N-Arachidonylethanolamine (anandamide) is an endogenous agonist of the cannabinoid CB1 and CB2 receptors and displays many of the same receptor-mediated physiological effects as Δ9-tetrahydrocannabinol (Δ9-THC), the active component of marijuana. As with any neurotransmitter, there must be tight control of anandamide receptor-mediated signaling and a means of rapid removal of the molecule from the system. Thus, the process by which anandamide is transported into the cell for metabolism has been a topic of much interest and has been implicated as a potential drug target in the treatment of several disease states that are reported to have an association with the endocannabinoid system. In this review, we will discuss the current models proposed for the mechanism of anandamide transport, the progress that has been made in the development of compounds that specifically inhibit anandamide transport, the observed effects of anandamide transport inhibition in vivo, and finally, potential therapeutic applications of compounds that inhibit anandamide transport.

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Keywords: Cannabinoid; Anandamide; Transporter; Δ9-Tetrahydrocannabinol; Marijuana; Amidohydrolyase

Abbreviations: Δ9-THC, Δ9-tetrahydrocannabinol; 2-AG, sn2-arachidonoyl glycerol; AEA, anandamide; CREAE, chronic relapsing experimental allergic encephalomyelitis; CNS, central nervous system; DAG, diacylglycerol; FAAH, fatty acid amide hydrolase; FAAH−/− mice, FAAH knockout mice; MAPP, methylarachidonylfluorophosphonate; GI, gastrointestinal; NAPE, N-arachidonylphosphatidylethanolamine; PLD, phospholipase D; PMSF, phenylmethylsulfonyl fluoride; SARI, selective AEA reuptake inhibitors; TRPV1, transient receptor potential vanilloid type 1 receptor; virdhamine, O-arachidonoyl-ethanolamine.

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1. Introduction

There has been a plethora of historical and contemporary interest in the potential medicinal benefits of *Cannabis sativa* or marijuana (Di Marzo, 1998). This is largely due to the effects of the major psychoactive component of marijuana Δ⁹-tetrahydrocannabinol (Δ⁹-THC; Gaoni & Mechoulam, 1971; Howlett, 1995). The actions of Δ⁹-THC on the central nervous system (CNS) particularly alterations in cognition and memory, sedation, and the euphoria leading to abuse potential are perhaps the most familiar. However, Δ⁹-THC and other cannabinoids have potential therapeutic activity in multiple conditions including pain, nausea and vomiting, appetite loss, convulsions, diarrhea, asthma, autoimmune diseases, fever, and glaucoma (Di Marzo, 1998; Piomelli et al., 2000; Cravatt & Lichtman, 2003). Indeed, during the last decade, a significant amount of attention has been given to drug development seeking to identify ligands that might have selective cannabimimetic activity for use in several of these disease states.

1.1. Cannabinoid receptors

For many years, cannabinoids were thought to mediate their effects through non-specific actions on the plasma membrane (Roth & Williams, 1979). However, two inhibitory G-protein coupled receptors, CB1 and CB2, have been cloned and identified as the pharmacological target of Δ⁹-THC and other cannabinoids (Matsuda et al., 1990; Munro et al., 1993). The CB1 receptor is found in the CNS and is linked to the inhibition of adenylyl cyclase (Matsuda et al., 1990) as well as N-type calcium channels and inwardly rectifying potassium channels (Felder et al., 1995). In addition, CHO cells transfected with the CB1 receptor cDNA exhibit non-receptor mediated effects for synthetic cannabinoids including release of both intracellular calcium and arachidonic acid (Felder et al., 1993). Autoradiography (Herkenham et al., 1991; Thomas et al., 1992; Westlake et al., 1994) and in situ hybridization (Matsuda et al., 1990) have identified the basal ganglia, hippocampus, cerebral cortex, and the molecular layer of the cerebellum as the brain regions with the highest levels of CB1 receptor expression consistent with the known CNS effects associated with marijuana use. The CB2 receptor is located peripherally, and similar to CB1 receptors, its activation is linked to the inhibition of adenylyl cyclase. However, unlike CB1 receptors, there has been no significant link established between CB2 receptor activation and inhibition of either N-type calcium channels or inwardly rectifying potassium channels (Felder et al., 1995). Localization studies have identified CB2 receptors in B lymphocytes (Lynn & Herkenham, 1994), macrophages (Munro et al., 1993), and mast cells (Facci et al., 1995) suggesting a role for this receptor in cannabinoid modulation of the immune response. There have been reports of a cannabinoid receptor that is not activated by plant-derived cannabinoids (Breivogel et al., 2001), and it is conceivable that there are cannabinoid receptors that are yet to be identified.

1.2. Anandamide as an endogenous cannabinoid

Identification of the CB1 and CB2 receptors along with characterization of their activity prompted the search for an endogenous ligand that would activate these receptors. *N*-Arachidonylethanolamine (anandamide; Fig. 1), an ethanolamine derivative of arachidonic acid, was isolated from porcine brain extracts by Devane et al. (1992) and found to possess competitive inhibitor properties for cannabinoid ligand binding and inhibition of the vas deferens twitch response, an assay for cannabimimetic activity. Anandamide was subsequently named “anandamide” after the Sanskrit word “ananda” meaning “bliss”. Extensive radioligand binding studies have confirmed that AEA is a potent ligand for CB1 receptors in rat brain (Childers et al., 1994) and in cells heterologously expressing the CB1 receptor (Felder et al., 1993; Vogel et al., 1993; Pinto et al., 1994). With respect to other eicosanoids, the effect of AEA is specific in that most other amides and esters of arachidonic acid including prostaglandin E2, A2, B2, and B1 fail to inhibit cannabinoid ligand binding (Pinto et al., 1994). In vitro AEA has been shown to possess many of the same pharmacological effects as Δ⁹-THC (Felder et al., 1993; Fride & Mechoulam, 1993). Furthermore, when administered exogenously to rodents, AEA was found to produce pharmacological and behavioral effects that were similar to that of Δ⁹-THC although with a shorter duration of action (Crawley et al., 1993; Fride & Mechoulam, 1993; Smith et al., 1994). The behavioral effects of AEA and other compounds include antinociception, hypothermia, hypomotility, and catalepsy all components of a tetrad of tests used to predict cannabimimetic
activity. These experiments led AEA to be characterized as an endogenous agonist of the cannabinoid receptors and thus, recognized as the first endocannabinoid. In addition to the above cannabimimetic actions, AEA has been shown to inhibit macrophage-mediated tumoricidal activity (Cabral et al., 1995), inhibit pituitary hormone secretion (Wenger et al., 1995), and modulate mast cell activation (Facci et al., 1995).

