5 fluorospectrophotometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm (bandwidth 10 nm). Calculations were based on external standards in the solvent (100–500 ng/ml) as described earlier (Chen et al., 1996).

**Semi-quantitative RT-PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR)**

Semi-quantitative RT-PCR was used in order to qualitatively assess the effect of injury and time post-CHI on the expression of each of the examined factors and to distinguish between factors which are affected by 2-AG and those that are not. Whenever a change in expression was observed following 2-AG treatment, qRT-PCR was used in order to further confirm and quantify this effect. For gene expression assays, mice were killed at the designated time points (2 h or 4 h after CHI), and cortex from injured hemisphere was stored in “RNA later” (Qiagen Inc. US) at −18°C until total RNA was isolated. All reagents used for RT-PCR were of molecular grade. Total RNA was isolated using TRI reagent (Molecular Research Center Inc. US). All RNA samples were confirmed free of DNA contamination using 260/280-nm optical density measurement. 10 µg of total RNA was transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen, US) with 100 pmol oligo(dT)15 as primers.

For RT-PCR, a PCR reaction mixture consisting of buffer, 2 µM Mg²⁺, 100 pmol of each primer and 2.5 U of Taq DNA polymerase (Bioline GmbH, Germany) was added to cDNA samples. The following primers with the respective annealing temperatures and Mg²⁺ concentrations were used for RT-PCR:

| IL-1β (a) forward: CATCAGCACCCTACAAGCAGA 60°C, 2 µM Mg²⁺ | (b) reverse: CAATCTACCCCCCAGTGTGTTGTTG 60°C, 2 µM Mg²⁺ |
| IL-6 (a) forward: TCTAGCAAAAGAGTTGCGAATGGC 60°C, 2 µM Mg²⁺ | (b) reverse: GAATGTCACAAACTGATAGTCTC 60°C, 2 µM Mg²⁺ |
| TNFα (a) forward: TGAGCGCTTCTCTCAATG 60°C, 2 µM Mg²⁺ | (b) reverse: TCTCAGTGTGTTGCGAAGCC 55°C, 2 µM Mg²⁺ |
| TACE (a) forward: GACTGCTGATGGCTGAAGGCG 57°C, 2 µM Mg²⁺ | (b) reverse: AAACCGAACAGGCAACCGTG 57°C, 2 µM Mg²⁺ |
| MMP-9 (a) forward: TCTCTGGACGTCAAAATGGTG 57°C, 2 µM Mg²⁺ | (b) reverse: ACTCTCTTATCCACCGGAGT 57°C, 2 µM Mg²⁺ |

β-actin (a) forward: AGGGAATCTGGCGTGTAC 52°C, 2 µM Mg²⁺ | (b) reverse: CATCTGCTGAAAGGTTGAC 52°C, 2 µM Mg²⁺ |
Samples were heated to 95°C for 2 min and cycled 20–36 times at 95°C for 1 min, 52–60°C for 1 min and 72°C for 1 min, after which additional extension step at 72°C for 5 min was included. All samples from one experiment underwent PCR at the same time and were carried out with a stock reaction mixture to minimize pipetting errors. PCR products were electrophoresed in 1% agarose gel and visualized using ethidium bromide (Promega, US) staining. Band size was recognized using DNA ladder (Bioline GmbH, Germany), and its optical density was measured using Bio-Rad video imaging system. Quantification was performed by expressing the optical density of the respective bands as percent of β-actin.

qRT-PCR was performed using Taqman® Gene Expression Assays-on-Demand (Applied Biosystems, US). The amplification reaction was carried out in an ABI 7700 sequence detection system (Applied Biosystems, US). Reaction was performed in a 20 μl reaction volume which contained 90 ng of cDNA, 10 μl of Taqman® universal PCR mix (Applied Biosystems, US) and 1 μl of the assay solution containing the specific primers and 6-FAM labeled probe. The thermal profile for PCR was 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. Relative quantity (RQ) values were analyzed using ABI 7700 sequence detection system software (Applied Biosystems, US) according to the ΔCt method which reflects the difference in threshold for each target gene relative to that of β-actin.

