ENDOCANNABINOID SYSTEM IN THE PLANARIAN MODEL

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In this study, the presence and possible function of endocannabinoid ligands in the planarian is investigated. The endocannabinoids ananadamide (AEA) and 2-arachidonoylglycerol (2-AG) and entourage NAE compounds palmitoylethanolamide (PEA), stearoylethanolamide (SEA) and oleoylethanolamide (OEA) were found in *Dugesia dorotocephala*. Changes in SEA, PEA, and AEA levels were observed over the initial twelve hours of active regeneration. Exogenously applied AEA, 2-AG and their catabolic inhibition effected biphasic changes in locomotor velocity, analogous to those observed in murines. The genome of a close relative, *Schmidtea mediterranea*, courtesy of the University of Utah *S. med* genome database, was explored for cannabinoid receptors, none were found. A putative fatty acid amide hydrolase (FAAH) homolog was found in *Schmidtea mediterranea*.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The Endocannabinoid System

The endocannabinoid system (ECS) is a complex endogenous signaling system comprised of cannabinoid receptors, their endogenous ligands, and ligand metabolizing enzymes. The pair of cannabinoid receptors termed CB_1 and CB_2 are G-protein-coupled receptors (GPCR) named for the exogenous ligand Δ^9-tetrahydrocannabinol (THC) famously found in *Cannabis sativa*. Their endogenous ligands, referred to as endocannabinoids, are polyunsaturated fatty acid-based lipid molecules in the eicosanoid super family. Discovery[1] and cloning[2] of the original CB receptor prompted the characterization of its endogenous ligand *N*-arachidonoylethanolamide (also known as anandamide, NAE 20:4, AEA)[3], another "peripheral" receptor later termed CB_2[4], then the abundant ligand 2-arachidonoylgylcerol (2-AG)[5, 6]. The ECS has been found to play a central role in immune function[7], motor function[8], memory, cognition[9], appetite[10], addiction[11], mental illness[12], inflammation[13], respiration[14], bone mass[15], reproduction[16], nociception[17], energy homeostasis[18], and even influencing the very death and survival of cells[19].

Other endogenous *N*-acylethanolamines (NAEs) and fatty acid esters of glycerol that do not bind at the CB receptors were found to mimic or enhance endocannabinoid activity likely by inhibiting their hydrolysis via enzyme substrate competition[20, 21]. This is termed an entourage effect. Additional orphan receptors are candidates for CB_3 and perhaps CB_4 designations. AEA and entourage ligands also affect non-CB targets, forming an increasingly complex web of interactions.

A great number of synthetic agonists, antagonists, inverse agonists, enzyme inhibitors and transport inhibitors have been developed as tools to elucidate the function and interactions of ECS components. Their pharmacological use has potential for treatment of conditions such as depression[22], neurodegenerative disorders[23], cardiovascular disease[24], obesity[25, 26], ADHD[26], pain[27], brain trauma[28] and cancer[29, 30].

Cannabinoid Receptors

CB_1 receptors are the most abundant receptor-type in the mammalian brain. Its activation accounts for the psychoactive effects of cannabinoids. A wealth of knowledge exists in the literature on the pharmacology and molecular biology of this receptor. All discussions of the ECS are with respect to CB_1.

CB_1 expression is most abundant and heterogeneously distributed in the central nervous system (CNS) with particularly high levels of expression in cerebellum, hippocampus and basal ganglia[31]. Placement is predominantly presynaptic and mainly localized to axons and nerve terminals[32]. CB_1 can also be found in the peripheral nervous system, immune cells[33] and a great many vertebrate organ systems[30, 34].

The CB_2 receptor expression is less widespread than that of CB_1. It is most common in immune tissues with particularly high levels in B cells and natural killer cells[33]. CB_2 has also been found in other tissues such as bone[35], the gastrointestinal tract[36] and reproductive organs[37]. For several years after initial characterization, CB_2 was considered absent in the CNS and referred to as the 'peripheral' cannabinoid receptor. More recently, studies have found CB_2 expressed at low levels in the brain, found in microglia (immune cells of the

brain)[38], and on a subset of neurons[39]. CB_2 expression increases under pathological conditions and following injury[40-42].