With regards to signal transduction, AEA exhibits partial agonist activity for inhibition of adenylate cyclase (Felder et al., 1993; Vogel et al., 1993; Childers et al., 1994; Pinto et al., 1994), inhibition of calcium channels (Felder et al., 1993; Mackie et al., 1993; Mackie et al., 1995), and inhibition of inwardly rectifying potassium channels (Mackie et al., 1995) in neuroblastoma cells naturally expressing CB1 receptors and in mammalian cells heterologously expressing CB1 receptors, actions consistent with Δ⁹-THC. AEA was also isolated from bovine brain extracts and shown to inhibit antagonist binding to L-type calcium channels (Johnson et al., 1993). In addition to effects on G-protein coupled CB1 and CB2 receptors, AEA is a partial agonist of the Ca²⁺-permeable ion channel transient receptor potential vanilloid type 1 (TRPV1) receptor (Di Marzo et al., 2001; Piomelli, 2001). TRPV1 receptors are sensitive to activation by noxious stimuli such as heat, protons, the vanilloid capsaicin, and, to a lesser extent, several endogenous lipid-like substances including AEA (Helliwell et al., 1998; Tominaga & Julius, 2000; Di Marzo et al., 2001). Effects on the TRPV1 receptor could be responsible for AEA’s analgesic and vasodilator effects (Zygumn et al., 1999; Jerman et al., 2002). In addition to the receptor-mediated effects, AEA also appears to share non-receptor-mediated effects on the release of intracellular calcium and arachidonic acid with synthetic cannabinoids (Felder et al., 1993). AEA induces MAP kinase activity in WI-38 fibroblasts by a mechanism that is linked to arachidonic acid release (Wartmann et al., 1995). Another action of AEA observed in striatal astrocytes is the inhibition of gap junction conductance and glutamate-stimulated wave propagation that appears to be mediated through a pertussis-toxin sensitive, non-CB1 receptor (Venace et al., 1995). With regards to cell-cell communication in the CNS, the modulatory effects of AEA include inhibition of long-term transformation and associatively produced long-term potentiation in certain gamma-aminobutyric acid (GABA)-ergic neurons in the hippocampus (Collin et al., 1995). The endogenous cannabinoids also appear to regulate the presynaptic release and/or uptake of norepinephrine, GABA, and dopamine (Ishac et al., 1996; Stefano et al., 1997; Romero et al., 1998).

The identification of AEA was not the first report of a fatty acid amide with biological activity. Palmitoylethanolamide had been isolated from mammalian brain tissue and egg yolks, and shown to have anti-inflammatory properties (Kuehl et al., 1957). Angiogenic activity has been ascribed to the fatty acid amide 13-docosenamide isolated from bovine mesentary (Wakamatsu et al., 1990). In the CNS, cis-9,10-octadecenoamide (oleamide) has been isolated from mammalian cerebrospinal fluid and demonstrated to have potent sleep-inducing properties (Cravatt et al., 1995). Indeed, there are a family of N-acylthanolamides that are co-released with AEA when brain neurons are stimulated (Di Marzo et al., 1994; Fontana et al., 1995). These include N-oleyl, N-stearoyl, N-linoleoyl, and N-palmitoyl-ethanola-
mide all of whose physiological roles have yet to be clearly elucidated. Homo-γ-linolenylethanolamide and docosatetraenoylethanolamide have also been found in pig brain and shown to inhibit cannabinoid radioligand binding to the CB1 receptor with potencies equivalent to AEA (Hanus et al., 1993; Pertwee et al., 1994). It has been suggested that acylethanolamides co-released with AEA may exert an “entourage” effect by augmenting the receptor-mediated signaling of AEA. For example, palmitoylethanolamide and oleoylethanolamide display some cannabimimetic properties that may be the result of their activity as inhibitors of AEA metabolism (Lambert & Di Marzo, 1999; Jonsson et al., 2001). Smart et al. (2002) showed that palmitoylethanolamide enhances the TRPV1-mediated effects of AEA on calcium influx into the cells (Smart et al., 2002). De Petrocellis et al. (2002) demonstrated that the “entourage” effect of palmitoylethanolamide enhanced TRPV1 activation by AEA resulted in antiproliferative effects on human breast cancer cells (De Petrocellis et al., 2002). Palmitoylethanolamide fails to inhibit cannabionid ligand binding to the CB1 receptor at concentrations up to 1 μM (Devane et al., 1992), but it does activate CB2 receptors expressed by mast cells thereby blocking mast cell activation (Facci et al., 1995). Whereas AEA fails to exhibit agonist properties for CB2 receptor-mediated mast cell activation, AEA can act as a CB2 receptor antagonist efficiently blocking the effects of palmitoylethanolamide (Facci et al., 1995).

A second class of endocannabinoids, the 2-acylglycerols, including sn2-arachidonoyl glycerol (2-AG) has been identified (Fig. 1). 2-AG is found in gut (Mechoulam et al., 1995) and in brain at levels 170 times greater than that of AEA (Stella et al., 1997). 2-AG is produced in the hippocampus upon stimulation and may play an important modulatory role in the formation and release of 2-AG. Whereas these studies confirm the existence of distinct pathways for the biosynthesis of endogenous N-acylethanolamines. There is strong evidence that formation of the other major cannabinoid, 2-AG, involves an sn1-specific diacylglycerol (DAG) lipase (Farooqui et al., 1986). The accepted hypothesis is that this enzyme would hydrolyze the sn1-ester bond of sn2-arachidonate-containing DAGs to form 2-AG. Whereas these studies confirm the existence of distinct pathways for the biosynthesis of endogenous cannabinoids, future studies will be required to fully understand the contribution of the individual pathways to the overall regulation of endocannabinoid signaling.

1.3. Biosynthesis of anandamide

Signaling in all neurotransmitter pathways is tightly controlled. This control is typically accomplished by regulating the levels of neurotransmitter available to the target receptor. Therefore, much work has been done focusing on the cellular biosynthesis of AEA and 2-AG. Unlike other neurotransmitters, AEA and 2-AG do not appear to be stored in vesicles for release into the synapse. Rather, the formation and release of these endocannabinoid molecules results from a stimulus-dependent cleavage from membrane phospholipid precursors. AEA is thought to be synthesized and released from the membrane precursor N-arachidonylphosphatidylethanolamine (NAPE). The enzyme N-acetyl-transferase catalyzes the transfer of arachidonate from the sn-1 position of a phospholipid to the primary amine of phosphatidylethanolamine by a calcium-dependent mechanism to form NAPE (Di Marzo et al., 1994; Cadas et al., 1997). The formation and release of AEA occurs by the calcium-dependent cleavage of NAPE by a phosphodiesterase that appears to display properties similar to the activity of phospholipase D (PLD) (Di Marzo et al., 1994). A novel PLD was isolated from rat heart and brain and proposed to be specific for the production of N-acylethanolamides such as AEA (Petersen & Hansen, 1999). The molecular characterization of this PLD, named NAPE-PLD, by Okamoto et al. (2004) has confirmed the enzyme to be primarily responsible for the formation of AEA and other endogenous N-acylethanolamines. There is strong evidence that formation of the other major cannabinoid, 2-AG, involves an sn1-specific diacylglycerol (DAG) lipase (Farooqui et al., 1986). The accepted hypothesis is that this enzyme would hydrolyze the sn1-ester bond of sn2-2-arachidonate-containing DAGs to form 2-AG. Whereas these studies confirm the existence of distinct pathways for the biosynthesis of endogenous cannabinoids, future studies will be required to fully understand the contribution of the individual pathways to the overall regulation of endocannabinoid signaling.