Measurement of low molecular weight antioxidants by cyclic voltammetry (CV)

Cortical slices (90–150 mg) from around the site of injury were homogenized in 10% (w/v) PBS (pH 7.4) and centrifuged at 1000 g for 10 min (4°C), and supernatant was analyzed by CV electrochemical apparatus (BAS 50 W; West Lafayette, IN) as described in detail by Kohen et al. (1999). An electrical linear potential gradient (0–1300 mV relative to a reference electrode) was applied at a rate of 100 mV/s across an electrode–solution interface (working electrode) to oxidize reducing species present in the sample. The working electrode was burnished before each measurement with a polishing kit (BAS-PK-1). Computerized analysis (BAS Windows Control Software, Version 2.3 EF-1661) obtained voltammetric waves in a peak-shaped mode. The potential (E_p) at which the peak current occurs on the voltage-axis (y) correlates with the reducing potency of compounds, such that the lower potential means greater reducing potency. Typically, three groups of LMWA are found in the brain, differing in their E_p, and each of the E_p recorded, represents a cluster of antioxidants (e.g., ascorbic acid, uric acid, beta-carotene etc.). The current intensity (I_s) is calculated from the y-axis and is proportional to the concentration of antioxidants being oxidized at a particular E_p. I_s values were normalized to protein concentration, as determined by Bradford (Sigma-Aldrich) (Kohen et al., 1999). This type of analysis reflects the total low molecular weight antioxidants (LMWA) present in the tissue samples, and changes in their levels reflect either consumption (due to overproduction of ROS) or recruitment (due to the tissue protective mechanisms) (Shohami et al., 1997b, Kohen et al., 1999).

Drugs

2-AG was synthesized in our laboratory according to a published procedure (Mechoulam et al., 1995). Emulphor was obtained from Sigma Israel. 2-AG was dissolved in anhydrous ethanol:emulphor:saline (1: 1: 18) to achieve 5 mg/kg, and 100 μl per 10 g body weight was injected i.p.

Statistical analysis

Data are presented as mean ± SD. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. Probability values (P) smaller than 0.05 were considered to be statistically significant.

Results

Effect of 2-AG on blood–brain barrier disruption

CHI causes major transient disruption of BBB integrity, which peaks at 4 h (Chen et al., 1996). Hence, we administered 2-AG immediately after CHI and determined the BBB permeability 4 h after the trauma. Fig. 1 shows that in the left, injured hemisphere 2-AG robustly reduced BBB disruption, as evidenced by 40% decrease in dye levels extracted from the brain (142.5 ± 22.8 vs. 241.3 ± 38.2 ng/g brain; P < 0.01). In the right hemisphere, not affected by CHI, no changes in dye levels were noted.

Effect of 2-AG on cytokines expression profile after CHI

Cytokines were semi-quantitatively examined using RT-PCR and their amount expressed relative to β-actin. TNF-α mRNA was not found in non-injured sham mice. 2-AG significantly inhibits TNF-α expression at 2 but not at 4 h (Fig. 2A, P < 0.05). The effect of 2-AG on TNF-α expression was confirmed and quantified by qRT-PCR, showing an inhibitory effect of 2-AG on this cytokine 2 h post-CHI (P < 0.05 vs. control at the same time point) but not at 4 h following injury (Fig. 2B).

Fig. 1. 2-AG reduces BBB permeability following CHI. 2-AG (5 mg/kg) or vehicle (control) was injected within minutes after CHI. Three hours later, Evans Blue (EB, 2% w/v in normal saline) was injected into the tail vein, and 1 h later, mice were anesthetized (i.p injection of 100 mg/kg ketamine hydrochloride), transcardially perfused with normal saline, and the brains were removed. EB was extracted as described in the text and its fluorescence measured (excitation wavelength 620 nm and emission at 680 nm). Calculations were based on external standards in the solvent (100–500 ng/ml) as described. 2-AG robustly decreases BBB disruption in injured hemisphere by nearly 40% (*P < 0.01), n = 7 mice per group.
enzymes are upregulated after CHI, and whether the protective effect 2-AG on the BBB is mediated by inhibition of these enzymes. Fig. 5 depicts the results of these experiment and shows that TACE expression increased between 2 and 4 h after CHI (\(P < 0.01\)), however, 2-AG had no effect on TACE level. MMP-9 levels were similar at 2 and 4 h, and no effect of 2-AG on this enzyme was noted.