CB_1 and CB_2 are GTP-binding protein-coupled receptors that share 44% protein identity and 68% homology in their seven transmembrane domains[4]. Most endogenous, plant derived and synthetic analog ligands share affinity for both receptors (see appendix A), though their effects vary among species[43]. Cannabinoid receptors are associated primarily with the pertussis toxin sensitive $G_{i/o}$ family of G-proteins. Stimulation of cannabinoid receptors inhibits adenylyl cyclase, activates mitogen-activated protein kinase (MAPK), activates A-type potassium channels and inhibits N and P-type calcium channels[44]. CB_1 also modulates Q-type calcium and inwardly rectifying potassium channels[45]. In addition to $G_{i/o}$, CB_1 couples to G_s to stimulate adenylyl cyclase production in some tissues[44]. In the CNS, CB_1 mediates retrograde signaling. Released from postsynaptic neurons, the signaling molecule travels backward over the synaptic cleft to activate presynaptic CB_1 receptors and transiently or persistently suppress neurotransmitter release[46].

Both CB receptors display reasonably high levels of constitutive activity. A portion of their population remains active in the absence of a bound ligand. The most common CB antagonistic agents: CB_1 selective SR141716A[47]′ and CB_2 selective SR144528[48] are actually inverse agonists. An inverse agonist reduces constitutive activity by preferentially binding to receptors in an inactive state, reducing signaling to below basal levels.

Cannabinoid Ligands

Cannabinoids can be divided into three general types: endogenous cannabinoids,

phytocannabinoids and synthetic cannabinoids. THC, and the non-psychoactive constituents, cannabinol (CBN), cannabidiol (CBD) and abnormal cannabidiol (Abn-CBD) are but a few of more than 60 *C. sativa* derived bioactive phytocannabinoids.

Many synthetic cannabinoids have been created to mimic the specific pathological and potential therapeutic properties of phytocannabinoids including THC analogs such as HU-210 and CP55940, and the aminalkylindole compound *R*-(+)- Win55212 (a stereospecific isomer hereafter referred to as WIN55212,2). $CB_{1/2}$ selective agonists are employed in targeted studies (see appendix A for a more extensive list). Once the existence of a cannabinoid receptor was confirmed, the presence of endogenous ligands was implied.

The NAE *N*-arachidonoylethanolamide, (anandamide, NAE 20:4, AEA), and the glycerolacylester 2-arachidonoylgylcerol (2-AG) are the most studied and best characterized endogenous cannabinoid ligands. Anandamide has been widely investigated since its description in 1992[3]. AEA is a promiscuous ligand. It is a full agonist at transient receptor potential cation channel vanilloid type 1 (TRPV1); it interacts directly with many molecular targets as discussed below. In 1995, 2-AG was isolated from canine gut[5] and rat brain[6] lipid extracts. Unlike AEA, it is present at relatively high levels in the central nervous system, roughly 100 times greater than anandamide (by most accounts).

2-AG is considered by some to be the natural ligand at the CB_1 receptor. The relatively new study of lipid rafts supports this hypothesis. Lipid rafts modulate the activity of GPCRs associated with these cholesterol-rich membrane microdomains and provide an organized platform for signaling complexes[49]. CB_1 receptors have been shown to localize within lipid rafts, CB_2 receptors do not. 2-AG is concentrated in CB_1 rich lipid rafts in dorsal ganglion, while

AEA is equally abundant elsewhere in the plasma membrane[50]. Lipid raft disruption fails to alter AEA metabolism while causing a marked increase in 2-AG synthesis as well as binding activity of CB_1 receptors[51].

Homo-γ-linolenoylethanolamide (HEA), docosatetraenoylethanolamide (DEA)[52], *N*-arachidonoyldopamine (NADA), 2-arachidonoylglycerylether (noladin ether, 2-AG ether)[53] and O-arachidonoylethanolamine (virodhamine)[54] also bind to CB receptors and are now listed among endogenous cannabinoids.