1.4. Termination of anandamide signaling

Termination of AEA signaling appears to be regulated by a two-step process: (a) AEA is transported into cells where, (b) it undergoes enzymatic hydrolysis by fatty acid amide hydrolase (FAAH) to form arachidonic acid and ethanolamine (Deutsch & Chin, 1993; Di Marzo et al., 1994; Cravatt et al., 1996; Beltramo et al., 1997; Hillard et al., 1997). This amidohydrolase activity has been shown to be relatively selective for long chain fatty acid amides and esters, sensitive to inhibition by the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), and found with highest activity in the globus pallidus and hippocampus in the CNS (Desarnaud et al., 1995; Ueda et al., 1995). The cloning of FAAH by Cravatt et al. (1996) has allowed for more detailed functional studies of this enzyme. These studies have demonstrated that FAAH has broad substrate
recognition catalyzing the hydrolysis of other fatty acid amides such as oleamid (Maurelli et al., 1995; Cravatt et al., 1996), 2-AG (Di Marzo et al., 1998; Goparaju et al., 1998), as well as AEA. Bracey et al. (2002) report the crystal structure of FAAH at 2.8 Å and conclude that, unlike other serine proteases of the same family, FAAH has a unique structural profile that allows it to integrate with cellular membranes giving its active site direct access to a membrane bilayer (Bracey et al., 2002). This bilayer is conceivably part of an intracellular compartment to which AEA has been trafficked. Therefore, AEA that is present in the membranes of intracellular compartments containing FAAH becomes readily available to the enzyme for serine-mediated hydrolysis.

In the rat brain, immunocytochemical studies have found the highest levels of FAAH in the cerebellum, neocortex, striatum, thalamus, and hippocampus, thus demonstrating a strong correlation with the known localization of CB1 receptors in the CNS. Additional immunocytochemical studies revealed that FAAH is localized to intracellular membrane compartments (Cravatt et al., 1996; Egertova et al., 1998). The antibody staining of rat FAAH in transfected Cos-7 cells appears to be perinuclear with FAAH possibly residing in the endoplasmic reticulum or mitochondria (Giang & Cravatt, 1997); however, detailed co-localization studies of native FAAH with known markers of intracellular compartments has yet to be reported. Immunofluorescence microscopy studies from our lab have confirmed the intracellular localization of FAAH in RBL-2H3 cells and have suggested that FAAH exhibits co-localization with cellubrevin, a protein that is associated with endocytic pathways (unpublished results). While 2-AG is a substrate for FAAH in vitro (Ueda et al., 2000), studies with FAAH knockout mice have indicated that FAAH is not essential for the metabolism of 2-AG in vivo (Lichtman et al., 2002). The enzyme monoacylglycerol lipase has been identified in brain and implicated as the primary enzyme responsible for 2-AG hydrolysis (Dinh et al., 2002). Termination of 2-AG signaling likely involves a key to understanding the mechanism of endocannabinoid inactivation. AEA uptake has been observed in multiple cell lines including C6 glioma (Deutsch & Chin, 1993), RBL-2H3 (Bisogno et al., 1997; Rakshan et al., 2000), U937 lymphoma (Maccarrone et al., 1998), CHP100 neuroblastoma (Maccarrone et al., 1998), N18TG2 neuroblastoma (Deutsch & Chin, 1993), and CCF-STTG1 astrocytoma (Beltramo & Piomelli, 1999). The uptake of AEA has also been shown to occur in primary neuronal cell cultures of cerebellar granule cells (Hillard et al., 1997), striatal neurons, astrocytes (Di Marzo et al., 1994; Beltramo et al., 1997), and cortical neurons (Fegley et al., 2004). There is a level of consistency between the data reported in the above studies leading to some consensus in the field regarding the process by which AEA transport occurs. AEA transport (a) can be inhibited by select fatty acid amide derivatives or specific AEA analogues in a dose-dependent manner; (b) is temperature-dependent; (c) occurs rapidly ($t_{1/2} = 2.5$ min); (d) is regulated by second messengers and signal transduction pathways; and (e) is saturable at $37$ °C (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997; Maccarrone et al., 1998; Maccarrone et al., 2000; Rakshan et al., 2000). AEA transport has also been shown to be independent of ion gradients and ATP hydrolysis (Beltramo et al., 1997; Hillard et al., 1997; Rakshan et al., 2000). Additionally, AEA transport is facilitated by nitric oxide donors in human umbilical vein endothelial cells suggesting that the uptake process can be regulated by a nitric oxide synthase-mediated mechanism (Maccarrone et al., 2000). Prompted by these studies, there have been considerable efforts made by numerous labs (personal communications), including ours, to identify the putative AEA transporter. To date, these efforts have been largely unsuccessful. However, there has been a role established for FAAH in maintaining an inward concentration gradient of AEA thereby promoting transport (Day et al., 2001; Deutsch et al., 2001).

It is agreed that AEA movement across the cell membrane is a saturable process; however, there is yet to be a consensus as to what cellular component is being saturated, as was eloquently discussed by Hillard and Jarrahian (2003). Based on current data, there are several models for AEA uptake by cells (Fig. 2). The cellular accumulation profile of AEA (discussed in the paragraph above) along with the observation that AEA uptake can be specifically inhibited by compounds that are not substrates for or inhibitors of FAAH (Ortar et al., 2003; Fegley et al., 2004; Ligresti et al., 2004) suggest that AEA transport occurs by a carrier-mediated process in which a protein carrier binds and translocates AEA from one side of the membrane to the other (Fig. 2A). It has also been suggested that AEA accumulation by cells is dependent solely on simple diffusion and that FAAH-mediated enzymatic cleavage of AEA, which maintains an inward concentration gradient, is the sole driving force behind accumulation (Glaser et al., 2003; Fig. 2B). Recently, Hillard and Jarrahian (2003) suggested that the cellular accumulation of AEA could be the result of intracellular sequestration of AEA (Hillard & Jarrahian, 2003; Fig. 2C). We have proposed an additional mechanism whereby AEA uptake
may occur via an endocytic process that targets AEA to intracellular compartments containing FAAH (Fig. 2D).

Inhibition of FAAH would effectively decrease the uptake of AEA by cells in all four of the models for AEA transport presented in Fig. 2. FAAH inhibitors could exert their effect on AEA transport by decreasing the inward concentration gradient of AEA (Fig. 2A-C) or by decreasing the efficiency of AEA clearance from recycling machinery (Fig. 2D).

Molecules that display no activity as FAAH inhibitors but specifically inhibit AEA transport presumably exert their effect by a different mechanism. In the model of AEA transport as a carrier-mediated process, transport could be inhibited by molecules that interact with a membrane-bound carrier protein and compete for the AEA carrier (Fig. 2A).

Similarly, molecules that interact with the proposed AEA binding protein resident in caveolae/lipid rafts could effectively inhibit AEA accumulation via a caveolae-related endocytic process (Fig. 2D). Furthermore, molecules that disrupt endocytic machinery could also decrease the cellular accumulation of AEA (Fig. 2D). In the models where sequestration of AEA occurs, those molecules that are sequestered into the same compartment as AEA could displace AEA from this compartment and thereby decrease the inward gradient for uptake (Fig. 2C and D).