**Effect of 2-AG on total reducing power of the brain**

Three classes of water-soluble antioxidants were found in the brain homogenates, having oxidation potentials of 330–360 mV (e.g., ascorbic acid, uric acid), 650–700 mV (e.g., carnosine, melatonin, NADH) and 1050–1100 mV (e.g., lipoic acid) (Shohami et al., 1997b). Fig. 6 shows that at 4 h after CHI, the class of weak antioxidants, having the highest oxidation potential (1050–1100 mV), is significantly enhanced after CHI.

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**In contrast to TNF-\(\alpha\), IL-1\(\beta\) mRNA was expressed in sham mice and was significantly upregulated at 2 h and 4 h following CHI (\(P < 0.05\) and \(P < 0.01\) vs. sham, respectively) (Fig. 3A). The effect of 2-AG on IL-1\(\beta\) mRNA levels was evident only at 4 h after CHI when significant inhibition of IL-1\(\beta\) expression was noted (\(P < 0.05\)). qRT-PCR verified that IL-1\(\beta\) levels continue to increase between 2 h and 4 h post-injury (\(P < 0.01\) 2 h vs. 4 h after CHI) and showed that indeed, 2-AG treatment leads to decreased expression of IL-1\(\beta\) 4 h following injury (\(P < 0.05\) vs. CHI at same time point) but not at 2 h post-CHI (Fig. 3B).

RT-PCR indicated that IL-6 mRNA levels after injury increase between 2 h and 4 h (\(P < 0.05\)). 2-AG treatment led to a decrease in IL-6 levels at 4 h (\(P < 0.01\)) but not at 2 h (Fig. 4A). Both these observations were confirmed and quantified using qRT-PCR (Fig. 4B, \(P < 0.05\) 2 h vs. 4 h after CHI and \(P < 0.05\) control vs. 2-AG at 4 h post-injury).

**Effect of 2-AG on TACE and MMP-9 after CHI**

Since the proteolytic enzymes TACE and MMP are implicated in the disruption of the BBB, we next examined whether these enzymes are upregulated after CHI, and whether the protective effect 2-AG on the BBB is mediated by inhibition of these enzymes. Fig. 5 depicts the results of these experiment and shows that TACE expression increased between 2 and 4 h after CHI (\(P < 0.01\)), however, 2-AG had no effect on TACE level. MMP-9 levels were similar at 2 and 4 h, and no effect of 2-AG on this enzyme was noted.

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2-AG further significantly increases the levels of these LMWAs, thus improving the tissue’s reducing capacity.

Discussion

The results of the present study extend our earlier findings on the neuroprotective properties of the endocannabinoid 2-AG after CHI and demonstrate an early (4 h) protective effect on the BBB. The significant reduction of the BBB permeability after treatment with 2-AG may explain its effect on edema, seen at 24 h (Panikashvili et al., 2001, 2005) and on functional recovery. The current findings also suggest that the mechanism by which 2-AG exerts its effect on the BBB may involve inhibition of the early (>4 h) inflammatory response.

Proinflammatory cytokines play a crucial role in traumatic brain injury. TNF-α is released early (within 1–4 h) after CHI (Shohami et al., 1997a; Stover et al., 2000) and acts upon its specific receptors. On binding, the cytosolic portions of both TNF receptors recruit multiple intracellular adapter proteins that activate the transcription factor NF-κB, starting with hydrolysis of the inhibitory protein IκB complexed with the heterodimer polypeptides p50 and p65. This allows the p65/p50 complex to translocate to the nucleus and to bind to NF-κB consensus sequences in enhancers of many genes, thus regulating the expression of genes encoding acute-phase proteins, cell adhesion molecules, cell surface receptors and cytokines (Baeuerle and Henkel, 1994). TNF-α and IL-6 are known as robust promoters of NF-κB activation (Wang et al., 2003), while NF-κB itself is responsible for expression of TNF-α and IL-6 (Libermann and Baltimore, 1990; Drouet et al., 1991).

