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Acute Neuronal Injury, Excitotoxicity, and the Endocannabinoid System

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Abstract

The endocannabinoid system is a valuable target for drug discovery, because it is involved in the regulation of many cellular and physiological functions. The endocannabinoid system constitutes the endogenous lipids anandamide, 2-arachidonoylglycerol and noladin ether, and the cannabinoid CB_1 and CB_2 receptors as well as the proteins for their inactivation. It is thought that (endo)cannabinoid-based drugs may potentially be useful to reduce the effects of neurodegeneration. This paper reviews recent developments in the endocannabinoid system and its involvement in neuroprotection.

Exogenous (endo)cannabinoids have been shown to exert neuroprotection in a variety of in vitro and in vivo models of neuronal injury via different mechanisms, such as prevention of excitotoxicity by CB_1-mediated inhibition of glutamatergic transmission, reduction of calcium influx, and subsequent inhibition of deleterious cascades, TNF-α formation, and anti-oxidant activity. It has been suggested that the release of endogenous endocannabinoids during neuronal injury might be a protective response. However, several observations indicate that the role of the endocannabinoid system as a general endogenous protection system is questionable. The data are critically reviewed and possible explanations are given.

Index Entries: Cannabinoid; anandamide; 2-arachidonoylglycerol; excitotoxicity; neuroprotection; neurodegeneration.

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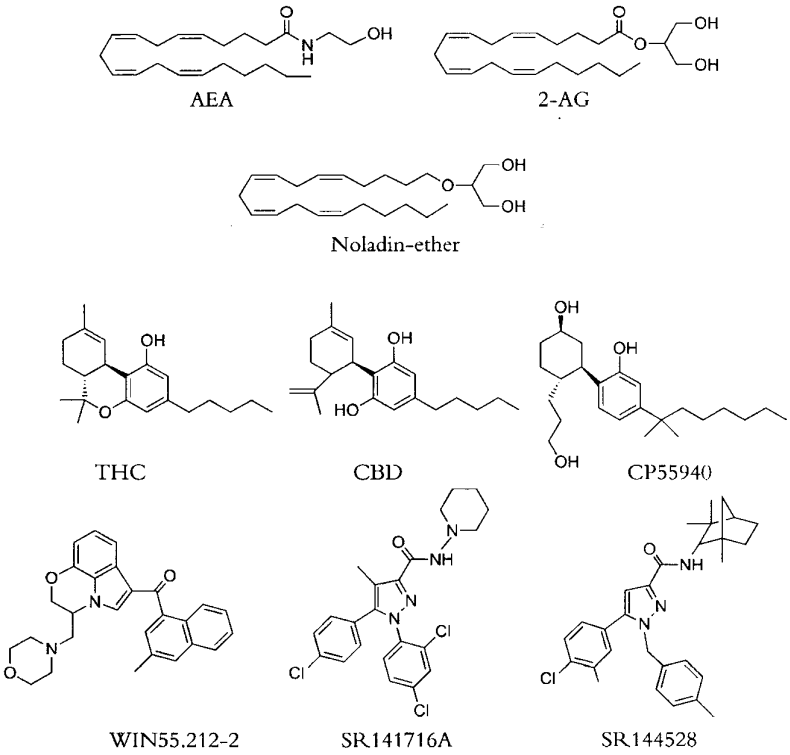

Fig. 1. Ligands of the cannabinoid receptors.

The Endocannabinoid System

Biosynthesis of Endocannabinoids

By definition *(1)*, "endocannabinoids" are endogenous substances capable of binding to, and activating at least one of the two high affinity cell membrane receptors for marijuana's psychoactive principle, (–)–Δ^9-tetrahydrocannabinol (THC) (*see* Fig.1). To date, three types of endocannabinoids have been described in animal nervous and/or non-nervous cells: 1) the anandamides, i.e., amides of ethanolamine with polyunsaturated fatty acids containing at least twenty carbon atoms and three 1,4-diene double bonds, of which the C20:4 homologarachidonoylethanolamide (AEA) *(2,3)* is the best known and most thoroughly studied one; 2) the polyunsaturated 2-acylglycerols, of which 2-arachidonoyl glycerol (2-AG) is the most active and abundant component *(4,5)*; and 3) the recently described 2-arachidonyl glyceryl ether, or noladin ether *(6)*, whose biosynthetic pathway remains controversial, with its pharmacological activity as an endocannabinoid not yet fully assessed (Fig.1). An entirely saturated AEA congener, palmitoylethanolamide (PEA), was proposed to act as an endocannabinoid at yet-to-be-characterized receptors, but the precise mechanism(s) underlying the activity of this compound in some assays is/are still a matter for speculation *(7,8)*. From of these compounds, biochemical mechanisms or the synthesis and inactivation have been described for

the anandamides, PEA, and 2-AG (*see* ref. *9* for review).

It is widely recognized that endocannabinoids are not stored in vescicles like other mediators but, by analogy with other eicosanoids, are produced "on demand". This peculiarity of AEA and 2-AG production by cells is the result of a biosynthetic mechanism relying on the existence of phospholipid precursors for these compounds, and of Ca^{2+}-sensitive and, possibly G-protein-activated, phosphodiesterases for the conversion of these precursors into their endocannabinoid products. The biosynthesis of endocannabinoids is immediately followed by their release, which occurs through the cell membrane by the laws of mass action according the concentration gradient across the membrane, possibly facilitated by one or more selective membrane transporters (*see* below). Hence, unlike other mediators and neurotransmitters, there cannot be enhanced endocannabinoid release outside the cell without enhanced endocannabinoid biosynthesis. Consequently, the amounts of AEA and 2-AG are measured in tissues under physiological or pathological conditions reflect the activities of the proteins which mediate their synthesis and degradation. The biosynthetic activities appear to be upregulated during cell damage *(10)*. Importantly, the cell (or mitochondrial) membrane-dependent biosynthetic mechanism of AEA and 2-AG allows them to be produced in principle from both axons and somas in neurons, and to activate receptors located either pre- or post-synaptically. In particular, it is known that AEA is produced from the hydrolysis of *N*-arachidonoyl-phosphatidylethanolamine (NarPE) *(11)*. This process is catalyzed by a specific phospholipase D-like enzyme, which has recently been partially purified and characterized (*see* Fig. 2) *(12,13)*. The same enzyme also catalyzes the formation of other acylethanolamides including PEA, from the corresponding *N*-acylphosphatidylethanolamines (NAPEs) (*see* ref. *14* for review). NarPE and other NAPEs are produced in turn from the *N*-acylation of phosphatidylethanolamine using, as acyl donors, the fatty acids coming from the *sn*-1 position of most phospholipids. This reaction is catalyzed by a *trans*-acylase (or *N*-acyl-transferase). The low amounts of arachidonic acid esterified on *sn*-1 of phospholipids explains why among NAPEs (and, subsequently, acylethanolamides), those with a *N*-arachidonoyl are the least abundant. Both phospholipase D and, particularly, *N*-acyl-transferase are Ca^{2+}-sensitive enzymes, which explains why AEA biosynthesis is triggered by Ca^{2+}-ionophores and neuronal membrane depolarization. The biosynthesis of 2-AG occurs by hydrolysis of *sn*-2-arachidonate-containing diacylglycerols by a *sn*-1-selective diacylglycerol lipase (*see* Fig. 3). The formation of these diacylglycerols relies on different types of phospholipid precursors, i.e., *sn*-2-arachidonate-containing phosphatidylinositol, phosphatidyl-choline or phosphatidic acid, and is catalyzed by Ca^{2+}-sensitive phospholipases C or Ca^{2+} (Mg^{2+})-sensitive phosphatidic acid hydrolase, respectively *(15–17)*.

Endocannabinoids and Their Membrane Receptors

Once released by stimulated cells, endocannabinoids act primarily at cannabinoid receptors. Although pharmacological and biochemical evidence is accumulating for the existence of more than two subtypes of such receptors, the only G-protein-coupled membrane proteins characterized so far (that are activated by AEA and 2-AG) are the CB_1 and CB_2 cannabinoid receptors *(18)*. Both endocannabinoids bind to both receptors, but only 2-AG is functioned as a potent full agonist at the human CB_2 receptor, whereas AEA is a weak partial CB_2 agonist *(19)*. While CB_2 receptors seem to be expressed almost uniquely in immune-competent cells, detectable levels of CB_1 receptors are found not only in central and peripheral (both sensory and autonomic) neuronal cells, but also in a number of other cell types, including: immune cells, astrocytes, gametes and reproductive tissue cells, endothelial cells from renal and vascular tissues, epithelial cells of various origin, etc. This