2.1. Anandamide transport by a carrier-mediated process

There is extensive evidence from structure-activity studies of AEA derivatives supporting the hypothesis that AEA transport occurs via a protein carrier-mediated process. These structure-activity studies reveal that ligand recognition by the carrier is stereoselective and demonstrates well-defined structural requirements (Khanolkar & Makriyannis, 1999; Melck et al., 1999; Piomelli et al., 1999; Jarrahian et al., 2000). The argument could be made that the structural requirements of a molecule for effective inhibition of the cellular accumulation of AEA represent the characteristics of a molecule that inhibits FAAH, and thereby, the mechanism that maintains an inward concentration gradient of AEA. However, several labs have reported substances that selectively inhibit the cellular uptake of AEA, and yet, demonstrate no effect on FAAH activity (Di Marzo et al., 2002; Lopez-Rodriguez et al., 2003a, 2003b; Ortar et al., 2003; Fegley et al., 2004; Ligresti et al., 2004). These findings indicate that the structural requirements of a molecule that are necessary to impart activity as an inhibitor of the cellular accumulation of AEA are not solely representative of the molecule’s activity as a FAAH inhibitor.
There have been other experimental observations that support the cellular uptake of AEA as a protein carrier-mediated process. The phenomenon of trans flux coupling is a result of an accumulation of substrate on the cis side of the membrane that causes an accumulation of carrier-proteins with their binding faces on the trans side of the membrane, and thereby movement of extracellularly applied substrate against the concentration gradient. Hillard and Jarrahian (2000) reported that the trans effect of flux coupling occurs for AEA in cerebellar granule neurons. This data is consistent with a model of AEA cellular accumulation that displays protein carrier-mediated movement of AEA across the cell membrane. Another study reported that AM404, an inhibitor of the AEA transport (Beltramo et al., 1997) and FAAH (Jarrahian et al., 2000), inhibits the efflux of AEA by both striatal and endothelial cells (Gerdenman et al., 2002; Maccarrone et al., 2002). If AM404 exerted its inhibitory effect on AEA transport by FAAH inhibition, then it would be expected that efflux of accumulated AEA would be facilitated by treatment of cells with AM404. The inhibition of AEA efflux by AM404 implies that AEA and AM404 are common substrates for a putative carrier protein at the membrane. This conclusion is further supported by evidence indicating that AM404 is a competitive-type inhibitor of AEA uptake (Rakhshan et al., 2000) and that AM404 is a substrate for the putative carrier (Piomelli et al., 1999).

Evidence to implicate a classic bi-directional membrane protein carrier in cellular accumulation of AEA has thus far only been reported in neurons and endothelial cells (Hillard & Jarrahian, 2000; Gerdenman et al., 2002). However, AEA accumulation that is sensitive to inhibition by select AEA analogues has been shown to occur in multiple cell lines, including cells that do not express detectable FAAH activity (Day et al., 2001; Ligresti et al., 2004). There could be membrane-associated scavenger or binding proteins that interact with AEA and serve as saturable cellular components of the AEA transport system. This model of AEA accumulation by cells is consistent with facilitative transport processes observed for other lipophilic molecules (Schaffer & Lodish, 1994; Kanai et al., 1995).

2.2. Facilitated diffusion mediated by fatty acid amide hydrolase

The cloning of FAAH by Cravatt et al. (1996) has allowed for a detailed pharmacological and functional characterization of this enzyme, and it has since been found to be the primary enzyme responsible for AEA metabolism (Ueda et al., 2000; Cravatt et al., 2001). The role of FAAH in the cellular uptake of AEA has been a topic of much interest and debate. Studies monitoring the accumulation of AEA by various cell lines have concluded that cellular expression of FAAH has a positive impact on the extent to which AEA accumulation occurs in cells. Deutsch et al. (2001) report that accumulation of AEA by FAAH-expressing neuroblastoma (N18) or glioma (C6) cells is blocked by 50% when cells are treated with the FAAH inhibitor methylarachidonylfluorophosphonate (MAFP; Deutsch et al., 2001). Deutsch et al. (2001) also report that cellular accumulation of AEA by laryngeal carcinoma (Hep2) cells, which do not display measurable endogenous FAAH activity, is not affected by treatment with 1 μM MAFP. Work performed in our laboratory by Day et al. (2001) has produced similar results. HeLa cells that lack detectable FAAH display a 2-fold increase in cellular uptake of AEA when they are transfected with FAAH (Day et al., 2001). The observed augmentation of AEA uptake in FAAH-transfected HeLa cells is blocked by PMSF, a FAAH inhibitor (Day et al., 2001). These data suggest that a role for FAAH in the cellular accumulation of AEA is to maintain an inward concentration gradient of AEA, and thus movement of AEA from outside to inside the cell.

Based on kinetic analysis of AEA uptake by N18TG2 neuroblastoma and CCF-STTG1 astrocytoma cell lines, Glaser et al. (2003) have suggested that AEA uptake by these cells is completely dependent on FAAH activity and that no protein-mediated transport process or transporter exists (Glaser et al., 2003). This conclusion is based on the observation that AM404, arvanil, and olvanil, all of which are FAAH transport inhibitors that display activity as FAAH inhibitors as well (for review, see Hillard & Jarrahian, 2000), significantly decrease AEA accumulation by cells at 5 min but have no impact on the cellular accumulation of AEA at early time points (Glaser et al., 2003). This observation was consistent for both neuroblastoma cells possessing FAAH activity, and astrocytoma cells lacking FAAH activity at 25 sec and 5 min time points or 45 sec and 5 min time points, respectively (Glaser et al., 2003). Glaser et al. (2003) contend that if a membrane carrier-protein was involved in the transport of AEA then there should be a saturable effect of transport inhibitors at the early time points of 25 and 45 sec. They conclude that the saturable inhibition of AEA accumulation at the 5-min time point is a result of the inhibition of downstream components of the AEA uptake process, that is, FAAH (Glaser et al., 2003). This model proposes that the saturable component of the system responsible for the cellular accumulation of AEA is FAAH. Analysis at early time points is valid with regards to the target. The question remains as to the order of the uptake process and what are the targets for the drugs used. If the inhibitors act at a target downstream of the initial uptake events, then no saturable activity would be observed at the early time points as reported. The role of FAAH as the sole driver of AEA uptake is not supported by data reported by Day et al. (2001) in which the uptake of AEA by wild-type HeLa cells is inhibited by the FAAH inhibitor MAFP, which is structurally similar to AEA (Day et al., 2001). Because HeLa cells do not display detectable levels of endogenous
FAAH activity, the inhibition of AEA uptake by MAFP in wild-type HeLa cells could be due to the interaction of MAFP with a presumed carrier protein at the plasma membrane. The $IC_{50}$ value for MAFP for inhibition of AEA uptake in HeLa cells was $\sim 9 \mu M$ (Day et al., 2001) suggesting that the $1 \mu M$ concentration used in the studies by Glaser et al. (2003) may have been insufficient to elicit significant inhibition at a putative membrane carrier. Most likely at nanomolar or very low micromolar concentrations, MAFP inhibits FAAH activity but is not an effective inhibitor of the putative carrier protein. AEA uptake by wild-type HeLa cells was not inhibited by PMSF, a compound that is not structurally similar to AEA (Day et al., 2001) suggesting that those compounds structurally similar to AEA can act at a target distinct from FAAH. Studies have reported that selective inhibitors of FAAH do not reduce AEA uptake in either cortical neurons (Kathuria et al., 2003) or astrocytes (Beltramo et al., 1997), both of which exhibit FAAH activity. Furthermore, Ortar et al. (2003) showed that aromatic analogues of $N$-oleylethanolamine and AEA were able to inhibit the cellular accumulation of AEA, yet showed no activity as inhibitors of FAAH in N18TG2 cell membranes. Studies reported by Ligresti et al. (2004) show that brain synaptosomes from FAAH knockout mice (FAAH$^{-/-}$) demonstrate saturable accumulation of radiolabeled AEA, although it is reduced compared to FAAH-expressing (FAAH$^{+/+}$) littermates (Ligresti et al., 2004). This observation is supported by a recent report by Fegley et al. (2004) demonstrating that cortical neurons from both FAAH$^{-/-}$ and FAAH$^{+/+}$ mice accumulate AEA by a temperature-sensitive, rapid, and saturable process that is inhibited by AM404 (Fegley et al., 2004). Moreover, this study reports a novel compound, AM1172, that blocks internalization of radiolabeled AEA by human astrocytoma cells and rodent cortical neurons, but is not a substrate or inhibitor of FAAH (Fegley et al., 2004). Further evidence that other factors besides FAAH activity mediate the cellular accumulation of AEA is found in a study by Centonze et al. (2004) in which both cocaine and the dopamine-2 (D2) receptor agonist quinpirole significantly decreased FAAH activity in striatum while having no effect on the transport of AEA (Centonze et al., 2004). Taken together, these data indicate that while FAAH can play an important role in facilitating the transport of AEA by cells, FAAH is not solely required for AEA uptake to occur. Finally, although presumably FAAH is sufficiently lipophilic to partition into the membrane bilayer, simple non-ordered diffusion of AEA through the aqueous cytoplasm to intracellularly located FAAH is unlikely rapid enough to account for the rate of AEA accumulation and metabolism that is observed. Furthermore, it is not clear what force would drive movement of AEA out of the membrane into the hydrophilic cytoplasm. An organized means of trafficking is almost certainly necessary for the efficient delivery of AEA to FAAH.