The transmembrane transport mechanism for AEA and 2-AG has been a matter of contention for quite some time, and has yet to be decisively resolved. It has been proposed that these molecules share a transport mechanism, are distinct, or that each has multiple means of transport[55]. AEA and 2-AG could theoretically move through the lipid bilayar by simple diffusion, or facilitated diffusion by the membrane bound FAAH[56]. The membrane-localized protein carrier hypothesis[57] is somewhat more popular and has been recently supported by the identification of fatty acid binding proteins (FABPs) as putative AEA transporters[58]. Little is known about the transport of entourage compounds, but it is believed to be distinguishable from the AEA mechanism[59, 60]. Effective AEA transport inhibitors have been developed[61].

Differences in cell type, cell location, or measured metabolic endpoint can lead to very different results from study to study for a given ligand. This is at least in part due to GPCR functional selectivity. A single GPCR has the ability to activate multiple pools of G-proteins. Different ligands are selective for different G-protein subunits, leading to activation of different pathways[62]. WIN55212,2 activates at least five different $G_{i/o}$ subunits[63].

Synthesis and Degradation

2-AG and anandamide are both arachidonic acid containing lipids derived from glycerophospholipids, however their biosynthetic pathways are very different. Anandamide is synthesized by *N*-acylation of phosphatidylethanolamine (PE) catalyzed by Ca^{2+}-dependent *N*-acyltransferase (NAT) followed by hydrolysis of the resultant NAPE[64]. Other pathways have been recognized[65]. 2-AG synthesis occurs by many proposed pathways varying by type of cell and stimuli. The accepted primary pathway involves sequential hydrolysis of arachidonic acid-containing inositol phospholipids by phopholipase C and diacylglycerol lipase (DAGL). Phospholipase C (PLC) may also synthesize 2-AG directly by interacting with phospholipase A_1[66]. Elevated AEA concentrations inhibit at least one 2-AG synthesis stimulating pathway[67]. 2-AG and AEA are formed locally from membrane phospholipids in response to elevations of intracellular calcium, released immediately from the cell, and after rapid selective reuptake are hydrolyzed intracellularly[68, 69].

AEA is hydrolyzed to arachidonic acid and ethanolamine once inside the cell, primarily by the intracellular membrane bound protein fatty acid amide hydrolase (FAAH)[70]. FAAH is preferential but not specific to AEA hydrolysis; it degrades other NAEs as well as 2-AG. Though widely distributed in mammalian tissues FAAH can be found in particularly high levels in CB_1 receptor rich regions[71]. NAE levels are increased sevenfold in tissues of FAAH knock out (KO) mice. FAAH KO mice also demonstrate acute sensitivity to anandamide[72]. Numerous available potent FAAH inhibitors are commercially available (see appendix A).

A second FAAH enzyme has been characterized only in primates. FAAH-2 is found in different locations inside the cell[73], yet appears to hydrolyze the same suite of compounds,

though less potently[74] than traditional FAAH. FAAH and FAAH2 are the only mammalian members of the otherwise common amidase signature family of proteins[75].

2-AG is hydrolyzed to arachidonic acid and glycerol primarily by the enzyme monoacylglycerol lipase (MAGL). Roughly 50% of 2-AG degradation in the murine brain is due to MAGL[76], the remaining by FAAH and at least two putative MAGL-like enzymes[77]. The location of 2-AG metabolizing enzymes indicates it, not AEA, is likely the natural retrograde messenger. MAGL expression is elevated in presynaptic axon terminals expressing CB_1[78], while DAGL is found on adjacent postsynaptic neurons[79]. Most research has been dedicated to the study AEA and consequently of FAAH, resulting in a paucity of potent or selective MAGL inhibitors[80].

N-acylethanolamine-hydrolyzing acid amidase (NAAA) is distinct from FAAH and found in lysosomes. Unlike FAAH, NAAA is maximally active at acidic pH and preferentially hydrolyzes Palmitoylethanolamide (NAE 16:0, PEA)[81]. Endocannabinoids also undergo oxidative metabolism by a number of fatty acid oxygenases including cyclooxygenase -2 (COX-2)[82, 83], 12-15-lipooxygenases [84], and cytochrome P450s[85]. Many of the resulting metabolites are CB receptor active[86].