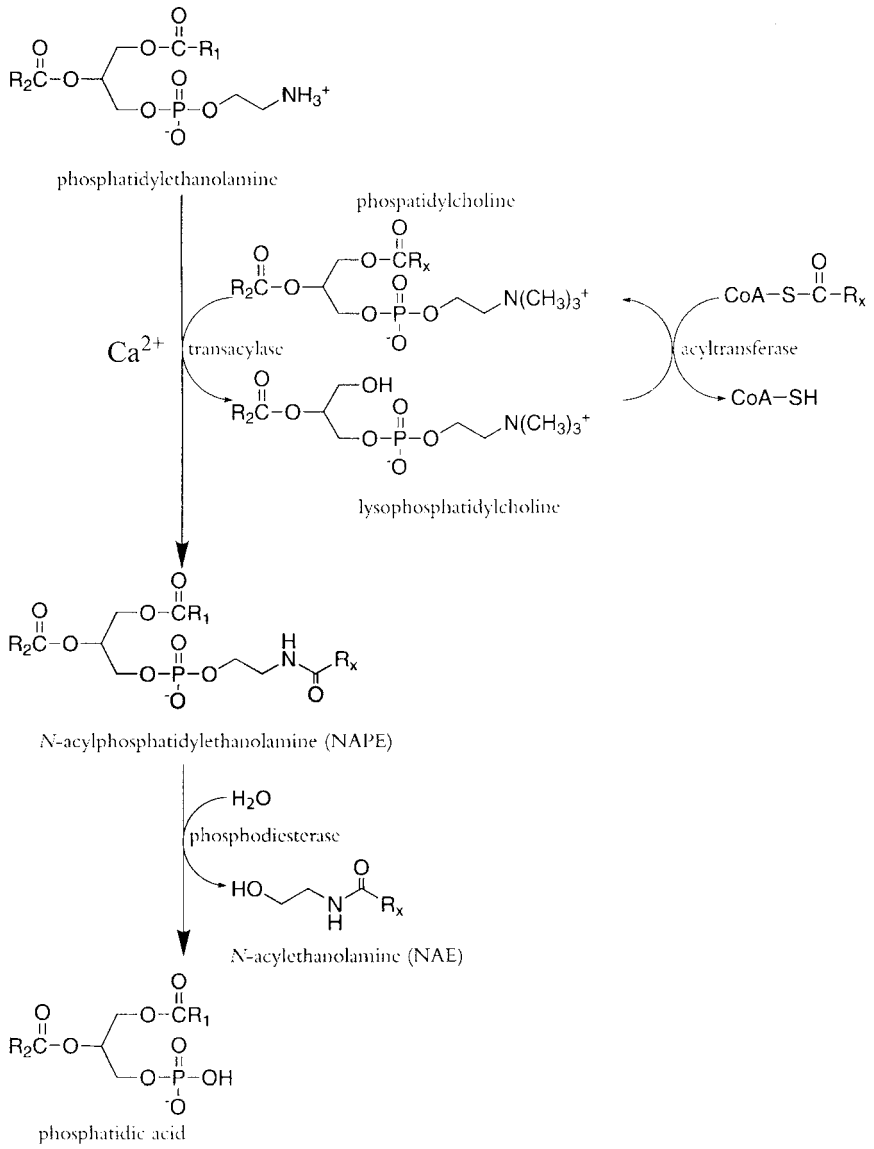

Fig. 2. Biosynthetic pathway of *N*-acylethanolamines, including anandamide (R_x = 20:4).

explains why it is possible to find this receptor subtype expressed not only in the CNS, where it is undoubtedly most abundant, but also in several peripheral organs *(18)*. In the brain, CB_1 is densest in areas controlling motor, cognitive, emotional, and sensory functions, i.e., the hippocampus, basal ganglia, cerebellum, cortex, thalamus, amygdala, and olfactory bulb. However, small nuclei with high density of CB_1 receptors are also found in other areas, for example those controlling pain, body temperature, sleep-wake cycles, and hormone function, such as the brainstem, the hypothalamus, and the pituitary gland *(20)*. Levels of

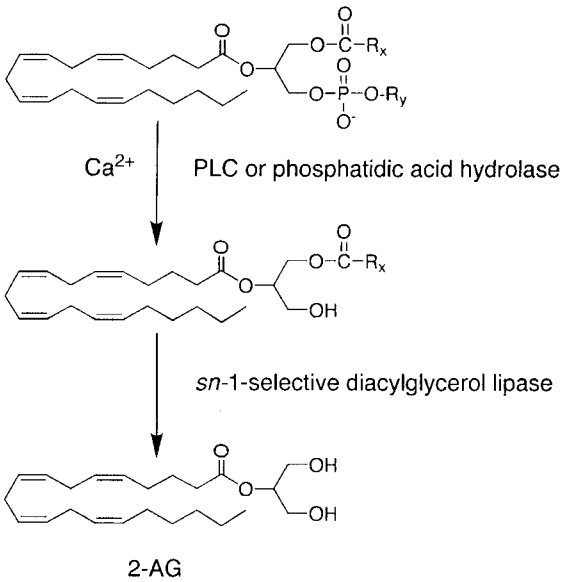

Fig. 3. Biosynthetic pathway of 2-arachidonoylglycerol. R_x = acyl chain, R_y = H, inositol, or choline.

CB_1 receptors measurable by immunohistochemistry or, indirectly, by *in situ* hybridization are also found in the spinal cord, dorsal horn and dorsal root ganglia, as well as in sensory fibers innervating the airways, the gut and the cardiovascular system, and in sympathetic fibers. The CB_2 receptors, by being expressed in cells involved in immune and inflammatory reactions in both central and peripheral nervous systems (including microglia, mast cells, and macrophages), might contribute to causing or inhibiting neuronal toxicity by up- or down-modulating the generation of mediators such as interleukins, nitric oxide (NO), and tumor necrosis factor (TNF)-α. (*See* ref. *21* for a review).

The existence of non-CB_1, non-CB_2 G-protein-coupled receptors (GPCRs) for endocannabinoids, and AEA in particular, has been suggested based on pharmacological and biochemical data. However, it should be noted that these putative proteins which might interact with endocannabinoids have not been identified or cloned yet. The first one was proposed to be present in astrocytes of mice and, to a smaller extent, rats and found to respond also to the non-cannabinoid agonist of CB_1 and CB_2 receptors, WIN55.212-2 *(22)*. Apart from inhibition of cAMP levels, this (or a very similar) receptor might regulate Ca^{2+} homeostasis in astrocytes by inhibiting gap-junctions *(23)*. Interestingly, a GPCR was also detected in several brain areas of CB_1 knock-out mice, with a distribution different from CB_1 receptors *(24,25)*. Also this site was activated functionally by AEA and WIN55.212-2, but not by other cannabinoid receptor agonists. In vascular endothelial cells a different GPCR for AEA and the non-psychtropic cannabinoid, abnormal cannabidiol, has been described *(26)*. This receptor participates in AEA-induced local vasodilation and could hence be involved, together with CB_1, in neuroprotection at the level of cerebral artery functionality.

Finally, several non-GPCR molecular targets for AEA have been proposed *(27)*. They are all membrane cation channels and are usually modulated specifically by AEA at low or submicromolar concentrations. In particular, TASK-1 K^+ channels *(28)* and T-type Ca^{2+} channels *(29)* are blocked by AEA, whereas vanilloid type 1 receptors (VR1), the sites of action for the pungent component of 'hot' red peppers, capsaicin, are activated by this endocannabinoid *(30,31)*. In heterologous expression systems the potency of AEA to induce typical VR1-mediated effects (e.g., cation currents, Ca^{2+}-influx and cell depolarization) is 5–20 fold lower than its average potency at CB_1 receptors. However, recent data, reviewed by Di Marzo et al. *(32)*, indicate that several in vitro and ex vivo pharmacological actions of AEA are due to activation of native vanilloid receptors, possibly under conditions of inflammation. Interestingly, the effects of AEA on both VR1 receptors and T-type Ca^{2+} channels seem to be exerted by binding to sites on the cytosolic side of these membrane proteins *(29,33)*. These effects, if shown to occur also with *endogenous* AEA, raise the intriguing possibility that this compound, when biosynthesized by neurons which express VR1 or T-type Ca^{2+} channels on their cell membrane,

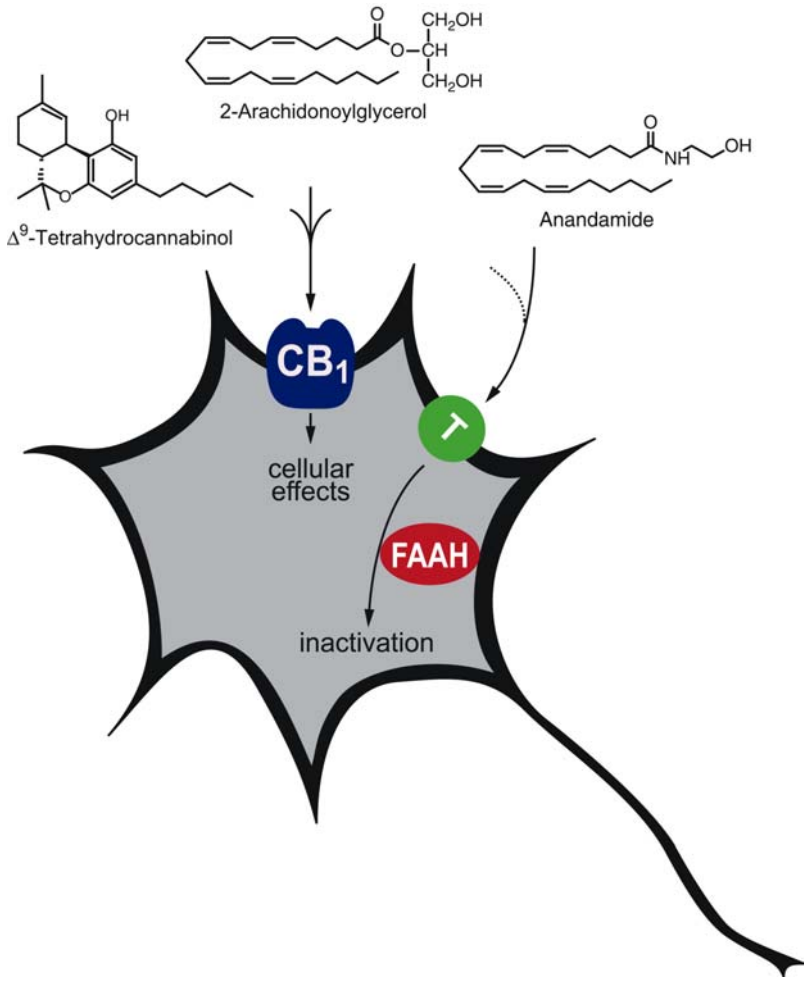

Fig. 4. The endocannabinoid system, including the cannabinoid (CB_1) receptor, transporter protein (T), and fatty acid amide hydrolase (FAAH), which inactivates the endogenous cannabinoids 2-arachidonolglycerol (2-AG) and anandamide (AEA). Adapted from ref. *(8)*.

activates these proteins *prior* to its release from cells and its action on cannabinoid receptors.

