Endocannabinoids and Neuroprotection

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(Published 23 April 2002)

Traumatic brain injury (TBI) releases harmful mediators that lead to secondary damage. On the other hand, neuroprotective mediators are also released, and the balance between these classes of mediators determines the final outcome after injury. Recently, it was shown that the endogenous brain cannabinoids anandamide and 2-arachidonoyl glycerol (2-AG) are also formed after TBI in rat and mouse, respectively, and when administered after TBI, they reduce brain damage. In the case of 2-AG, better results are seen when it is administered together with related fatty acid glycerol esters. Significant reduction of brain edema, better clinical recovery, and reduced infarct volume and hippocampal cell death are noted. This new neuroprotective mechanism may involve inhibition of transmitter release and of inflammatory response. 2-AG is also a potent modulator of vascular tone, and counteracts the endothelin (ET-1)-induced vasoconstriction that aggravates brain damage; it may thus help to restore blood supply to the injured brain.

Traumatic Brain Injury

Traumatic brain injury (TBI) accounts for an estimated 2 million cases per year in the United States alone and is one of the leading causes of mortality and disability in young individuals throughout the world (*1*). Although much research on TBI has been conducted, no specific therapy is available (*2-4*). The typical features of TBI include posttraumatic changes in neurological and cognitive functional status, increase in the cerebral water content (edema), increased permeability of the blood brain barrier, axonal injury, and neuronal cell loss in specific brain regions (*5*). Various chemical mediators are released after TBI, and the temporal changes in the concentrations of these mediators reveal impairment of brain ionic homeostasis and massive glutamate release from presynaptic terminals within minutes to hours after injury. Reactive oxygen species (ROS) are produced, and inflammatory cytokines originating in brain-resident cells (microglia, neurons, and astrocytes) accumulate in the surrounding tissue. In addition, endothelium-derived active mediators are also released, affecting the local vascular tone, with a net result of early (~1 hour after trauma) ischemia (reduced blood flow at the injured area), followed by late (>24 hours after trauma) hyperemia (excessive blood flow at the injured area) (*3*, *5*).

Experimental models have been designed to investigate the pathophysiology of TBI and to design and test new therapies (*4*, *6*). Rat and mouse models that mimic some basic features of human TBI have been developed that use a weight-drop device falling onto a closed skull [closed head injury (CHI)] (*7*). Using this model, we were able to demonstrate many of the above-mentioned features of clinical CHI (*7*), detect the accumulation of calcium at the injury site (*8*), and observe the activation of the eicosanoid pathway (*9*) and the release of harmful mediators (including ROS and inflammatory cytokines) (*10*, *11*). Local ischemia and reduced metabolism were also demonstrated within hours after CHI (*12*, *13*).

The concept of opposing responses to injury and healing has emerged, with emphasis on the role of inflammatory mediators in both processes. Endogenous protective agents can be formed that initiate a protective cascade of reactions after TBI to oppose the detrimental effects of harmful mediators. Adenosine, melatonin, sex hormones, and vasoactive intestinal peptide-related peptides have a neuroprotective role (*5*, *14-16*), and endogenous antioxidants can reduce the harmful effects caused by post-TBI oxidative stress (*3*, *10*). Endocannabinoids have also been found to be neuroprotective. The final outcome of TBI will therefore depend on the balance between the degenerative and repair mechanisms that are both intrinsic to the brain. One of the key factors that may aggravate the outcome of CHI is local vasoconstriction, which limits the blood supply to the injured area. This vasoconstriction is mainly controlled by endothelin-1 (ET-1), a mediator involved in ischemia (*17-19*). The release of this substance has been observed in patients with stroke, trauma, or vasospasm (*20*, *21*). In animal models of brain ischemia, the release of ET-1 has been linked to hypoperfusion and tissue damage, which were ameliorated by treatment with an ET receptor antagonist (*22-25*). Recently, renewed attention has been drawn to the possible role of additional endothelium-derived mediators, such as nitric oxide (NO), as factors that can control changes in vascular reactivity. Both ET-1 and NO play a major role in sustaining homeostasis of blood vessels by regulating vascular tone and blood flow, which are disturbed in trauma and ischemia (*19*, *26-28*).