### 2.3. Sequestration of anandamide by cells

Hillard and Jarrahian (2003) have presented evidence that there may be cellular components that can sequester AEA. This hypothesis is based on the observation that radiolabeled substrates reach intracellular concentrations that far exceed the concentration of the extracellular media in RBL-2H3 cells, C6 glioma cells, N18 neuroblastoma cells, Hep2 cells, and cerebellar granule neurons when they are treated with radiolabeled-AEA (Hillard & Jarrahian, 2000; Rakhshan et al., 2000; Deutsch et al., 2001). These data would seem to contradict evidence that free AEA moves across the plasma membrane in a manner dependent on the FAAH concentration gradient (Hillard et al., 1997). Hillard and Jarrahian (2003) conclude that because there is no convincing evidence of a process that actively transports AEA against a concentration gradient, one explanation for the concentrative behavior of AEA is the existence of two distinct intracellular pools of AEA: free AEA and AEA that is sequestered in a saturable cellular component. Sequestered AEA is not readily available to diffuse across the plasma membrane, and therefore, positively impacts the inward concentration gradient of AEA that is maintained by FAAH (Hillard & Jarrahian, 2003). Two mechanisms have been suggested to account for the sequestration of AEA by cells (Hillard & Jarrahian, 2003). First, AEA might associate with specific membraneous compartments that serve as a saturable reservoir for the lipophilic AEA. It could be argued that compounds that inhibit the cellular accumulation of AEA, yet display no activity as FAAH inhibitors, exert their effect by taking up space in this reservoir (Hillard & Jarrahian, 2003). Second, intracellular binding proteins may bind AEA in a saturable manner and allow for competitive binding of other molecules (Hillard & Jarrahian, 2003). This scenario also adequately accounts for the phenomena of AEA sequestration by cells and the accumulation of intracellular AEA beyond its concentration gradient.

### 2.4. Anandamide uptake by endocytic processes

We suggest that the mechanisms of AEA sequestration by cells described above may occur as part of caveolae-related endocytic process. Lipid rafts are plasma membrane microdomains that are enriched in cholesterol, sphingolipids, arachidonic acid, and plasmenylethanolamine (Brown & London, 2000; Pike et al., 2002). Caveolae have a composition similar to lipid rafts and have been identified as flask-shaped invaginations in the plasma membrane that are non-clathrin coated (Pike et al., 2002; Razani et al., 2002). A family of integral membrane proteins, the caveolins, serve as the major structural protein for caveolae (Razani et al., 2002). For long chain fatty acids, many proteins such as albumin, fatty acid binding proteins, and caveolin-1 have been reported to serve as intracellular shuttles of these lipids to various subcellular organelles.
(for review, see Stremmel et al., 2001). The diverse functions of caveolae include endocytosis, potocytosis, cholesterol transport, and organization of key signal transduction components (Sowa et al., 2001). Caveolae-related endocytosis has been observed and linked to caveolae, and is distinct from clathrin-dependent endocytosis that involves the internalization of specialized membrane domains known as clathrin-coated pits (Johannes & Lamaze, 2002). This process has been termed caveolae-related endocytosis as opposed to caveolae-dependent endocytosis due to its occurrence in lipid raft domains that lack the protein caveolin-1 (Sharma et al., 2003). The intracellular transport and uptake of the interleukin-2 receptor, simian virus 40, cholera toxin B-subunit, cholesterol esters, and albumin exhibits a dependence on intact caveolae/lipid raft-related endocytic pathways (Pelkmans et al., 2001; Puri et al., 2001; Schubert et al., 2001; Johannes & Lamaze, 2002).

AEA uptake demonstrates pharmacological specificity, is temperature-dependent, rapid, saturable at 37 °C, and energy-independent (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997; Maccarrone et al., 1998, 2000; Rakhshan et al., 2000). A caveolae-related endocytic mechanism for the cellular uptake of AEA could meet the above criteria and could also be facilitated by, but not dependent on, FAAH activity. Furthermore, endocytosis could be dependent on a saturable pool of binding proteins/carriers that allow for AEA transport inhibition by AEA analogues that display no FAAH activity and would account for the observed sequestration of AEA in cells by suggesting a saturable capacity of caveolae/lipid rafts to accumulate AEA.

Recent data from our lab suggest that AEA may be internalized by a caveolae-related endocytic process (McFarland et al., 2003, 2004). Such a process is known to be responsible for the intracellular transport of many different hydrophobic molecules in multiple cell types (Razani et al., 2002). For example, ceramide is a hydrophobic signaling molecule that is transported from the plasma membrane to the Golgi by a caveolae-related endocytic process (Puri et al., 2001; Sharma et al., 2003). Using multiple methods to inhibit endocytic processes, we have implicated a caveolae-related endocytic process in the specific uptake of AEA by RBL-2H3 cells. Depletion of cholesterol by nystatin/progesterone pretreatment, and thereby disruption of caveolae and lipid rafts, reduced the specific uptake of AEA by ~50% (McFarland et al., 2003). Pretreatment of cells with both N-ethylmaleimide (NEM) and the tyrosine kinase inhibitor genistein, known methods of inhibiting caveolae-related endocytosis (Schnitzer et al., 1995; Aoki et al., 1999; Puri et al., 2001), also reduced the cellular accumulation of AEA (McFarland et al., 2004). Inhibition of clathrin-dependent endocytosis by chlorpromazine pretreatment or incubation in potassium free buffer had no effect on the cellular accumulation of AEA. We propose the existence of a binding protein in caveolae that undergoes transport into cells by endocytosis in response to AEA. In cells expressing FAAH, AEA will be efficiently transported to intracellular compartments containing FAAH where metabolism occurs and thereby free the binding protein/carrier to recycle to the plasma membrane for participation in further transport events (Fig. 3). In our model, FAAH activity does not facilitate the uptake of AEA by maintaining an inward concentration gradient per se, but rather by clearing AEA from the saturable pool of binding proteins/carriers after intracellular transport. It is also interesting to note that albumin, which readily binds AEA, undergoes caveolae-related endocytosis (Schubert et al., 2001). The hypothesis that AEA transport occurs by the association of AEA to a protein in caveolae or lipid rafts followed by caveolae-related endocytosis of AEA is consistent with other proposed mechanisms of AEA transport in that it is facilitated but not dependent on FAAH activity, suggests a protein other than FAAH that is necessary for specific uptake, and accounts for the sequestration of AEA by cells. Interestingly, Sarker and Maruyama (2003) reported that treatment of various cell lines with methyl-β-cyclodextrin, which depletes cholesterol and thereby disrupts lipid rafts, inhibited AEA-induced cell death further supporting a role for specialized membrane microdomains in AEA signaling (Sarker & Maruyama, 2003).