Inactivation of Endocannabinoids

Mechanisms for the termination of the signal carried by endocannabinoids have been identified (*see* Fig. 4) *(11,34)*. When AEA and 2-AG act by binding to sites on the extracellular side, one simple way of inactivating them is to allow their diffusion away from these sites by decreasing their extracellular concentration. This is achieved by means of a transporter (or two distinct albeit very similar transporters [*35*], named "anandamide membrane transporter" (AMT). The AMT has been only partially characterized from many neuronal and non-neuronal cells as a temperature-sensitive, saturable, and Na^+-insensitive selective carrier protein for the facilitated transport across the membrane (*see* ref. *36* for a comprehensive review). Recently, it has been shown that AMT also mediates the cellular uptake of noladin ether in both glioma C6 and basophilic RBL-2H3 cells *(37)*. Since this(ese) protein(s) transport(s) endocannabinoids according to the

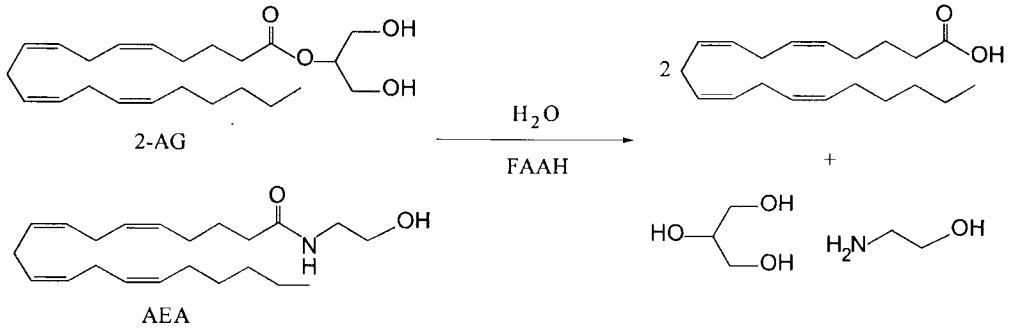

Fig. 5. Reaction scheme of inactivation of endocannabinoids.

concentration gradient across the membrane, the AMT has been suggested to: 1) facilitate AEA *release* from neurons immediately after AEA biosynthesis, by working in the opposite direction to cellular uptake *(38)*; and 2) require intracellular metabolism to carry on clearing AEA (and possibly 2-AG) from the extracellular milieu. Indeed, both AEA and 2-AG are subject to intracellular metabolism, mostly through hydrolysis of their amide or ester bond, respectively, which can be catalyzed by the enzyme "fatty acid amide hydrolase" (FAAH) (Fig. 5) (see refs. *39,40* for a comprehensive review). This enzyme is distributed in CNS neurons in a complementary way with CB_1 receptors, in support of its role as an endocannabinoid inactivating enzyme, a role further confirmed by recent studies carried out in FAAH "knockout" mice *(41)*. However, in the absence of FAAH, other metabolic reactions can occur for the endocannabinoids. For example:

1. 2-AG can either be hydrolyzed by other monoacylglycerol lipases, or directly esterified into phospholipids and di- or tri-acylglycerols *(34,42,43)*.
2. AEA can be hydrolyzed by another amidase *(44)* which shows preference for the other cannabimimetic fatty acid amide, PEA.
3. AEA and 2-AG can be oxidized by:
   a. Cyclooxygenase-2 (COX-2), and subsequently metabolized to prostaglandin ethanolamides or glycerol esters, which are inactive on cannabinoid receptors; or
   b. Various lipoxygenases to hydroperoxy- and hydroxy-derivatives, which in some cases are still capable of binding to cannabinoid receptors *(45)*.
4. Noladin ether, whose chemical nature as an ether makes its enzymatic hydrolysis very unlikely, can be directly esterified into phospholipids *(37)*.

Of these alternative reactions, the COX-2 reaction is relevant to neuroprotection as this enzyme is known to be expressed during conditions leading to neuronal damage and inflammation, and this might lead to excessive degradation of endocannabinoids, with a possible worsening effect on brain injury. FAAH expression/activity can also be down-regulated in cells potentially involved in cell damage, for example:

1. By lipopolysaccharide (LPS) in lymphocytes, as during septic shock *(46)*;
2. By lipoxygenase derivatives in mast cells *(47)*; and
3. By "entourage" fatty acid amides and esters, i.e., cannabinoid-receptor inactive congeners of AEA and 2-AG that are present in cells and are in some cases co-biosynthesized with the endocannabinoids *(48–50)*.

The activity of monoacylglycerol lipase responsible for 2-AG hydrolysis in rat circulating macrophages and platelets can also be down-regulated by LPS *(42)*. Finally, regulation of endocannabinoid inactivation during cell injury may also occur at the level of AMT, which is stimulated by NO in several cell types *(51,52)*. Interestingly, the activation of the AMT by NO leads to inhibition of those effects of AEA and 2-AG that are mediated by CB_1

receptors, but instead to the enhancement of those effects of AEA that are due to VR1 activation *(33)*.

Endocannabinoid Signalling

Both CB_1 and CB_2 receptors inhibit cAMP formation via G_i-proteins and activate mitogen-activated-protein (MAP) kinases *(18)*. In addition, CB_1 receptors activate ion channels such as A-type and inwardly rectifying potassium channels, and inhibit voltage sensitive N-type and P/Q-type calcium channels, and D-type potassium channels *(9,53,54)*. The coupling to A- and D-type potassium channels is thought to be regulated via cAMP. Activation of CB_1 receptors may also lead to arachidonic acid release and closing of 5-HT_3-receptor ion channels. Under certain conditions, the CB_1 receptor may couple to G_s-proteins, which activate adenylate cyclase and reduce outward potassium K^+ current *(55)*. CB_1- and CB_2-receptor activation by AEA and 2-AG in N18TG2 neuroblastoma and NG108-15 neuroblastoma-glioma hybrid cells have been shown to induce a rapid transient increase in intracellular free calcium via its release from IP_3-sensitive calcium stores *(56,57)*. It should be noted that CHO-cells transfected with either CB_1 or CB_2 receptors failed to exhibit changes in IP_3 or phosphatidic acid in response to AEA or WIN55212-2, under conditions in which other exogenously expressed receptors coupled to phospholipases C could evoke such responses *(18,58,59)*. AEA has been shown to reduce NMDA-receptor mediated calcium responses in rat cortical and cerebellar slices, which could be blocked by pertussis toxin and agatoxin (P/Q-type calcium channel blockers) *(60)*. It was suggested that voltage-sensitive calcium channels were activated in response to the depolarization associated with NMDA-induced calcium influx, so that their inhibition by cannabinoid receptor activation reduced the overall calcium current *(60)*. Noteworthy, AEA potentiated NMDA-induced calcium currents in cortical, cerebellar, and hippocampal slices in the presence of SR141716A. This capacity of AEA to increase calcium currents was also observed in *Xenopus* oocytes expressing NMDA receptors, which suggested that AEA could directly interact with NMDA receptors *(60)*. In the same expression system AEA was shown to directly inhibit AMPA receptor currents in a CB_1-receptor independent manner *(61)*. Furthermore, it was recently shown that CB_1 receptors in cultured cerebellar granule neurons could operate through a phospholipase C-sensitive mechanism to enhance NMDA-elicited calcium release from inositol-1,4,5-triphosphate-gated intracellular stores *(62)*.

Cannabinoids and Glutamatergic Neurotransmission

Cannabinoids inhibit neurotransmitter release via specific presynaptic CB_1 receptors *(63)*. In this review, the authors focus on glutamate, which is the principal excitatory neurotransmitter in the CNS. It is present in millimolar concentrations in the brain and in micromolar concentrations in the extracellular fluid. Its clearance from the extracellular space is mediated almost exclusively by neuronal and astrocytic glutamate transporters *(64)*. The postsynaptic effects of glutamate are mediated by ionotropic *N*-methyl-D-aspartate (NMDA), kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolproprionate (AMPA)-receptors, and by metabotropic receptors.

In rat hippocampal cultures glutamatergic transmission could be blocked by cannabinoids at subnanomolar concentrations in a receptor-mediated manner *(65)*. The inhibition was stereo-selective with the following rank order CP55.939> CP55.940> WIN55.212-2> AEA, and could be blocked by pertussis toxin. WIN55.212-2 blocked stereo-selectively AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) elicited by presynaptic stimulation with an extracellular electrode, but did not affect the presynaptic action potential or currents elicited by direct application of kainate *(65)*. It was suggested that this CB_1-mediated inhibition of excitatory synaptic transmission was developmentally regulated

in rat hippocampus, being most prominent in neonatal rats *(66)*. Furthermore, other studies have indicated that CB_1 receptors are involved in the regulation of long-term potentation in the hippocampus *(67)*. By contrast, histological studies could not detect CB_1 receptors on glutamatergic terminals in the hippocampus. A possible explanation was recently put forth *(68)*. Electrophysiological measurements in hippocampal slices of CB_1 knock-out mice suggest that a novel cannabinoid-sensitive receptor could be responsible for the inhibition of glutamatergic neurotransmission *(68)*. This receptor could be the non-CB_1 non-CB_2 G-protein coupled receptor for AEA and WIN55.212-2 detected in several areas of CB_1-receptor knockout mouse brain, including the hippocampus *(25)*. Nevertheless, concrete proof of a new CB-type receptor in the hippocampus awaits its molecular characterization.