The Cannabinoids

The structure of the active constituent of marijuana, Δ^9-tetrahydrocannabinol (THC), was elucidated in 1964 (*29*). However, its mechanism of action remained an enigma until a specific cannabinoid receptor was identified in the brain and later cloned. This receptor, now named CB_1, is found in brain areas such as the cerebellum, basal ganglia, hippocampus, cerebral cortex, and nucleus accumbens—areas known to be affected by THC—which are associated with motor coordination, learning, memory, higher cognitive functions, and reward pathways (*30*). The cannabinoid receptor CB_1 is also present in the periphery, particularly in the female reproductive system. A second receptor, CB_2, was identified in the periphery, mostly in cells with immune functions (*30*). Evidence for the existence of new, only partly characterized cannabinoid receptors has been published (*31*).

Because the psychotropic activity of THC is strongly enhanced in synthetic derivatives of THC, which are even more lipophilic than the already highly lipophilic natural product, we assumed that the putative endogenous compounds that bind and stimulate the cannabinoid receptors are also lipid moieties. Indeed, all three known types of endogenous cannabinoids are derivatives of arachidonic acid (or related fatty acids), namely (i) arachidonoyl

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ethanolamide (anandamide) (*32*), (ii) 2-arachidonoyl glycerol (2-AG) (*33*, *34*), and (iii) 2-arachidonyl glyceryl ether (noladin ether) (*35*) (Fig. 1). Anandamide and 2-AG bind to both cannabinoid receptors, CB_1 and CB_2, whereas noladin ether is specific for CB_1. Anandamide and 2-AG are not stored, but apparently are formed when needed. They are rapidly taken up by cells, where they are hydrolyzed by a fatty acid amide hydrolase (FAAH), which acts on both the amide anandamide and the ester 2-AG (*36*). The latter is also hydrolyzed by monoacylglycerol lipase(s) (Fig. 2).

Most of the activities of the endocannabinoids parallel those of THC; however, the effects of endocannabinoids is considerably shorter in duration because of their cellular uptake and hydrolysis by FAAH. Many pharmacological effects are produced by the endocannabinoids in the central and peripheral nervous systems, as well as in the immune, cardiovascular, and reproductive systems. However, the physiological roles of the endocannabinoids have not been fully clarified. There is strong evidence that the endocannabinoids reduce pain, block working memory, enhance appetite, regulate some immune responses, and affect cardiovascular and reproductive functions (*30*, *31*, *37*).

Many studies have focused on 2-AG as a vasoactive substance that can participate in ischemia and trauma events (*38-40*). 2-AG causes hypotension, which has been attributed to its possible hyperpolarizing properties (*41*, *42*). The induction of this potential vasomodulator in endothelium was suggested to take place in parallel to NO, through the activation of cholinergic receptors (*42*). Accumulated experimental data indicate that endothelium-dependent relaxation of some vascular beds is not contingent only on endothelium-derived relaxation factor (EDRF), but also on an endothelium-derived hyperpolarizing factor (EDHF) that is not NO or any known prostanoid (*43*). NO, which is the most effective EDRF and also affects EDHF (in some animal vessels), functions with ET-1 in the endothelium and may regulate the endothelium-dependent microvascular and capillary responses in the brain. We hypothesized that 2-AG, functioning as a potential vasorelaxant, may also interact with ET-1, the most powerful known vasoconstrictor, to provide an additional or alternative regulatory pathway for endothelium-dependent vascular reactivity (*44*).

Evidence for Neuroprotective Properties of Endocannabinoids

Some of the activities of 2-AG reported in the literature prompted us to expect that 2-AG might have neuroprotective properties. Presynaptic Ca^{2+} accumulation, through activated *N*-methyl-D-aspartate receptor channels, is an early post-TBI event, which leads to the activation of phospholipase C (PLC) and the production of diacylglycerol (DAG) and 2-AG (*45-47*).