Entourage Compounds

Many endogenous compounds exert in vivo cannabimimetic effects though they have little or no affinity for $CB_{1/2}$ receptors. These are commonly referred to as 'entourage' compounds. Entourage compounds potentiate AEA and 2-AG either by competitive hydrolysis,

inhibition of catabolizing enzyme expression[21], or interacting with their shared non-CB molecular targets.

N-acylethanolamines were first recognized as bioactive lipids five decades ago when PEA was discovered to have anti-inflammatory properties[87]. Of the NAEs found in animal tissue, PEA, stearoylethanolamide (NAE 18:0, SEA) and oleoylethanolamide (NAE 18:1, OEA) are prevalent in most tissues, specifically the brain, and are the best studied. Linoleoylethanolamine (NAE 18:2, LEA) is present at high levels in the small intestine[88]. Like AEA, its congeners are formed from NAPE and their corresponding fatty acids and hydrolyzed by FAAH and FAAH-2. In addition PEA is preferentially hyrodrolyzed by NAAA[81]. Total levels of NAEs in animal tissues generally run in the nmol/g range with AEA comprising less than 5%[89].

2-AG is accompanied by several glycerol esters in brain, spleen and gut. These glycerol esters do not bind to $CB_{1/2}$ receptors, but do potentiate the actions of 2-AG[20].

Other Receptor Interactions

In the years following the characterization of CB_1 and CB_2 receptors evidence began to emerge that they alone did not mediate all the effects of cannabinoids. Anandamide-elicited hypotension and mesenteric vasodilation could not be mimicked by several potent synthetic $CB_{1/2}$ agonists[90]. AEA but not THC continued to elicit stereotyped behavioral responses in CB_1-/-, CB_2-/- mice[91]. AEA and Win55212,2 stimulated [^{35}S]GTPγS binding in the absence of CB receptors[92]. As research continued additional molecular targets were identified and the inherent complexity of the ECS began to emerge (see Table A.3 for summary).

TRP channels are six-domain trans-membrane channels mostly expressed in nociceptive sensory neurons which gate the passage of several cations including Ca^{2+} following physical or chemical noxious stimuli. At least five distinct TRP channels are gated directly or indirectly by AEA and its congeners[93].

Vanilloid receptor type1 (TRPV1) is a non-selective ion channel best known for its response to capsaicin, a compound common to hot chili peppers. AEA, OEA and LEA are full agonists at TRPV1[94, 95]. Their effects can be potentiated by SEA[96] and PEA[97], which are not TRPV1 agonists[98]. AEA binds to TRPV1 on the same intracellular binding site as capsaicin between second and third transmembrane domains[99]. CB_1 and TRPV1 receptors are often found localized, along with FAAH in the same or neighboring cells[100]. CB_1 receptor activity is required to maintain TRPV1 in a sensitized state and permit the nociceptive response to capsaicin[101].

AEA antagonizes TRPM8[102], a channel activated by cooling compounds such as menthol, spearmint and the synthetic agonist icilin. AEA can stimulate TRPA1 mediated enhancement of intracellular Ca^{2+}[103]. TRPA1 can be activated by the FAAH inhibitor URB597[104]. TRPV2[105] and TRPV4[106] are also implicated as targets for some cannabinoids.

The vasodilator effects of AEA are replicated by abnormal-cannabidiol (abn-cbd), independently of CB_1, CB_2 and TRPV1 yet sensitive to SR141716A blockade[107]. This still mysterious 'receptor' (referred to as the endothelial cannabinoid receptor, abn-cbd receptor or CB_x,) is a pertussis toxin- sensitive GPCR[108], unaffected by $_L$-name or COX-2 inhibitors[109].