Recently, it was shown that WIN55.212, HU210, and AEA inhibited excitatory synaptic transmission in rat striatal neurons *(69,70)*. HU210 reduced the amplitude of evoked EPSCs in a dose-dependent matter. WIN55.212-2 significantly increased the paired-pulse facilitation of synaptically evoked EPSCs, while having no effect on the sensitivity of postsynaptic neurons to AMPA *(69)*. Superfusion of WIN55.212-2 elicited a membrane hyperpolarization accompanied by a decrease in input resistance. The WIN55.212-2-mediated synaptic inhibition was blocked by the $G_{i/o}$-protein inhibitor, pertussis toxin, and reversed by SR141716, but not by $GABA_A$ receptor antagonist bicuculline or $GABA_B$ receptor antagonist SCH50911 *(69)*. Pretreatment with the N-type Ca^{2+} channel antagonist ω-conotoxin (GVIZ) selectively abolished the WIN-55.212-2 mediated synaptic inhibition. These results suggest that cannabinoids depress corticostriatal glutamatergic synaptic transmission through the activation of presynaptic CB_1 receptors to inhibit N-type Ca^{2+} channels activity, which in turn reduces glutamate release. When Ca^{2+} was replaced by Sr^{2+} in the extracellular solution, application of HU-210 significantly reduced the frequency, but not amplitude, of evoked, asynchronous quantal release events *(70)*. Spontaneous synaptic release events occurring between stimuli were similarly decreased in frequency but not in amplitude. Thus, while CB_1-activation may lead to reduced glutamate release via reduced Ca^{2+} influx, a more direct and Ca^{2+}-independent inhibition of the release process may also be implicated *(70)*.

CB_1-mediated inhibition of glutamatergic transmission has also been found in rat cerebellar slices *(71)* and substantia nigra pars reticulata *(72)*, in rat substantia gelatinosa neurons of the spinal cord *(73)*, mouse nucleus accumbens *(74)* and in slices of the periaqueductal gray *(75)*, as well as in rat prefrontal cortex pyramidal neurons *(76)*. In the latter study it was shown that SR141617A acutely increased glutamatergic transmission and favoured Long Term Potentiation at the expense of Long Term Depression *(76)*. In contrast, in vivo microdialysis in rat prefrontal cortex revealed that i.p. injection of WIN55.212-2 led to a release of glutamate, which was counteracted by SR141716A *(77)*.

The findings described above were taken a step further by the results of a number of newer studies in which endogenously-released endocannabinoids were shown to mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals (*see* Fig. 6) *(78–85)*. It is now thought that endocannabinoids are responsible for depolarization-induced suppression of inhibition and excitation (DSI and DSE). In hippocampal neurons, depolarization of postsynaptic neurons and resultant elevation of intracellular Ca^{2+} led to transient suppression of inhibitory transmitter release, while in cerebellar purkinje cells postsynaptic elevation of intracellular Ca^{2-} concentrations caused transient suppression of excitatory transmitter release. These effects could be mimicked by cannabinoid receptor agonist WIN55.212-2, and were completely blocked by application of the CB_1 antagonists SR141716A and AM281 *(78–84)*. DSI was not present in CB_1 knock-out mice, and pharmacological and kinetic data suggested that CB_1 activation inhibited presynaptic Ca^{2+} channels

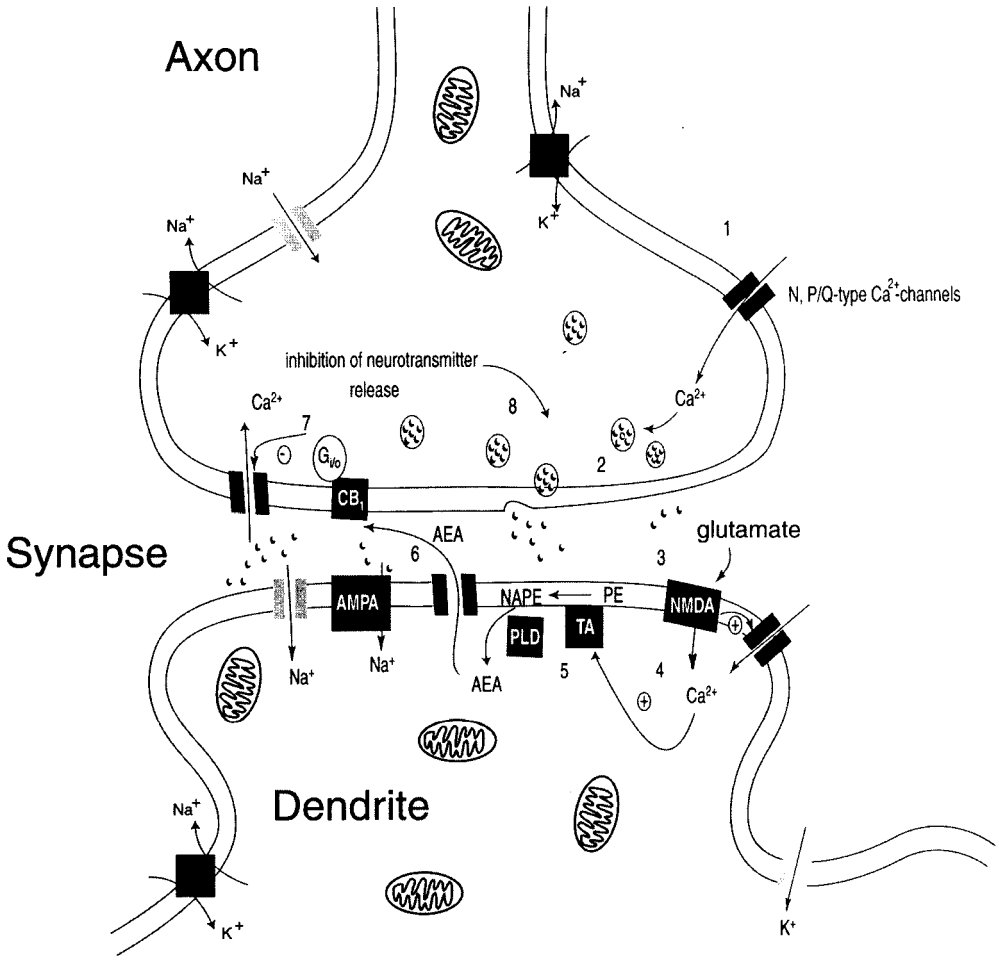

Fig. 6. Model of hypothetical retrograde AEA-action. **(1)** Presynaptic depolarization leads to calcium influx, which in turn activates glutamate exocytosis. **(2)** Glutamate diffuses through synaptic cleft and activates postsynaptic glutamate receptors. **(3)** Activation of NMDA, AMPA and other glutamate-subtype receptors leads to postsynaptic depolarization and calcium influx. **(4)** Elevated postsynaptic calcium levels activate a transacylase (TA), which converts phosphatidylethanolamine (PE) into *N*-arachidonoylphosphatidylethanolamine (NAPE). **(5)** NAPE is hydrolysed by a phospholipase D (PLD), which yields AEA. AEA is released from the postsynaptic cell and diffuses back to presynaptic CB_1 receptors. **(6)** Upon activation of CB_1 receptors by AEA, $G_{i/o}$-proteins are released, which inhibit N-, and P/Q-type voltage-sensitive calcium channels. **(7)** Closing of voltage-sensitive calcium channels results in a reduced release of neurotransmitters, such as glutamate or GABA. **(8)** Adapted from ref. *(8)*.

through direct G-protein inhibition *(78)*. Metabotropic glutamate receptor antagonists, a $GABA_B$ receptor antagonist and postsynaptically applied botulinum toxin—which inhibits vesicular glutamate release from the postsynaptic cell—were ineffective. Interestingly, flash photolysis of caged Ca^{2+} was sufficient to induce DSI. However, stimulation of metabotropic glutamate receptors, without elevation of calcium levels, could also induce cannabinoid-dependent DSE in cerebellar purkinje cells *(80)*. It is still unclear which endo-

cannabinoids are responsible for DSI and DSE. The mechanism of their synthesis and release, which could be triggered either by depolarization of the postsynaptic neuron or by phospholipid remodelling induced by metabotropic glutamate receptor activation, is also unclear. Interestingly, the induction of a form of striatal synaptic plasticity, long-term depression (LTD), was dependent on activation of the CB_1 receptor. LTD was facilitated by blocking cellular endocannabinoid uptake, and postsynaptic loading of AEA produced presynaptic depression *(85)*. The endocannabinoid necessary for striatal LTD was suggested to be released postsynaptically as a retrograde messenger.

Acute Neuronal Injury and Excitotoxicity

Stroke, Traumatic Brain Injury, and Excitotoxicity

Stroke and traumatic brain injury (TBI) are leading causes of death and permanent disability, and a socio-economic factor of major importance in developed countries. Excitotoxicity takes center stage in the pathologic sequelae after stroke and TBI. In exitotoxicity, cell death is initiated by the over-stimulation of excitatory amino acid receptors by high concentrations of extracellular glutamate. *(86–92)*. Excitototoxicity can be triggered by ATP depletion after stroke, by direct mechanic damage, lysis of neighboring cells, and edema-induced ischemia after traumatic brain injury *(93)*. Excitotoxicity has also been implicated in relatively slowly progressing neurodegenerative disorders like amyotrophic lateral sclerosis and Huntington's or Parkinson's diseases. However, the evidence for the participation of excitotoxicity is less clear and indirect, but neurons that are already weakened might not survive glutamate concentrations that would normally not be lethal. This latter form of 'slow excitotoxicity' is relevant to diseases that involve mitochondrial, metabolic, or genetic abnormalities and is beyond the scope of this review (reviewed in ref. *94*).