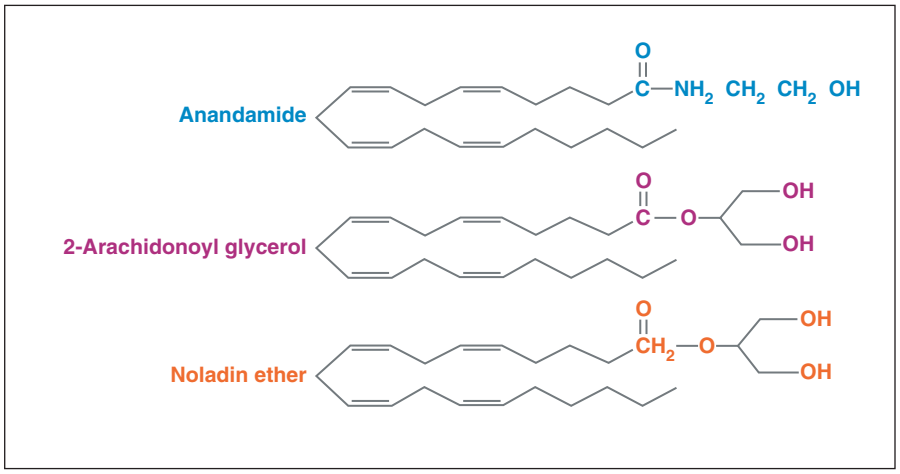

Fig. 1. Chemical structures of the endocannabinoids anandamide, 2-AG, and noladin ether.

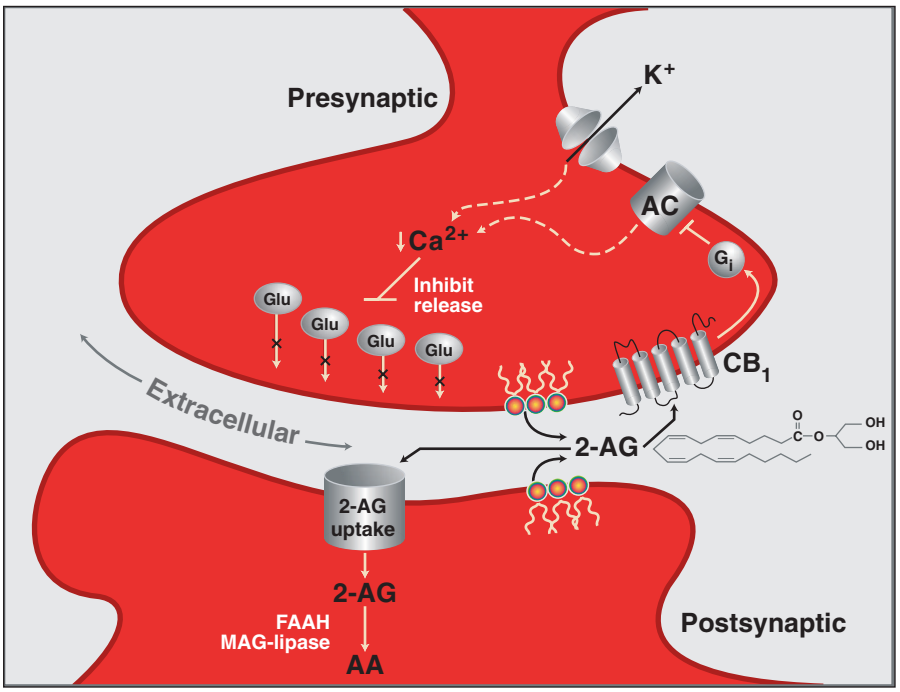

Fig. 2. An illustrative model of a neuronal protective mechanism of 2-AG [modified after (*75*)]. 2-AG is synthesized or released from phospholipids (PL) of the pre- or postsynaptic membrane, diffuses to reach the presynaptic terminal, and binds to the CB_1 receptor. The activated receptor, through a G_i-coupled mechanism [or other mechanisms (Fig. 3)], may inhibit adenylate cyclase (AC), open potassium channels, and subsequently lead to reduced Ca^{2+} accumulation. This results in the inhibition of transmitter (for example, glutamate) release. After dissociating from the receptor, 2-AG is taken up by a specific carrier into the postsynaptic cell, where it is hydrolyzed by FAAH or by monoacylglycerol (MAG) lipase(s) to yield arachidonic acid (AA), which will subsequently be either reincorporated into the membrane or oxidized to yield eicosanoids.