Although caveolin-1 is not found in neurons, caveolin-1 is found in brain tissue (possibly glia; Cameron et al., 1997). Lipid-raft membrane domains lacking caveolin-1 are also prominent in brain tissue and are capable of endocytosis in the absence of caveolin-1, and therefore, could be involved in endocannabinoid uptake and signaling in the brain (Cameron et al., 1997; Sharma et al., 2002). Caveolae or lipid raft-related endocytosis occurs ubiquitously in cells. Thus, this model of AEA transport may account for the reason why AEA accumulation occurs in numerous, and drastically different cell types.

2.5. Inhibition of anandamide transport

Based upon reported studies concerning the process(es) governing the cellular accumulation of AEA, we can hypothesize that there are multiple mechanisms by which a small molecule could inhibit AEA uptake. Inhibition of FAAH will decrease intracellular metabolism of AEA, and thus, either decrease the inward concentration gradient of AEA or the efficient clearance of AEA from binding proteins potentially associated with the endocytic pathway. A compound that acts as a competitive substrate of the putative AEA carrier protein, whether a transporter or a binding protein, should also effectively block the cellular accumulation of AEA. Several classes of chemical compounds have been identified as AEA reuptake inhibitors and are summarized in Table 1. The characterization of such compounds is made difficult by the fact that the putative AEA carrier has yet to be actually
identified. Many of the compounds identified thus far as AEA reuptake inhibitors have also been shown to interact with either FAAH or the TRPV1 receptor. However, molecules have been reported that inhibit the cellular accumulation of AEA, but display no detectable activity as FAAH inhibitors (Jarrahian et al., 2000; Di Marzo et al., 2002; Lopez-Rodriguez et al., 2003a, 2003b; Fegley et al., 2004; Ligresti et al., 2004). In general, these compounds are all derivatives of long chain fatty acids with the most potent compounds possessing \( K_i \) values for AEA uptake inhibition in the low micromolar range. Structure activity relationship studies have reported that a transport inhibitor’s interaction with the AEA carrier is dependent on the ability to adopt a U-shaped, or bent conformation (Piomelli et al., 1999). This bent conformation is imparted by the presence of cis-double bonds in the middle of the fatty acid chain (Piomelli et al., 1999; Reggio & Traore, 2000). The AEA binding site of the transporter also appears to tolerate very bulky additions to the head group of the fatty acid chain (Piomelli et al., 1999; Jarrahian et al., 2000). A hydrogen bonding acceptor near the head group region is necessary for activity as an AEA uptake inhibitor (Piomelli et al., 1999). However, hydrophobic character in the region of the head
group appears to favor binding to the carrier as well (Jarrahian et al., 2000; Reggio & Traore, 2000).

3. In vivo studies of anandamide transport and metabolism

Understanding of the molecular mechanism(s) of AEA transport, while not yet completely characterized, has validated the system as a potential drug target for positively modulating cannabinergic tone. Characterization of how inhibition of AEA transport and metabolism affects other cellular/neuronal systems, and animal models of disease will be crucial to the development of therapeutically relevant compounds. Studies examining the AEA transport and behavioral profiles of FAAH−/− mice along with work examining the effects of AEA transport inhibitors in rodent models of disease have revealed a great deal about the therapeutic potential of compounds that inhibit AEA transport.

3.1. Fatty acid amide hydrolase knockout mice

As discussed above, molecules that inhibit FAAH activity dramatically decrease the cellular accumulation of AEA. A decrease in AEA metabolism by FAAH should result in increased cannabinoid-induced behavioral effects because AEA will not be efficiently cleared from the extracellular spaces. In vivo studies performed in rodent models have shown that exogenously administered AEA exerts only mild and transient cannabimimetic behavioral effects. This is likely due to the rapid removal from the system and metabolism of AEA (Cravatt et al., 2001). Cravatt et al. (2001) reported that mice lacking FAAH (FAAH−/− mice) exhibit a dramatic deficiency in their ability to metabolize AEA and display CB1 receptor-mediated responses of analgesia, hypomotility, catalepsy, and hypothermia when treated with exogenous AEA. Endogenous brain levels of AEA are found to be increased 15-fold in FAAH−/− mice, and FAAH−/− mice display a reduced pain response that is reversible by a CB1 receptor antagonist (Cravatt et al., 2001). These findings validate FAAH as a potential drug target for modulation of pain perception and indicate that inhibitors of FAAH may be effective tools for modulating AEA-linked cannabinoid tone in the CNS. However, AEA also displays proconvulsant activity in FAAH−/− mice causing them to demonstrate an increased seizure susceptibility (Clement et al., 2003). This is probably due to the capability of AEA to inhibit the

<table>
<thead>
<tr>
<th>Head group modification of fatty acid backbone</th>
<th>Representative compound</th>
<th>Transport inhibition approx. IC50 or K (μM)</th>
<th>Transient receptor potential vanilloid type 1 receptor activity approx. EC50 or K (μM)</th>
<th>Inhibition of fatty acid amide hydrolase approx. IC50 (μM)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenols</td>
<td>AM404</td>
<td>1–5</td>
<td>110 nM</td>
<td>0.3–0.9</td>
<td>Beltramo et al., 1997; Jarrahian et al., 2000; Zygmunt et al., 2000</td>
</tr>
<tr>
<td></td>
<td>VDM11</td>
<td>10–11</td>
<td>ND</td>
<td>50</td>
<td>De Petrocellis et al., 2000</td>
</tr>
<tr>
<td>Catechols</td>
<td>Arvanil</td>
<td>3.6</td>
<td>0.5 nM</td>
<td>32</td>
<td>Melck et al., 1999; De Petrocellis et al., 2000</td>
</tr>
<tr>
<td></td>
<td>UCM707</td>
<td>0.8</td>
<td>5 μM</td>
<td>30</td>
<td>Lopez-Rodriguez et al., 2003a,b</td>
</tr>
<tr>
<td>Five-membered heterocycles</td>
<td>OMDM2</td>
<td>3</td>
<td>10 μM</td>
<td>50</td>
<td>Ortar et al., 2003</td>
</tr>
<tr>
<td>Tyrosinol derivatives</td>
<td>AM1172</td>
<td>2–2.6</td>
<td>ND</td>
<td>ND</td>
<td>Fegley et al., 2004</td>
</tr>
<tr>
<td>Benzonamides</td>
<td></td>
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<td>ND=not detectable.</td>
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release of multiple types of neurotransmitters, and thus, exert both inhibitory and disinhibitory effects in the brain depending on the cellular localization of the CB1 receptor. Inhibition of FAAH activity may also impact the production and metabolism of molecules in other pathways. For example, FAAH−/− mice dosed with AEA show an increased production of prostamides compared to normal mice that may indicate increased cyclooxygenase-2 activity (Weber et al., 2004). The effects of FAAH inhibition on other signaling pathways will certainly have to be assessed as the field further characterizes the usefulness of FAAH inhibitors as therapeutically effective pharmacological tools, and the role of FAAH in AEA uptake is further defined.