The ionotropic AMPA- and kainate-receptors are activated upon glutamate binding. The resultant influx of Na^+ after over-stimulation of these ion channels disturbs cellular ion homeostasis and leads to a rapid dissipation of the membrane potential. Membrane depolarization triggers the opening of voltage-gated Ca^{2+} channels and relieves the voltage-sensitive Mg^{2+}-block from the NMDA-receptor, resulting in a massive increase of intracellular calcium concentration ($[Ca^{2+}]_{ic}$). In addition, the dissipation of the Na^+ gradient disrupts transport processes, such as the uptake of synaptic glutamate *(95)*, but the functionality of the Na^+/Ca^{2+} exchanger will also be disturbed. This will further increase $[Ca^{2+}]_{ic}$ *(96)*. The rise in intracellular Na^+ leads to electrogenic Cl^- uptake and osmotic cell-swelling, also termed 'cytotoxic edema' *(97)*. Osmotic cell-swelling may result in cell death by lysis of the cell, and resultant loss of its constituents into the extracellular space. However, the increase in $[Ca^{2+}]_{ic}$ alone is also sufficient to induce neuronal death *(98)*. Thus, cells might die from acute Na^+ and Cl^--dependent cell-swelling or succumb to a delayed calcium-induced degeneration. Increases of $[Ca^{2+}]_{ic}$ may lead to metabolic derangement by inappropriately activating numerous enzymatic pathways which, together with excessive mitochondrial Ca^{2+} sequestration, leads to cell death *(99)*. Together the experimental evidence points out that excitoxic neuronal injury is an important determinant of cell fate in the acute phase of neurodegenerative disorders and may also sensitize cells for delayed injury mechanisms, such as inflammatory attack and apoptosis *(88)*.

Neuroprotection by Cannabinoids

In Vitro Studies

The events that lead to excitotoxic cell death can be initiated at almost every level: by ATP depletion secondary to oxygen and glucose deprivation; by mitochondrial disorders *(100)*;

by exogenous administration of glutamate-receptor agonists *(101)*; by removing the voltage-sensitive Mg^{2+} blockade from the NMDA-receptor *(102)*; by blocking glutamate-uptake *(103)*; by pharmacologically inhibiting Na^+/K^+-ATPase thereby directly inducing depolarisation, etc. Regardless of the point of initiation, the neurotoxic events self-amplify, and ultimately lead to cell death. These principles have been extensively used to model acute neuronal injury caused by stroke or TBI, both in vivo and in vitro.

In vitro models of acute neuronal injury relevant to stroke can roughly be divided into organotypical cultures in which the cells more or less maintain normal *in situ* relationships, and dissociated neuronal cultures or neuronal/glial co-cultures. Organotypical cultures or brain slices are widely used, and are the closest to the in vivo situation. Injury is induced by exposing slices of whole brain, or often just of hippocampus or cortex, to culture medium devoid of oxygen ('anoxic/hypoxic injury'), or of oxygen and glucose simultaneously ('in vitro ischemia') *(104,105)*. Alternatively, addition of glutamate, NMDA, or other excitotoxins to the culture medium are used to induce excitotoxic neuronal injury *(106)*. Identical injury techniques are applied to dissociated cell-cultures *(105,107–112)*.

CB-Dependent Protection

Depending on the model, plant and synthetic cannabinoids have been shown to exert neuroprotection via different mechanisms *(see also* ref. 21). For example:

1. Cerebellar granule neurons were rescued from glutamate toxicity by administration of WIN55.212-2, Δ^8-THC, 11-OH-THC, and palmitoylethanolamide 15 min after glutamate exposure (*see* Table 1, no. 1) *(109)*. The mechanism of protection is unknown, but it was suggested that palmitoylethanolamide interfered with downstream consequences of an excitotoxic stimulus upon binding to CB_2-like receptors. However, the involvement of such a CB_2-like receptor was not demonstrated.
2. In another study it was shown that WIN55.212-2 and CP55.940, via the CB_1 receptor, protected hippocampal neurons in vitro against secondary excitotoxicity, which was induced by reduction of Mg^{2+}-levels (Table 1, no. 2) *(110)*. Presynaptic CB_1 mediated inhibition of Ca^{2+}-spiking and glutamate release were assumed to be responsible for the neuroprotective effects *(110)*. In line with this hypothesis was the observation that the cannabinoids could not rescue the cells from direct glutamate exposure (Table 1, no. 2) *(110)*.
3. In contrast, THC could protect cultured mouse-spinal neurons against direct kainate toxicity in a CB_1-dependent manner (Table 1, no. 3) *(108)*. It should be noted that in studies *(110)* and *(108)* the distribution of the CB_1 receptor was different. In spinal neurons the CB_1 receptor was located on cell bodies *(108)*, whereas a CB_1-like receptor was thought to be located at presynaptic sites in hippocampal neurons *(68,110)*. It is noteworthy that AEA could reduce NMDA-induced calcium influx via CB_1-mediated closing of voltage-sensitive calcium channels in rat brain slices *(60)*. Thus, CB_1-mediated closing of voltage-sensitive calcium channels may be responsible for the observed neuroprotection in spinal neurons.
4. In addition, it was shown that CP-55.940 could protect cortical neurons against glutamate exposure at low concentrations in a CB_1-dependent manner via closing of N- and P/Q-type calcium channels (Table 1, no. 6) *(113)*. It was shown that the protective effects of CP-55.940 in vitro could only be observed when the cAMP levels were elevated.

CB-Independent Neuroprotection

In other studies, cannabinoids were shown to protect neurons independently of CB receptors. For example:

1. WIN55.212-2 protected cerebral cortical neurons from in vitro hypoxia and glucose deprivation in a CB_1- and CB_2-independent manner (Table 1, no. 4) *(114)*.
2. Exogenous AEA and 2-AG protected cultured cerebral neurons in the same model also in a CB-independent manner (Table 1, no. 7) *(112)*. The mechanism of neuroprotection of the synthetic and endogenous cannabinoids was not investigated in these studies.
3. In another study, cannabidiol and THC were shown to protect, via their anti-oxidative properties, rat cortical neurons exposed for a short time (10 min) to toxic levels of glutamate,

AMPA, and kainate receptor ligands (Table 1, no. 5) *(111)*. The neuroprotection was unaffected by CB-receptor antagonists. THC and cannabidiol were anti-oxidants, as was demonstrated by cyclic voltammetry. Furthermore, THC and cannabidiol prevented H_2 O_2-induced damage in neuronal cultures *(111)*.

4. In addition, various cannabinoids with a phenol group were recently shown to be anti-oxidants in cell-free biochemical assays, and protected neuronal cells exposed to H_2O_2 (Table 1, no. 8) *(115)*.

The CB_1 receptor was not required for neuroprotection against oxidative stress, because the phenolic cannabinoids protected to the same extent cerebellar cells from CB_1 knockout mice and non-transfected HT22 cells, and neurons derived from wild-type mice and CB_1-transfected HT22 cells *(115)*. Interestingly, it has been reported that 2-AG suppress the formation of reactive oxygen species and tumor necrosis factor-α (TNF-α), a neurotoxic inflammatory mediator, by murine macrophages in vitro following stimulation with lipopolysaccharide (LPS) *(116)*.

Thus, the mechanism of neuroprotection by cannabinoids differs in the various in vitro models and can involve: a) activation of yet to be characterized CB_2-like receptors; b) CB_1-mediated inhibition of glutamatergic transmission via closing of N-, and P/Q-type calcium channels; c) reduction of calcium influx, at both the pre- and postsynaptic level, followed by subsequent inhibition of deleterious cascades; d) anti-oxidant activity; and e) inhibition of TNF-α formation.

In Vivo Neuroprotection of Classical and Synthetic Cannabinoids

In vivo ischemia models can be subdivided on the basis of the extent of the ischemic territory into focal and global ischemia models. Global ischemia models are almost always temporary and are presumed to relate to cardiac arrest and near-drowning. Global ischemia is induced by occluding both carotid arteries alone (two-vessel occlusion, [*117*]) or together with both vertebral arteries (four-vessel occlusion, [*118*]), and can be combined with hypoxia and hypotension. Several variations exist, including three-vessel occlusion in which the basilar artery is occluded instead of the two vertebral arteries *(119)*. Focal ischemia can be induced permanently or transiently by occluding one or more intracranial vessels, and relates more closely to human ischemic stroke. In animal species lacking a complete Circle of Willis (such as the mongolian gerbil), unilateral ischemia can be induced by simply occluding a carotid artery *(120)*. The territory of the middle cerebral artery (MCA) is the most common site of thrombotic stroke in humans, and stroke in this territory is frequently associated with a severe outcome. The MCA is therefore considered the artery of choice for occlusion in most focal cerebral ischemia models. Neuronal injury in the acute phase after transient (clinically relevant) global ischemia develops mainly during reperfusion after a relatively short, intense insult. Selectively vulnerable brain regions such as the hippocampus, suffer earlier and more severe damage, but compared to focal ischemia the overall insult is quite uniform. Focal ischemia is characterized by a much more heterogeneous injury pattern, often subdivided into an ischemic core with a dramatically reduced blood flow, and a marginally perfused border-zone, or 'penumbra', receiving collateral blood supply. Neuronal injury in the core is extremely severe and irreversible after prolonged ischemia (> approx 1 h) *(121)*. In the penumbral region, neuronal injury and cell death occur over a period of hours to days after the insult and are more susceptible to therapeutic intervention than cell death processes in the infarct core. A similar distinction can be made in experimental traumatic brain injury. In the majority of these models, injury is induced by a brief fluid pressure-pulse to the intact dura 'fluid percussion', [*122*],) by dropping a weight on the exposed skull ('closed-head injury', [*123*]) or by rigid indentation of the cortical surface ('cortical-impact injury', [*124*]). Neuronal injury, occurring at the moment of impact, results from