Excess quantities of glutamate in the extracellular space lead to uncontrolled shifts in sodium, potassium, and calcium concentrations that disrupt ionic homeostasis and lead to severe cell swelling and death. However, 2-AG-dependent activation of presynaptic CB_1 receptors on nerve terminals may modify neurotransmission and specifically inhibit glutamate release, thus limiting its excitotoxicity (Fig. 2) (*48*-*50*). A recent study demonstrated that cannabinoids inhibit presynaptic glutamate release via a receptor distinct from CB_1, which is nevertheless sensitive to inhibition by a selective CB_1 receptor antagonist, SR141716A (*51*).

Anandamide protects cerebral rat cortical neurons from in vitro ischemia (*52*), and a synthetic cannabinoid agonist, WIN-55,212, has been reported to protect the rat brain against ischemia (*53*). 2-AG suppresses the formation of ROS and the expression of tumor necrosis factor-α (TNF-α) in macrophages in vitro and suppresses TNF-α expression in macrophages in vivo (*54*). Both ROS and TNF-α are important contributors to the pathophysiology of brain injury (*11*). Finally, THC protects the rat brain against toxicity induced by the Na^+-K^+-adenosine triphosphatase inhibitor ouabain (*55*).

Human endothelial cells have been used to study the interplay between ET-1 and 2-AG (*44*). 2-AG reduced ET-1-stimulated Ca^{2+} mobilization in a dose-dependent manner. This response was not mediated by NO synthase, cyclooxygenase, or lipoxygenase activities, because the 2-AG-regulated effect was not affected by their respective inhibitors: N^G-nitro-1-arginine methyl ester, indomethacin, and nordihydroguaretic acid. SR141716A prevented the 2-AG-mediated decrease of ET-1-stimulated Ca^{2+} mobilization in a dose-dependent manner, indicating that CB_1 receptors are, at least in part, involved in this event (*44*). In addition, these data strongly suggested for the first time that cerebromicrovascular and capillary endothelium expresses cannabinoid CB_1 receptors. Liu *et al*. (*56*) also documented the presence of CB_1 messenger RNA (mRNA), binding sites, and activated signaling pathways in human endothelial cells derived from peripheral vessels. The results indicated that CB_1 receptors are coupled to the mitogen-activated protein kinase signaling cascade, which may influence gene expression related to cell growth and proliferation.

2-AG in TBI

We studied the temporal changes in 2-AG concentration after CHI in mice and the effect of exogenous 2-AG on the outcome of CHI (*57*), and found a ~10-fold increase in the amount of 2-AG in the injured hemisphere, peaking at 4 hours after CHI and persisting for at least 24 hours. Treatment with exogenous 2-AG, given 1 hour after CHI, was also effective: Clinical recovery was facilitated and edema formation and infarct volume were reduced (*57*). Moreover, a significant reduction of hippocampal cell death was observed in the CA_3 region of the 2-AG-treated mice, as compared to controls. The CB_1 antagonist SR141716A reversed, in a dose-dependent manner, the effect of 2-AG (*57*). These findings confirmed our assumption that 2-AG has neuroprotective properties after TBI.

A species difference in neuroprotection by endocannabinoids has been noted. Anandamide, but not 2-AG, accumulates after CHI in young rats (*58*), in contrast to findings in adult mice. Indeed, using a cortical impact model for trauma in rats, we found a decrease in 2-AG, which was reversed by treatment with DFU [5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2 (5H)-furanone], a selective inhibitor of cyclooxygenase-2. The restoration of 2-AG concentrations after this treatment was associated with better recovery (*59*). Van der Stelt *et al*. reported that exogenous anandamide protects the rat brain against neuronal injury caused by ouabain (*60*). However, the endogenous levels of either 2-AG or anandamide did not change during ouabain-induced neuronal damage. Furthermore, the CB_1 antagonist SR141716A and the inhibitor of endocannabinoid re-uptake VDM11 did not significantly modify ouabain-induce neuronal damage. The authors interpreted these data as suggesting that endocannabinoids do not tonically protect the neonatal rat brain from ouabain-induced neuronal damage.