Matrix metalloproteinases (MMPs) play major role in physiological extracellular matrix turnover in pathological processes. Increases in MMP activity were reported in brain disorders such as multiple sclerosis, Alzheimer’s disease and after head trauma (von Gertten et al., 2003). Downregulation of certain metalloproteinases (MMP-9) might exert neuroprotection (Mori et al., 2002). Matrix metalloprotease-9 (MMP-9) participates in the disruption of the blood–brain barrier during hemorrhagic transformation and exacerbates brain injury after cerebral ischemia. However, the consequences of long-term inhibition or deficiency of MMP-9 activity could be deleterious (Tang et al., 2004). Because MMP-9 is involved in BBB impairment, and TACE is involved in TNF shedding from the membrane-bound, inactive pro-TNF, we decided to examine the effect of 2-AG on these two enzyme. The present results suggest that the inhibition of cytokines expression and the
BBB protection are not associated with inhibition of the proteolytic enzymes TACE and MMP-9.

The augmentation of LMWA levels which is shown at 4 h after CHI may also contribute to the neuroprotection exerted by 2-AG. Higher levels of ROS-neutralizing molecules allow better coping of the tissue with oxidative stress, and protection of the BBB (Beit-Yannai et al., 1997). We have proposed that ROS and TNFα act synergistically at the early post CHI period, leading, among other effects, to BBB disruption (Tremblower et al., 1999; Ginis et al., 2000). Thus, taken together, inhibition of cytokines along with higher LMWA protects the BBB from the early effects of CHI.

Different cannabinoids were shown to have antiinflammatory properties in numerous conditions associated with CNS inflammation. Lyman et al. (1989) and Wirguin et al. (1994) showed that either Δ⁹-THC or Δ⁸-THC suppresses the development of EAE (experimental autoimmune encephalomyelitis) with less inflammation in spinal cord and reduction in neurological deficits of treated animals. The synthetic cannabinoid WIN55212-2 ameliorated the symptoms of another model of MS–TMEV (Theiler’s murine encephalomyelitis infection) syndrome and decreased CNS mRNA encoding for TNF-α, IL-1β and IL-6 in these mice (Croxford and Miller, 2003). The non-psychotropic synthetic cannabinoid, dexanabinol (HU-211) was also shown to inhibit the expression of TNF-α after CHI (Shohami et al., 1997a).

In addition to the above reports on the effect of cannabinoids on the expression of inflammatory cytokines, they were also shown to affect the activation of the transcription factor NF-κB and related promoters. Puffenburger et al. (2000) showed that THC suppresses LPS induced proinflammatory cytokines expression in rat microglial cells. Cannabinol and 2-AG were shown to inhibit IL-2 expression in activated thymocytes through inhibition of NF-κB (Herring and Kaminski, 1999; Ouyang et al., 1998). The neuroprotective cannabinoid dexanabinol inhibits NF-κB translocation into the nucleus and its transcriptional activity through reducing mRNA accumulation of its target genes TNF-α and IL-6 (Juttler et al., 2004). The endocannabinoid anandamide inhibited TNF-α induced NF-κB activation (Sancho et al., 2003) and suppressed release of NO and TNF in astrocytes in response to TMEV (Molina-Holgado et al., 1997). 2-AG inhibited the in-vitro production of TNF-α by mouse macrophages, as well as in mice (Gallily et al., 2000), and suppressed LPS stimulated release of IL-6 by macrophages (Chang et al., 2001). The endocannabinoid system may also contribute to neuroprotection by promoting de-novo synthesis of antiinflammatory cytokines such as IL-10 (Smith et al., 2000) and IL-1 receptor antagonist (Molina-Holgado et al., 2003). In our previous publications, we reported that neuroprotection exerted by 2-AG is NF-κB and CB1 receptor mediated. Based on the present findings, we may add that 2-AG also inhibits, at an early stage (2–4 h), the expression of the main proinflammatory cytokines, TNF-α, IL-6 and IL-1β, and this is accompanied with reduction of BBB permeability.

Hence, the 40% reduction in Evans blue extravasation and BBB protection by 2-AG may account for, at least in part, the reduction in edema, as its vasogenic component is mediated via disrupted BBB and suppression of TNF-α and IL-6 release. This notion is supported by our earlier studies that demonstrated less BBB disruption after treatment with TNF-binding protein or with HU-211 (Shohami et al., 1996; 1997a).

In conclusion, the present study sheds additional light on the antiinflammatory properties of the endocannabinoid 2-AG and on the mechanism by which it exerts its neuroprotection within the early period after traumatic brain injury.
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