Table 1
Neuroprotection by (Endo) Cannabinoids in Different In Vitro and In Vivo Models of Neuronal Injury

No.	Compound	Dose	Time of application relative to injury	Type	Model and toxin used
1	WIN55.212-2 Δ^8-THC 11-OH-THC PEA	24.5 μM[a] 2.8 μM[a] 0.88 μM[a] 54.6 μM[a]	15 Min post-toxin	In vitro	Glutamate
2	WIN55.212-2 CP55940	100 nM[b] 100 nM[b]	Coapplication	In vitro	Reduced [Mg^{2+}]
3	Δ^9-THC	0.5 μM[b]	Coapplication	In vitro	Kainate
4	WIN55.212-2 AEA THC	30 nM[b] 100 nM[b] 10 μM[b]	Coapplication	In vitro	Hypoxia and glucose deprivation
5	Δ^9-THC CBD	3.7 μM[a] 30 μM[b]	Coapplication	In vitro	Glutamate AMPA-ligand Kainate-ligand
6	CP55940	10 nM[b]	Coapplication	In vitro	Glutamate
7	AEA 2-AG	100 nM[b] 1000 nM[b]	Coapplication	In vitro	Hypoxia and glucose deprivation
8	THC, HU-210 CP55940,	0.1–10 μM	Coapplication	In vitro	H_2O_2
9	AEA	100 μM[a]	15 Min post-toxin	In vitro	Glutamate
10	AEA PEA	10 μM[b] 10 μM[b]	Pre- and post-toxin	In vitro	Glutamate
11	WIN55.212-2	0.1–1 mg/kg[b], i.p.	40 Min pre-injury	In vivo	Global ischemia; four vessel occulusion
12	WIN55.212-2	1 mg/kg[b], i.p.	30 Min post-injury	In vivo	Focal ischemia, MCA-occlussion
13	Δ^9-THC	0.1–10 mg/kg[b], i.p.	7-d pre-injury	In vivo	Ischemic reperfusion Bicarotid occlusion Reduced blood pressure
14	Δ^9-THC	1 mg/kg[b], i.p.	30 Min pre-injury	In vivo	Ouabain-induced Excitotoxicity
15	CP55940	4 mg/kg[b], i.p.	5 Min post-injury	In vivo	Global ischemia; bicarotid occlusion
16	2-AG	0.1–10 mg/kg[b], i.v.	15 Min post-injury	In vivo	Traumatic brain injury
17	AEA	1–10 mg/kg[b], i.p.	30 Min pre-injury	In vivo	Ouabain-induced Excitotoxicity

[a] ED_{50} values.

[b] Highest (effective) concentration tested.

immediate, irreversible mechanical damage and is followed by secondary damage caused by injury mechanisms that are at least in part comparable to those in the ischemic penumbra. For example, closed-head injury to the infant rat brain induces ultra-structural neurodegenerative changes in the hypothalamus that are identical to acute changes induced in the brain by either hypoxia/ischemia or glutamate injection. Excitotoxicity is central to determining neuronal demise in both these settings *(90)*.

Cannabinoids and Stroke Models

To date, the neuroprotective effects of cannabinoids have only been addressed in a

Table 1 *(Continued)*

Dose of toxin and duration of toxin application	Species	Neuron type	Protection	Time of assessment after injury	CB dependent?	Ref.
500 μ*M*, 5 min	Rat	Cerebellar granule	Yes	24h	CB_2-like	*(109)*
18–24 h	Rat	Hippocampal	Yes	0 h	CB_1	*(110)*
100 μ*M*, 24 h	Mouse	Spinal	Yes	24 h	CB_1	*(108)*
8 h	Rat	Cortical	Yes Yes No	16 h	CB_1- and CB_2-independent	*(114)*
250 μ*M*, 10 min 1.5 μ*M*, 20 h 10 μ*M*, 20 h	Rat	Cortical	Yes	18–20 h	Anti-oxidant	*(111)*
200 μ*M*, 15 min	Rat	Cortical	Yes	18–20 h	CB_1	*(113)*
8 h	Rat 2-AG	Cortical 1000 n*M*[b]	Yes	16 h	CB_1- and CB_2-independent	*(112)*
60–250 μ*M*, 12h	Rat, mice	Cerebellar, HT22	Yes	12h	CB_1-independent	*(115)*
500 μ*M*, 5 min	Rat	Cerebellar Granule	No	24 h		*(109)*
100 μ*M*, 1 or 24 h	Chick	Telencepalon	No	24 h		*(142)*
15 min	Rat	Hippocampal	Yes	3 d	CB_1	*(114)*
permanent	Rat	Cortical	Reduction in infarct volume	24 h	CB_1	*(114)*
12 min	Rat	Striatal, cortical	Yes	3 wk	N.D.	*(125)*
0.5 nmol intrastriatal	Rat	Hippocampal, striatal, cortical	Reduction in infarct volume	15 min and 7 d	CB_1	*(129)*
10 min	Gerbil	–	Improvement of locomotion and EEG	1, 3 and 7 d	CB_1	*(127)*
	Mouse	Hippocampal	Reduction in infarct volume	1 and 7 d	In part CB_1	*(138)*
0.5 nmol intrastriatal	Rat	Hippocampal, Istriatal, cortical	Reduction in infarct volume	15 min and 7 d	In part CB_1	*(141)*

few in vivo models of neuronal injury. However, the results show a large degree of variation in models of cerebral ischemia.

1. Chronic Δ^9-THC administration has been shown to reduce the impact of an ischemic insult evoked by a reduced blood pressure and 12 min bilateral carotid artery occlusion (Table 1, no. 13) *(125)*. THC was injected i.p. at either a low (0.1 mg/kg) or high (10 mg/kg) dose every 12 h for 7 d prior to ischemia. Three weeks after neuronal injury rat brains were analysed via a histological procedure. No protection was observed in the hippocampus at either dose, whereas animals treated with the high dose showed significantly less neocortical injury. The

striatum was protected in all THC-treated animals. The involvement of the CB_1 receptor was not studied.

2. In contrast, no protective effect could be found for the CB receptor agonist WIN55.212-2 in rats when the middle cerebral artery was occluded for 2 h. Surprisingly, CB_1-receptor antagonists were protective, which may suggest that endogenously-released endocannabinoids are toxic *(126)*. The CB_1 antagonists, SR141716A and LY320135, reduced the size of the infarct and improved neurological outcome *(126)*.
3. Yet, in another study, WIN55.212-2 afforded dose-dependent and CB_1-mediated protection to hippocampal and cortical neurons in rats with a permanent middle cerebral artery occlusion or global ischemia (Table 1, nos. 11 and 12) *(114)*. WIN55.212-2 (1 mg/kg, i.p.) could reduce the infarct volume in rat brains when administered up to 30 min after permanent focal ischemia (Table 1, no. 12) *(114)*.
4. Finally, in gerbils subjected to transient global ischemia, CP-55,940 (4 mg/kg i.p.) reduced the ischemia-induced hyperlocomotion and improved electroencephalographic (EEG) spectral power after 24 h (Table 1, no. 15) *(127)*. These CP-55.940-induced effects were still observed after 7 d. Co-administration of SR141716A completely blocked the effect of CP-55.940, which indicated that the CB_1 receptor was involved.

It should be noted that in each of these stroke models different perturbations in cerebral blood pressure and flow were induced, which may explain the varying results. It has recently been shown that endocannabinoids generated in monocytes and platelets contributed to hypotension in acute myocardial infarction *(128)*. It might be possible that (endo)cannabinoid-induced vasorelaxation is affecting the extent and pathway of neuronal demise in a different manner in each of the stroke models. Therefore, the authors have tested the neuroprotective effects of THC in a model of acute neuronal damage without directly affecting blood pressure and flow to the brain *(129)*.

Cannabinoids and Excitotoxicity Model

In our in vivo model, acute neuronal damage was elicited in neonatal rats by inhibiting the Na^+/K^+-ATPase thereby producing secondary excitotoxicity (Table 1, no. 14, [*129*]). THC (1 mg/kg, i.p.) could reduce the volume of cytotoxic edema in a CB_1-dependent manner already 15 min after blockade of the Na^+/K^+-ATPase. After 7 ds the infarct volume in THC-treated rats was ~40% smaller compared with control animals. The effect was abolished by co-administration of SR14716A. The authors have suggested that a CB_1-mediated reduction in calcium influx and a reduced glutamate release are responsible for the neuroprotection in the hippocampus, striatum, and cortex *(129)*. This suggestion was based on several studies which have shown that both synthetic and plant cannabinoids, as well as AEA, can inhibit glutamatergic transmission via presynaptic CB_1-mediated closing of voltage-sensitive calcium channels in different areas of rat brain such as striatum, hippocampus *(65,69,70)*, substantia nigra pars reticulata *(72)*, as well as in prefrontal cortex pyramidal neurons *(76)*.

Endogenous Neuroprotection by Endocannabinoids?

To Be Protective?

It has been suggested that the release of endocannabinoids during neuronal injury might be an endogenous protective response *(130)*. If this is the case, inhibitors of endocannabinoid clearance may provide useful therapeutics for neurodegenerative diseases. The lines of evidence are the following:

1. 2-AG, AEA, and its precursor *N*-arachidonoylphosphatidyl-ethanolamine are normally found in low concentrations in the brain, but their levels increase in a calcium-dependent manner *post-mortem* and upon (severe) neuronal injury *(131–135)*. AEA levels were increased in cultured primary neo-cortical neurons treated with NMDA or glutamate *(136)*. High levels of NAPEs were found in neonatal rat brains after

receiving a striatal injection of NMDA (25 nmol) *(137)*. In the latter model, total acylethanolamide (NAE) levels were increased 46-fold in cerebral cortex after 24 h and AEA concentrations were elevated by a factor of 13 *(135)*. In addition, a large increase in 2-AG was found in a closed head injury model in mice *(138)* and in rats treated with picrotoxinin *(139)*.