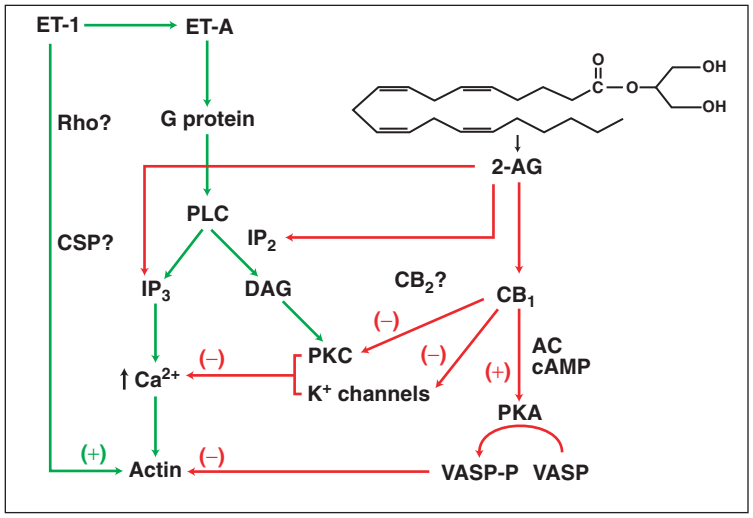

Fig. 3. Schematic diagram of ET-1 and 2-AG pathways based on published and personal observations. rho, family member of the small GTPases; CSP, contractile stimulating protein; ET-A, endothelin receptor type A; VASP-P, phosphorylated VASP; +, stimulatory effects; –, inhibitory effects.

Mechanisms by Which 2-AG May Exert Neuroprotection

Inhibition of Glutamate Release. Many experimental and clinical studies on neuroprotection have been performed using antagonists selective for the various glutamate receptor subtypes (*61*, *62*); however, none of the antagonists have made a breakthrough into clinical use. The protective effect of 2-AG may therefore lie in its activity as an inhibitor of neurotransmitter release (mediated by the inhibition of Ca^{2+} mobilization) (Fig. 2) rather than by acting at a specific subtype(s) of glutamate (or other) receptors.

Inhibition of Inflammatory Cytokines and ROS. The detrimental roles of inflammatory cytokines and ROS within hours after TBI are now well recognized (*11*). Indeed, cannabidiol and THC appear to be antioxidant neuroprotectants that presumably act on ROS (*63*). The inhibition of TNF-α and ROS production, as mediated by 2-AG, in macrophages (*54*) provides support for the beneficial use of 2-AG after CHI. The mechanism by which 2-AG inhibits TNF-α production is not fully elucidated. However, a synthetic cannabinoid, dexanabinol, which is devoid of psychotropic activity but shares the neuroprotectant action of 2-

AG (*64*), inhibits the activation of the transcription factor nuclear factor kappa B (NF-κB), which is involved in the induction of cytokine gene expression (*65*). Whether 2-AG has similar effects on this nuclear factor and whether such activity is associated with neuroprotection remain unclear.

Improved Glucose Utilization. One signaling pathway that might explain the neuroprotective effect of 2-AG involves the lipid second messenger ceramide. Activation of the CB_1 receptor triggers the generation of ceramide, which has been linked to the regulation of cell metabolism (*66*). In this study, cannabinoids stimulated the uptake and consumption of glucose and the production of ketone bodies through a CB_1-mediated mechanism. This pathway may provide another mechanism of neuroprotection that needs to be elucidated. Indeed, it has been reported that ketone bodies have neuroprotective effects in neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases (*67*).