The orphan receptor GPR55 was first cloned in 1999 and found to be widely expressed in human brain[110]. More recently it has become a much debated candidate for a new cannabinoid receptor. The dispute arises from conflicting experimental results that appear to vary by cell type and pathway. The first published evidence using a GTPγS binding assay[111] identified OEA, AEA, PEA and 2-AG as agonists with PEA and 2-AG having particularly high efficacy. Subsequent studies brought conflicting results when monitoring specific GPR55-mediated Ca^{2+} signaling activity[112, 113] and extracellular signal-regulated kinase (ERK) phosphorylation[114]. GPR55 is activated by abn-cbd but has been eliminated as a CB_x candidate[115].

GPR119 was first identified by bioinformatic approach and subsequently assigned as a close relative of the cannabinoid receptors[116]. Expressed chiefly in the pancreas and gastrointestinal tract, GPR119 activation is surmised to promote insulin secretion and play a role in obesity and diabetes[117, 118]. OEA is a potent agonist as determined by a yeast based assay, followed by PEA and SEA. 2-AG does not activate GPR119 while AEA and LEA illicit only a modest response[119].

Peroxisome proliferator-activated receptors (PPARs) belong to a family of nuclear receptors comprising three isoforms: α, δ, and γ. PPARs target genes regulating metabolism, energy homeostasis and cell differentiation[120]. OEA, AEA, LEA, PEA[121], and oxygenated 2-AG metabolites[122] activate PPARα[123, 124]. OEA binds with high affinity and initiates transcription of genes involving lipid metabolism[125]. AEA binds directly to PPARγ[126, 127]. 2-AG induces PPARγ activation[128]. PPARγ is a therapeutic target for cancer treatment. OEA binds to PPARδ[125], though its function has not been studied.

Cannabinoids can also modulate ion function directly, independent of a receptor mechanism. AEA inhibits K^+ channels and N, T, L, P type Ca^{2+} channels, 2-AG also inhibits L and P type Ca^{2+} channels[129]. AEA, 2-AG as well as PEA inhibit Na^+ channels[130]. AEA can directly interact with a variety of neurotransmitter gated ion channels including NMDA[131], serotonin 5-HT_3[132] and 7α-nicotinic acetylcholine[133] receptors.

ECS Functions in Human and Murine Physiology

The ECS in cooperation with entourage compounds and related receptor targets directs several diverse and significant biological functions, including appetite and energy homeostasis, synaptic plasticity, injury recognition and inflammatory response as well as management of cell-fate related processes such as proliferation, differentiation and death, all of which manifest in observable behavioral responses. Below I discuss some of these roles as observed in their best studied models; humans and murines.

Cannabimimetic Behavior

The metrics antinociception, hypomotility, hypothermia and catalepsy, collectively referred to as the 'tetrad test' have long been the defining characteristics of cannabinoid activity. Phytocannabinoids[134] and synthetic analogs[135] produce these effects as well as anandamide[136] (albeit less potently than THC).

Early experimentation found that THC and cannabidiol induce catalepsy in mice, rats and gerbils[137]. The 'ring test' was developed in 1971 for the quantitative measurement of catalepsy expressed as 'immobility index'[138]. Immobility index is simply the percentage of

time the subject remains motionless after placed on a suspended wire ring. Spontaneous motor activity is measured by number of lines crossed within a grid placed in an open field. Cannabinoid induced hypomotility is associated with modulation of neurotransmitters including serotonin, norepinephrine[134], and glutamate[139]. CB_1 antagonists reverse cannabinoid induced hypomotility and actually double locomotor speed[135]. Specific stereotyped turning behaviors can be induced by cannabinoid microinjections into associated brain regions[140]. Tetrad test outcomes for SEA are similar excepting prolonged catalepsy and delayed hypothermic effect[59]. PEA induces neither catalepsy nor hypothermia[141]. The specific roles of 2-AG in behavior are little understood.

FAAH KO mice have wild type 2-AG levels[142] and are supersensitive to anandamide in tetrad tests with more pronounced effects that can be attenuated by SR141716A[72]. Movement disorders and hypothermia are not associated with FAAH disruption alone. FAAH inhibitors are analgesic but produce neither catalepsy nor hypomotility[143, 144]. Mice exposed to selective MAGL inhibition (without increase in anandamide) exhibit analgesia and hypomotility[142]. Combined inhibition results in hypomotility, catalepsy and an extremely potent analgesic effect[143].