2. CB_1 expression is enhanced in the cortical mantle zone in rats after 20 min occlusion of the middle cerebral arteries *(140)*. Western blotting and immunohistochemistry showed that the increased CB_1 expression began after 2 h, and persisted for 72 h or more after ischemia. CB_1 transcripts and [^{3}H]CP55.940 binding capacity were also elevated in the border regions of the cortex in mice subjected to mild concussive head trauma *(135)*.
3. The neuroprotective effects of plant and synthetic cannabinoids have been reported (*see* above). However, only one in vitro study described a neuroprotective effect of endocannabinoids (Table 1, no. 7) *(112)*. Only very recently, in fact, it has been shown that 2-AG exerts a protective role after closed head injury of mouse brain by acting at least in part via the CB_1 receptor (Table 1, no. 16) *(138)*. 2-AG administered to mice after closed head injury reduced brain edema, improved clinical recovery, reduced infarct volume, and reduced hippocampal cell death compared to controls. The reduction in brain edema by 2-AG was dose-dependently attenuated by SR141716A. At the same time, we have shown in a longitudinal MRI-study that exogenously-applied AEA dose-dependently reduced neuronal damage in neonatal rats injected i.c. with the Na^+/K^+-ATPase inhibitor ouabain (Table 1, no. 17) *(141)*. Fifteen minutes after injury, AEA (10 mg/kg), administered 30 min before ouabain injection, reduced the volume of cytotoxic edema in a manner insensitive to the CB_1-receptor antagonist. Seven days after ouabain treatment, the lesion volume was 64% smaller in AEA-treated rats. When assessed at this time point, the neuroprotective actions of AEA were abolished by SR141716A.

Or Not to Be Protective?

In order to propose a cause–effect relationship between enhanced endocannabinoid levels during neuronal injury and neuroprotection, further data are necessary such as the demonstration that CB_1-receptor antagonism or inhibition of endocannabinoid degradation produce neuroprotection *per se*. Several observations indicate that the role of the endocannabinoid system as a general endogenous protection system is questionable.

1. It has been demonstrated that the extent of the increase of AEA or 2-AG levels varies in different in vivo models of neuronal damage and is dependent on the type of cell death and the species *(137)*. As stated above, high levels of AEA were found in NMDA-injected neonatal rats, but only moderate and low increases were observed in a closed head injury model in mice and in an apoptotic model, respectively. On the other hand, 2-AG increased during closed head injury in mice, but no increase of this compound was found after traumatic brain injury or NMDA-injection in rats *(135)*. In our secondary excitotoxicity model in neonatal rats, in which neurodegeneration was elicited by ouabain, AEA and 2-AG concentrations were not elevated *(141)*.
2. Some in vitro studies do not support a neuroprotective action of endocannabinoids.
   a. AEA did not protect cerebellar granule neurons in a post-glutamate paradigm, whereas THC and palmitoylethanolamide did (Table 1, no. 9) *(109)*.
   b. AEA was also ineffective against prolonged glutamate exposure of chicken telencephalon neurons (Table 1, no. 10) *(142)*.
   c. Hippocampal neurons exposed to THC died in a CB_1-dependent manner, probably due to an activated arachidonic acid pathway *(143)*.
3. Observations in four different in vivo models indicate that endogenously released or constitutive endocannabinoids do not protect the brain against acute neuronal injury.
   a. In a mouse traumatic brain injury model, application of SR141716A (20 mg/kg) did not increase the volume of edematous tissue and the application of entourage compounds did not reduce the volume of edematous tissue *(138)*.
   b. No increase in infarct volume upon application of SR141716 was found in global and focal ischemia models (MCA-occlusion) *(114)*.

c. In our secondary excitotoxicity model compared to control animals, application of AEA-uptake inhibitor, VDM11, or SR141716A alone neither affected lesion volumes at d 0 nor at d 7 *(129,141)*.

4. In an ischemic reperfusion model, application of SR141716A was neuroprotective *(126)*. In this study neuronal damage was assessed after 24 h, whereas in most of the other in vivo studies neuronal injury was quantified after 3 d or more (Table 1, no. 11–17).

Some Possible Explanations

Why can exogenously applied (endo)cannabinoids prevent neuronal loss, while endogenously released endocannabinoids do not seem to be able to reduce neuronal damage? Several explanations can be put forward, which can hold true independently or reinforce each other.

1. As described above, the neurodegenerative insult may not always lead to an up-regulation of endocannabinoid biosynthesis, the occurrence of which seems to be dependent on the species and on the type of injury. For example, traumatic brain injury in mice resulted in a substantial increase in 2-AG levels *(138)*, whereas in neonatal rats no increase was found *(137)*. Intrastriatal injection of NMDA, but not ouabain, in neonatal rats led to an increase in AEA *(137,141)*. Recently, it has been shown that the simultaneous application of glutamate and carbachol (an acetylcholine receptor agonist), but not of either agent alone, caused a marked increase in AEA biosynthesis in cortical neurons *(144)*. Thus, membrane depolarization was necessary for the biosynthesis, but was insufficient *per se* to initiate AEA biosynthesis. To date, although enzyme activities for the biosynthesis of endocannabinoids have been identified, the regulation of these proteins and of their activity remains largely unexplored. Isolation or cloning of the enzymes responsible for endocannabinoid formation may help to understand the regulation of endocannabinoid biosynthesis in response to neuronal injury, and to develop specific inhibitors to be used as tools to investigate the role of endocannabinoids in neuroprotection.
2. The distribution and localization of the CB_1 receptor is also of importance. The CB_1 receptor is highly expressed in several areas of the CNS at presynaptic and postsynaptic sites, as well as on cell bodies. As noted before, presynaptic CB_1 receptors can block secondary excitotoxicity in in vitro models by inhibiting glutamate release. However, these presynaptic CB_1 receptors are ineffective against a direct glutamate-induced elevation of postsynaptic calcium concentrations *(69,70)*, such as that occurring in glutamate toxicity models *(145)*.

   It is noteworthy that endocannabinoids have been shown to act as retrograde messengers in the hippocampus and cerebellum *(78,79,82,84)*. They are released from the postsynaptic membrane and have to diffuse back to the presynaptic CB_1 receptors. Upon activation of these receptors, the release of GABA or glutamate is inhibited. If during the in vivo toxic stimulus glutamate has been released (or exogenously applied) in large quantities, then endogenously-released endocannabinoids might reach the site of injury too late to exert a protective action, and only when the damage has already been inflicted. In this way the endocannabinoid system may not be able to function as an endogenous protection system. Conversely, pretreatment with exogenous (endo)cannabinoids may inhibit the toxic stimulus-induced glutamate exocytosis in advance, thereby preventing the spreading (and in this way) reducing the effect of the toxic stimulus.
3. The ability of endocannabinoids to influence downstream effects of increased calcium concentrations is also dependent on the cell-type, strength, duration, and, stimulus-type. In most studies cannabinoids have been shown to reduce intracellular calcium concentrations via CB_1-mediated closing of voltage-sensitive calcium channels, but several studies have indicated that cannabinoids may also increase calcium concentrations *(27)*. For example, CB_1-receptor activation by AEA and 2-AG in N18TG2 neuroblastoma and NG108–15 neuroblastoma-glioma hybrid cells has been shown to induce a rapid transient increase in intracellular-free calcium via its release from IP_3-sensitive calcium stores *(56,57)*. It has been shown that synthetic cannabinoids can enhance peak amplitude of NMDA-elicited signals in a CB_1-dependent manner via intracellular calcium release in cerebellar granule neurons *(62)*. Moreover, AEA was shown to directly modulate NMDA receptors in the presence of SR141716, thereby potentiating calcium currents *(60)*.

4. Another possibility is that the inflicted damage in the in vivo models of acute neuronal injury is too severe. It has been shown that severe excitotoxic injury leads to a loss of CB_1-receptor expression *(137)*. If so, endogenously-released endocannabinoids may only be effective in the border zone of the damaged brain area, or in mild-to-moderate brain injury in which the expression of the CB_1 receptor is not lost *(114,129,137,140,141)*. Thus, the extent of the neurodegenerative process may be a major determinant of the ability of endocannabinoids to exert neuroprotection.