Counteracting the Vasoconstrictive Effect of ET-1. The interplay between ET-1 and 2-AG may explain the mechanism by which 2-AG maintains cerebral blood flow in the endothelium, thus providing protection after CHI (Fig. 3). The mechanisms involved in this interplay involve Ca^{2+} mobilization, cytoskeleton rearrangements, and changes in vasodilator-stimulated phosphoprotein (VASP) concentration or phosphorylation state changes that are mediated by adenosine 3′,5′-monophosphate-(cAMP)-dependent protein kinase A (PKA) (*68*). 2-AG inhibits ET-1-induced Ca^{2+} mobilization, and this inhibition is partially blocked by bisindolymaleimide (BIS), a selective inhibitor of protein kinase C (PKC), suggesting that this kinase may be involved in the regulation of 2-AG- and ET-1-mediated effects.

The capacity of inhibitors of calcium-dependent potassium channels or potassium-sensitive channel blockers (for example, quinine, apamine, or charybdotoxin) to partially reverse the 2-AG-induced inhibition of ET-1-stimulated Ca^{2+} mobilization suggest that these channels may also function in the ET-1 response. Along the same line, the role of such channels has been demonstrated for a novel non-CB_1 and non-CB_2 receptor on mesenteric endothelial cells, the activation of which elicits NO-independent mesenteric vasodilation, possibly by means of the release of EDHF (*69*). In addition, high K^+ concentrations, in part, prevented the 2-AG reduction of ET-1-inducible Ca^+ mobilization and cytoskeleton rearrangement in the endothelium. Taken together, these observations suggest that 2-AG may function as a hyperpolarizing agent (*44*).

Further characterization of the signal transduction pathway distal to the ET-1 receptor showed that 2-AG also reduced Ca^{2+} mobilization by mastoparan-7 (Mas-7, the active analog of mastoparan, a peptide venom from wasps that increases intracellular Ca^{2+} concentrations) and decreased the ET-1-stimulated production of inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3). These changes in calcium mobilization and phosphoinositide production were associated with a reduction in the density and diameter of ET-1-induced cytoskeletal filaments. In addition, 2-AG enhanced the phosphorylation of VASP; this phosphorylation was blocked by H8 (a preferential PKA inhibitor), but not by H7 (a preferential PKG inhibitor) or BIS (a PKC inhibitor) (*44*). These findings clearly indicate that 2-AG is capable of abrogating ET-1-induced Ca^{+2} mobilization and altering cytoskeleton assembly in the brain endothelium, in a manner similar to that of NO but by different mechanisms.

Ca^{2+} and phosphoinositides play a regulatory role in assembling and disassembling actin and are themselves modulated by upstream signals that include small guanosine nucleotide triphosphatases (GTPases), protein kinases, and ion channels (*70*, *71*). In addition, the association of VASP with actin filaments may both impede and promote actin assembly (*72*). Even though the exact mechanisms responsible for either ET-1- or 2-AG-mediated effects on the endothelium remain unknown, it is clear that cytoskeletal filaments can be altered by these vasoactive substances. These results, therefore, strongly suggest that cytoskeletal structures play an important physiological role in endothelium function. This contention is in agreement with the reported prevention of ischemic injury by the modulation of the structure of actin filaments in the endothelial cytoskeleton (*73*).

Most important, the functional interplay between 2-AG and ET-1 provides an alternative pathway that may dominate when the NO-mediated guanosine 3′,5′-monophosphate-dependent pathway of relaxation is impaired (for example, by trauma, ischemia, hypertension, diabetes mellitus, or hypercholesterolemia) (*27*, *43*, *74*). The existence of both such pathways may prove to be useful in designing strategies for the treatment of vascular diseases.

Recent research has shown that the endocannabinoids represent a new class of neuroprotective agents that have multiple mechanisms of action apparently involving the inhibition of neurotransmitter release and inflammation, as well as improving the blood supply to the injured brain.

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Citation: R. Mechoulam, M. Spatz, E. Shohami, Endocannabinoids and Neuroprotection. *Science's STKE* (2002), http://www.stke.org/cgi/content/full/OC_sigtrans;2002/129/re5