3.2. Anandamide reuptake inhibitors in vivo

AEA reuptake inhibitors have been used in vivo in an effort to demonstrate their ability to inhibit cellular accumulation of AEA and thereby stimulate cannabimimetic signaling. The AEA reuptake inhibitor AM404 produces physiological effects similar to AEA in vivo such as decreased plasma prolactin secretion, increased hypothalamic tyrosine hydroxylase activity, decreased activity of tyrosine hydroxylase in the substantia nigra, increased time spent in inactivity, and marked motor inhibition (Gonzalez et al., 1999). AM404 has also been shown to inhibit the specific uptake of AEA in vivo and to potentiate the receptor-mediated effects of exogenously administered AEA (Beltramo et al., 1997; Calignano et al., 1997). In vitro, however, AM404 is an agonist of the TRPV1 receptor (Zygmunt et al., 2000) and is a competitive inhibitor of FAAH (Jarrahian et al., 2000). Thus, while AM404 appears to be an effective AEA transport inhibitor in vivo, it is not optimal for the accurate characterization of the physiological effects specific to inhibition of AEA transport. With myriad potential therapeutic uses of an AEA reuptake inhibitor, significant efforts have focused on the development of compounds selective for the AEA transport process, selective AEA reuptake inhibitors (SARI).

A new specific inhibitor of AEA uptake, UCM707, has recently been characterized and revealed to be the most potent inhibitor of AEA transport yet described (Lopez-Rodriguez et al., 2003a, 2003b). UCM707 displays little to no affinity for FAAH, TRPV1 receptors, CB1 receptors, or CB2 receptors in vitro (Lopez-Rodriguez et al., 2003a, 2003b). In vivo rodent studies examining dose-response effects on antinociception and motor activity show that UCM707 is able to potentiate the receptor-mediated actions of a subeffective dose of AEA. However, UCM707 administration alone showed little effect (Lopez-Rodriguez et al., 2003a, 2003b). This could possibly be due to the fact that SARI will only augment the action of endogenous cannabinoids that are present in the system. It is reasonable to hypothesize that AEA synthesis and release needs to be triggered by a disease state or dysregulated physiology in order to see relevant physiological effects of SARI.

4. Potential therapeutic implications of anandamide transport inhibition

The physiological effects of Δ9-THC, the active principal of marijuana, and other cannabinoids have been of interest since ancient times. Cannabinoids have been implicated in the treatment of pain, appetite loss, nausea, asthma, autoimmune disease, arthritis, fever, and glaucoma (Di Marzo, 1998; Piomelli et al., 2000; Cravatt & Lichtman, 2003). Over the last decade, the isolation of AEA and other endogenous cannabinoids showing activity at the cannabinoid receptors has allowed for the characterization of the pathways that regulate endocannabinoid signaling (Devane et al., 1992; Sugiura et al., 1995; Hanus et al., 2001; Porter et al., 2002). Emerging evidence suggests that compounds inhibiting the removal of AEA (i.e., transport and metabolism) from the receptor-mediated signaling pathways by either FAAH inhibition or inhibition of the putative AEA carrier may be therapeutically relevant in the treatment of specific disease states.

4.1. Analgesia

It has been reported by Richardson et al. (1997) that the cannabinoid receptor antagonist SR 141716A produces a hyperalgesic state when administered to mice suggesting a role for the endogenous cannabinoids in nociception (Richardson et al., 1997). Antisense oligonucleotides directed against CB1 receptor mRNA produced a significant reduction in spinal cannabinoid binding sites that was accompanied by the induction of a hyperalgesic state (Richardson et al., 1998). Interestingly, hyperalgesia induced by SR 141716A is blocked in a dose-dependent manner by the administration of the NMDA receptor antagonists D-AP-5 or MK-801 suggesting that there is tonic activation of the spinal endocannabinoid system that is involved in modulating pain perception (Richardson et al., 1998). A decrease in this cannabinergic tone results in NMDA-dependent hyperalgesia (Richardson et al., 1998). These data convincingly reveal the importance of the role of endocannabinoid signaling in nociception. Furthermore, FAAH−/− mice have been shown to possess a baseline threshold of pain perception that is increased compared to FAAH+/+ mice (Cravatt et al., 2001). Administration of exogenous AEA is not necessary to produce the hyperalgesia in FAAH−/− mice as they possess endogenous AEA levels in brain that are 15-fold higher than those observed in FAAH+/+ mice (Cravatt et al., 2001). Several inhibitors of AEA transport have been shown to enhance the analgesic response to a subeffective dose of AEA (Beltramo et al., 1997; Lopez-Rodriguez et al., 2003a, 2003b; de Lago et al., 2004). Also, the AEA transport inhibitor VDM11 blocks AEA-mediated activation of the pain receptor TRPV1 by preventing AEA from accessing its intracellular binding site on the receptor (De Petrocellis et al., 2001).
Taken together, these studies indicate that treatment with both FAAH inhibitors and compounds that inhibit AEA uptake might show therapeutic benefits as analgesic agents. A dysregulation of the antagonistic relationship between endocannabinoid and NMDA receptor signaling pathways could result in a constant state of hyperalgesia reminiscent of chronic pain. Thus, positive regulation of endocannabinoid signaling might be therapeutically useful in the treatment of chronic pain. However, we must consider that the above studies were performed on animals that do not exhibit a disease state that would classically be associated with chronic pain. It is possible that during a disease that results in chronic pain, the endocannabinoid mediators of analgesia produced by the body, such as AEA, are not able to maintain a cannabinergic tone that is sufficient to prevent hyperalgesia. This could be because another signaling pathway that positively regulates nociception, such as NMDA receptor-mediated signaling, has been up-regulated, and a presumed increase in endocannabinoid production is unable to compensate for it. Another possibility is that there is a malfunction of the endocannabinoid signaling pathway itself resulting in decreased cannabinergic tone and thereby hyperalgesia. Inhibitors of AEA transport alone, although they do not appear to induce a hypoalgesic state in healthy animals, might demonstrate greater “stand-alone” effects with potential therapeutic benefit in the treatment of chronic pain if administered to animals that have altered physiology and thus, possibly exhibit a cannabinergic tone that is insufficient to prevent a hyperalgesic state.