   The other way around, it is possible that if the endocannabinoid system is malfunctioning, (e.g., endocannabinoid biosynthesis is inhibited, CB_1 receptor is inactive or its expression is lost), then glutamate homeostasis is unbalanced and excitotoxicity may be initiated. For example, in a genetic model of Huntington's disease, CB_1 mRNA was decreased prior to the development of either Huntington's disease phenotype or neurodegeneration *(146)*. Also a loss of CB-receptor binding capacity was evident before degeneration of the nerve terminals was seen. More recently, endocannabinoid levels were found to be significantly decreased in the striatum of an animal model of HD *(147)*. It is tempting to speculate that the early down-regulation of CB receptors induces excitotoxicity and subsequent neurodegeneration. Furthermore, it has been speculated that the CB_1 knock-out mice, which die suddenly without any obvious sign of disease, might succumb to neurological problems such as seizures *(148)*.
5. The lack of neurotoxic effects by CB_1-receptor antagonists, which argues against a tonic neuroprotective action of endocannabinoids, may result from neuroprotective actions of AEA and 2-AG via molecular targets other than the CB_1 receptors. For example, palmitoylethanolamide reduced glutamate toxicity in a CB_1-independent manner *(109)*. This observation has not been extended yet to an in vivo model, but palmitoylethanolamide is a potent anticonvulsant in electroshock and chemically-induced seizures in mice *(149)*. Furthermore, AEA and 2-AG have been shown to reduce neuronal damage both in vitro and in vivo, partially via a CB-independent pathway. It is unclear which molecular targets are responsible for these effects. As described above, recent data demonstrate that AEA is capable of interacting with many other proteins, such as vanilloid receptors, L-type calcium channels, Shaker related and TASK-1 K^+ channels, and non-CB_1 non-CB_2 GPCRs for AEA in mouse brain and vascular endothelial cells, some of which may contribute to a reduction in cellular swelling. The inhibition of gap junctions and intracellular calcium signaling in striatal astrocytes by the non-CB_1 G-protein-coupled AEA-receptor, or the inhibition of L- and T-type calcium channels might help preventing glutamate exocytosis and the spreading of excitotoxicity *(23,150)*. Recently, a new CB-type receptor was suggested to be responsible for WIN55.212-2-induced reduction in glutamatergic transmission in the hippocampus of CB_1 knock-out mice *(25,68)*. Thus, endocannabinoids may provide neuroprotection through other molecular targets. However, it should be noted that the AEA-uptake inhibitor, VDM11 *(141)* and entourage compounds of 2-AG did not affect lesion volumes *(138)*, which argues against an endogenous endocannabinoid tone-controlling acute neuronal damage via any extracellular molecular target.

   Conversely, it is also possible that by acting at non-CB receptors, endocannabinoids exert a neurotoxic effect that partially masks the tonic neuroprotective effect mediated by CB receptors. In particular, activation of the VR1 receptor by AEA, if not followed by immediate desensitization of these receptors, might result in an excessive influx of Ca^{2+} into neurons, with subsequent neuronal damage and apoptosis (*see* below). VR1 stimulation has been shown to lead to glutamate release in the hypothalamus *(151)*, and chronic activation of VR1 leads to cell death *(152)*. AEA and PEA can also sensitize VR1 to activation by noxious inflammatory stimuli (e.g., decreased pH, bradykinin) or neurotoxins (e.g., resiniferatoxin), again leading to neuronal-cell toxicity *(153,154)*.

Cannabinoids and Apoptosis

Apoptosis is a form of programmed cell-death that has been implicated in neuronal degeneration after ischemic brain injury in animal models of stroke *(155,156)*. Activation of the apoptotic signaling pathways occurs in

animal- and cell-culture models of stroke. These pathways may involve increased levels of intracellular oxygen radicals and calcium, as well as the induction and activation of proteins such as caspases and calpains. AEA has been associated with the induction of apoptosis both in vitro and in vivo *(157–160)*. This is in line with previous studies in which THC-induced apoptosis in glioma tumors *(159)*, glioma cells *(161)*, primary neurons *(143)*, hippocampal slices *(143)*, and prostate cells *(162)*.

An anti-proliferative action of AEA has also been reported in human-breast carcinoma cells, arrested at the G_1/S transition *(163)*. This effect of AEA was due to a CB_1-like receptor-mediated inhibition of adenylate cyclase and activation of extracellular signal-regulated kinase (ERK) *(164)*. The signalling pathways linked to these two enzymes led to a lower expression of both the high-molecular weight form of the prolactin receptor *(163)* and the high affinity *trk* neurotrophin receptor in the cells *(165)*, thus resulting in growth arrest. The anti-tumor effect of AEA has been recently demonstrated also in vivo *(166)*, where it implicates inhibition of *ras* oncogene-dependent tumor growth.

Several pathways have been implicated in the pro-apoptotic activity of AEA and THC. The programmed death of glioma cells in vitro seems to involve activation of both CB_1 and CB_2 receptors followed by ceramide accumulation and Raf-1/ERK activation *(159,167)*. On the other hand, inhibition of glioma growth in vivo was shown to be dependent on the activation of CB_2 receptors *(168)*. In rat cortical astrocytes and human astrocytoma cells, AEA activates CB_1 receptors leading to sphingomyelin breakdown through adaptor protein FAN, suggesting a CB_1 receptor-mediated pro-apoptotic signaling independent of $G_{i/o}$ proteins *(169)*. In the same cells, CB_1-receptor activation also leads to long-term activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), and it is suggested that a threshold might exist above which endocannabinoid-induced JNK and p38 MAPK activation would lead to cell death *(170)*. Generally, it may be suggested that AEA binding to CB_1 receptors modulates the balance among ERK, JNK, and p38 MAPK thereby regulating the cell proliferation and death. In this respect, it has been shown that cannabinoids are able to modulate, again through CB_1 receptors, the activity of the phosphoinositide 3′-kinase (PI3K)/protein kinase B pathway, which serves as a pivotal anti-apoptotic signal *(171)*. Recent observations have shown that AEA induces apoptosis in human neuroblastoma and lymphoma cells by binding to, and activating vanilloid receptors *(172)*. This effect of AEA occurs through a series of events including increased intracellular calcium concentration, activation of the arachidonate cascade through the cyclooxygenase and the lipoxygenase pathways, uncoupling of mitochondria and release of their cytochrome c, and activation of caspases 3 and 9 *(172)*. Some of these events are typical of different, unrelated pro-apoptotic stimuli *(173)*, suggesting that AEA shares signalling pathways with other inducers. Interestingly, activation of CB_1 (in neuroblastoma cells) or CB_2 (in lymphoma cells) receptors significantly reduces AEA-induced apoptosis *(172)*, implying that in these cells cannabinoid receptors have a protective role against the apoptotic potential of AEA. The relative involvement of cannabinoid and vanilloid receptors in the induction of apoptosis by AEA has also been recently investigated in rat glioma cells, where a causative role for oxidative stress and calpain activation has been demonstrated *(174)*. This is in line with the fact that AEA-induced apoptosis in rat pheochromocytoma cells requires the production of superoxide anions *(158)*.

At the moment it is unclear what role is played by (endo)cannabinoid-induced apoptosis during neuronal degeneration. It has been suggested that AEA may facilitate the induction of apoptosis of injured neurons and neighboring cells, thereby inhibiting the spreading of a necrotic event *(10)*. However, anti-apoptotic strategies have been identified

as potentially beneficial in limiting ischemic neuronal injury *(155)*. Therefore, it seems unlikely that the pro-apoptotic activity of (endo)cannabinoids may help to improve neuronal outcome after injury. On the contrary, it might exacerbate neuronal damage by causing death of non-lethally injured neurons. Further research is necessary to assess the mechanism of AEA-induced apoptosis and to investigate its role during stroke, TBI, and excitotoxicity.

Perspectives

Recent developments, such as the generation of CB_1 and of CB_2 knock-out mice, have provided insights into the (patho)physiological roles of the endocannabinoid system. As the biochemical pathways underlying endocannabinoid formation, action, and inactivation are being revealed, several tools for the manipulation of endocannabinoid levels and activity through selective inhibition by synthetic compounds of the metabolic enzymes and molecular targets of AEA and 2-AG are available; i.e., competitive AMT inhibitors, reversible and irreversible FAAH inhibitors, as well as selective agonists and antagonists for CB_1 and CB_2 receptors. However, much is still to be done to render these inhibitors and ligands more selective. Furthermore, some important fundamental aspects of the endogenous-cannabinoid system remain to be discovered. For example, the proteins responsible for the biosynthesis of endocannabinoids and their transport into cells have not been cloned or purified yet. The regulation of the biosynthetic and inactivation pathways of AEA and 2-AG is also largely unknown. It is likely that novel CB receptor subtypes, as well as novel endogenous ligands, will be found. The understanding of the complex interplay of the endocannabinoid system with other neurotransmitters in the CNS and their function as retrograde messengers will greatly enhance our knowledge about the physiological roles of the endocannabinoid system. This may provide useful information to exploit the cannabinoid system for therapeutic intervention in diseases. Crystal or NMR structures of the proteins of the cannabinoid system may help elucide the nature of the ligand–protein interaction, thereby facilitating the design of selective and potent molecular probes for each of these proteins.

The studies reported in this review indicate that agonists of CB_1 receptors might also be useful to improve the outcome after acute brain damage. It is interesting to note that THC and CP55.940 are partial agonists that can reduce excitotoxicity without completely blocking glutamatergic transmission. Complete inhibition of glutamate neurotransmission may lead to serious complications such as hallucinations *(175)*. Nevertheless, many questions have to be answered before a CB_1 agonist can be used as a neuroprotective agent, such as: 1) What type of human brain injury can be treated? 2) To what extent do cannabinoids improve functional and neurological outcome after brain damage? 3) What is the therapeutic time-window? 4) Do cannabinoid-induced vasorelaxation, psychotropic side effects, and pro-apoptotic activity pose a problem? At the moment it is not clear whether CB_2 agonists, which do not cause psychotropic effects, are also able to reduce neuronal damage in vivo, but other anti-inflammatory drugs have been proven to reduce neuronal injury and improve functional recovery. It is noteworthy that dexanabinol (HU-211), a non-psychotropic cannabinoid which does not bind to CB receptors, is a powerful neuroprotective agent. Its action is mediated via several mechanisms, i.e., direct antagonism of the NMDA receptor, anti-oxidative properties and blockade of TNF-α production *(176)*. The fact that dexanabinol has recently entered phase III clinical trials against brain trauma, substantiates the hope that cannabinoid-based drugs will be useful as therapeutic agents for acute brain damage. If the extent of neuronal damage is not too large, compounds that mimic or strengthen a putative tonic beneficial action of endocannabinoids might provide valid therapeutic avenues for novel, efficacious neuroprotective drugs.

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