4.2. Anxiety

Cannabinoids exert effects on emotions such as anxiety in humans and animal models with seemingly contradictory actions. Δ⁹-THC precipitates feelings of both euphoria and anxiety in humans (Zuardi et al., 1982). This finding is consistent with data demonstrating that cannabinoids exert both anxiogenic and anxiolytic effects in animals (Sulcova et al., 1998; Berrendero & Maldonado, 2002). What factors dictate the effects of Δ⁹-THC and other cannabinoids on anxiety remain poorly understood. As eloquently discussed by Gaetani et al. (2003), the varying effects of cannabinoid receptor agonists on anxiety could be due to cannabinoid receptor expression in diverse regions of the brain that have contrasting effects on anxiety-like responses (Herkenham et al., 1990; Freund et al., 2003). Alternatively, the cellular localization of CB1 receptors with both GABAergic and glutamatergic axon terminals in the forebrain, where AEA inhibits the release of either neurotransmitter, creates a situation in which cannabimimetic activity could have markedly different effects on anxiety depending on the balance between the two pathways (Hajos et al., 2001; Freund et al., 2003; Gaetani et al., 2003). Kathuria and coworkers recently utilized the compound URB597 to test the hypothesis that inhibition of FAAH will increase available levels of endogenous AEA and thereby potentially produce an anxiolytic effect in rats (Kathuria et al., 2003). URB597 inhibits FAAH activity with an IC₅₀ value of 0.5 nM displaying no significant activity at the CB1 receptor, CB2 receptor, monoglycerol lipase, or as an inhibitor of AEA transport (Kathuria et al., 2003). URB597 decreased isolation-induced vocalizations of rat pups removed from their nest and demonstrates properties in the elevated-maze test similar to those of the anxiolytic benzodiazepines (Kathuria et al., 2003). Both of these results indicate that administration of the FAAH inhibitor URB597 produces an anxiolytic effect in rats. Interestingly, URB597 administration to rats did not induce the prototypical physiological responses to CB1 receptor agonists of catalepsy, hypothermia, or hyperphagia (Freund et al., 2003; Kathuria et al., 2003). Endogenous brain levels of AEA were shown to be increased in animals treated with URB597, and the anxiolytic effects of this compound could be blocked by the CB1 receptor antagonist SR 141716A (Kathuria et al., 2003). These findings support further exploration of the ability of compounds that inhibit FAAH-mediated hydrolysis of AEA or block the uptake of AEA to modulate anxiety by augmenting endogenous AEA receptor-mediated signaling.

4.3. Spasticity

Anecdotal evidence has existed for some time that cannabinoids provide benefit in the treatment of muscle spasticity associated with diseases such as multiple sclerosis. The potential link between cannabimimetic activity and antispastic effects was tested by Baker et al. (2000) through administration of the CB1 receptor agonist R(+)-WIN 55,212 and Δ⁹-THC to mice with chronic relapsing experimental allergic encephalomyelitis (CREAE), which is an autoimmune model of multiple sclerosis. CB1 receptor activation was found to effectively reduce spasticity in this murine model of disease (Baker et al., 2000). Furthermore, the antispastic effects of these CB1 receptor agonists were inhibited by SR 141716A (Baker et al., 2000). Similar effects on spasticity in CREAE mice were observed when the endocannabinoids AEA and 2-AG were administered exogenously (Baker et al., 2001). Research by de Lago et al. (2004) report that the novel compounds OMDM-1 and OMDM-2 act as inhibitors of AEA transport and thereby potentiate the receptor-mediated activity of endogenous AEA (de Lago et al., 2004). These compounds also effectively reduced hindlimb spasticity in mice with CREAE (de Lago et al., 2004). Future studies will likely further confirm the role of CB1 receptors as modulators of spasticity in multiple sclerosis and the potential of compounds that inhibit AEA uptake to produce a similar modulatory effect.

4.4. Neuropsychiatric disorders

The areas of the brain in which the CB1 receptor is most prominently expressed are the basal ganglia and cortex
CB1 receptors are expressed endogenously on enteric nerves, and their activation has been shown to produce a gastroprotective effect (Di Carlo & Izzo, 2003). Also, there are detectable levels of the endocannabinoid AEA expressed throughout the GI tract (Di Carlo & Izzo, 2003). Currently, the cannabinoids naboline and $\Delta^2$-THC are used as anti-emetic agents (Di Carlo & Izzo, 2003). It is possible that both inhibitors of FAAH, and AEA uptake would show some therapeutic benefit as anti-emetic agents due to the presence of endogenous AEA in the gut. At the very least, one would expect inhibitors of AEA uptake or metabolism to augment the activity of cannabinoids and cannabinoid-like pharmaceutical agents working as anti-emetics.

Another therapeutic potential for regulation of endocannabinoid signaling relates to proliferation of prostate carcinoma (PC-3) cells. AEA causes inhibition of prostate cancer cell proliferation via a cannabinoid receptor-mediated mechanism (Melck et al., 2000; Mimeault et al., 2003; Sanchez et al., 2003). Ruiz-Llorente et al. (2004) have demonstrated that AEA is accumulated by PC3 cells in a manner that is consistent with carrier-mediated uptake and that PC3 cells endogenously express FAAH (Ruiz-Llorente et al., 2004). Further studies are necessary, but it is possible that SARI could augment the endocannabinoid regulation of prostate cancer cell proliferation.

5. Discussion

There has been a tremendous amount of effort put forth in attempts to characterize the mechanisms associated with termination of endocannabinoid signaling. AEA transport appears, depending on which cell line is used and how uptake experiments are performed, to occur by either a carrier-mediated transport or a form of facilitative diffusion. Here we discussed four possible models of how AEA cellular accumulation may occur including a bidirectional membrane-bound protein carrier, simple diffusion dependent on FAAH activity, sequestration of AEA by cells, and caveolae-related endocytosis. Experimental evidence can be found to support each model, and it is conceivable that all four hypotheses are plausible explanations of how AEA transport occurs. If there are a variety of mechanisms mediating AEA transport it would help to explain the multiple and diverse results obtained from various cell lines that accumulate AEA. The major limiting factor in the characterization of AEA transport is that the molecular identity of the putative AEA carrier remains unknown. Medicinal chemistry has provided compounds that show specificity for the AEA carrier and little interaction with FAAH, CB1 receptors, CB2 receptors, and TRPV1 receptors. Such compounds have been useful tools in characterizing AEA transport both in vitro and in vivo and validating transport inhibition as a viable drug target. Identification of the AEA carrier will allow for a more complete understanding of AEA transport and will undoubtedly be useful in the design of compounds to inhibit this process.

Natural and synthetic cannabinoids have become approved for use in the treatment of certain disease states, usually employed for their anti-emetic effects and their efficacy as analgesics. However, there are social stigmas and concerns associated with the use of cannabinoids as part of an approved therapeutic regimen. This is due largely to the abuse potential of agents that directly activate cannabinoid receptors.
receptors. Compounds that augment endocannabinoid signaling by inhibition of transport or metabolism potentially offer a new means of pharmacological intervention. The pharmacodynamic effect of such compounds is dependent upon, and limited by, the production of endogenous AEA. Therefore, it is not expected that the abuse potential of these drugs would be significant. Much like the monoamine transport inhibitors that are currently used in the drug therapy of depression, inhibitors of AEA uptake and metabolism would only augment the body’s endogenous signaling pathways. Thus, compounds that inhibit AEA uptake and metabolism might only be able to significantly impact cannabinoid receptor-mediated signaling during times when there is a stimulus for the production of endogenous AEA or a need for increased cannabinergic tone. The cannabinoid receptor-mediated signaling pathways have been linked to several disease states. Research suggests that targeting and reducing the removal AEA from the system by treatment with SARI could represent an effective means of therapeutic intervention in many disease states such as chronic pain, multiple sclerosis, and neuropsychiatric disorders. Therapeutically relevant observations have also been made from models in which FAAH-mediated metabolism of AEA in the system has been decreased. FAAH inhibitors show anxiolytic activity in rats, and FAAH knockout mice are hypoalgesic suggesting that FAAH inhibitors might be useful in the treatment of both anxiety and chronic pain. Future studies may show modulation of AEA transport and metabolism to be a useful tool in the treatment of psychiatric disorders and control of GI function. Overall, there is strong evidence that drugs that alter both FAAH and the putative AEA carrier will show therapeutic relevance in several disease states and merit further study to fully understand the contribution of the endocannabinoid system to disease.

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References