Anxiety is a behavioral manifestation of stress, a response to an internal or external threat which triggers multiple physiological responses to maintain homeostasis. There are several standardized tests for assessing anxiety levels in rodents, including the light/dark box, elevated plus maze and social interaction test. In each test the anxious behavior is associated with avoidance of an unknown location or situation. Unfortunately, anxiety-related effects of various agonists are species dependent and pose a problem in animal model testing. For

instance WIN55212,2 reduces anxiety in rats yet produces the opposite effect in mice[145]. To complicate things further, low doses of cannabinoids can actually stimulate motor activity, exploration and reduce anxiety while higher doses generally produce the opposite effects[146]. THC is anxiolytic at a 0.3mg/kg dose and precipitates anxious behavior at 5.0 mg/kg[147].

While cannabinoid agonists may have a biphasic effect on anxiety behaviors, AEA uptake inhibitors and FAAH disruption are more reliable antidepressants and anxiolytics. AM404 is anxiolytic in the elevated plus maze test[148] and inhibits defensive burying behavior in response to a predator odor[149]. FAAH inhibition, genetic and pharmacological, reduces spontaneous[150, 151] and precipitated[152] anxious behavior in mice.

The cannabimimetic effects of AEA can still be observed in $CB_{1/2}$ deficient mice and are not blocked by SR141716A[91]. The hypomotility component can be completely reversed by a TRPV1 antagonist[139] while anxiety-like behaviors cannot[153].

Cannabinoid Withdrawal

Researchers have used many behavioral endpoints to measure cannabinoid withdrawal in murines, the most common and pronounced of which are 'wet dog shakes' and front paw tremor. Cannabinoids are generally unable to induce observable withdrawal spontaneously so most all studies have treated the subjects with SR141716A to precipitate symptoms.

Even a toxic dose of anandamide cannot induce spontaneous withdrawal symptoms in rats nor can it be precipitated by SR141716A. After discontinuation of a 4-day regimen of increasing doses (starting at 50 proceeding to 100 mg/kg/day) the subjects display an aversion to touch and a heightened startle response that observers attribute to rebound[154] (a return

of symptoms more severe than before when a medication is discontinued). Simple THC discontinuation also fails to produce spontaneous withdrawal symptoms but does result in reduction of dopaminergic neural transmission[155].

Some studies have reported that lower doses (≤ 10mg/kg) of SR141716A precipitate withdrawal from THC, elevating anxiety-like behavior[156], increasing adenylyl cyclase activity in vivo, and withdrawal-associated behaviors[157]; however other studies could only illicit this behavior at higher doses of SR141716A challenge[158]. Acute administration of either FAAH or MAGL inhibitor attenuates precipitated withdrawal symptoms[159]. As opposed to anandamide and THC, WIN 5512,2 is able to induce modest spontaneous withdrawal behaviors as well as other stereotyped behavior not associated with THC withdrawal[160].

Appetite

Absence[161] or blockade[162] of CB_1 receptors results in decreased appetite and body weight. The inverse agonist SR141716A has since been marketed as the obesity management drug Rimonabant (Rimonabant production was suspended after users experienced increased depression and anxiety as well as other cannabimimetic side effects[163]).

AEA levels and metabolism in the gastrointestinal tract are inversely correlated to SEA[164], PEA, LEA and most notably OEA[123]. AEA stimulates appetite[165] during periods of perceived hunger, once satiated AEA decreases and OEA levels increase[166]. OEA is 'anorexigenic'[167]. OEA stimulates fat utilization via PPARα[125] and likely stimulates insulin secretion via GPR119[119]. CB receptors mediate intestinal inflammation and motility[168]. Gastrointestinal TRPV1 activation induces visceral discomfort[169].

Analgesia

One physiological role of endocannabinoids is pain suppression, mediated at least in part by $CB_{1/2}$ mechanisms. AEA and 2-AG levels are elevated in brain areas associated with nociception[170]. Cannabinoids, uptake inhibitors, both FAAH and MAGL inhibitors all exhibit analgesic effects in acute, inflammatory and neuropathic pain models. CB_1 selective SR14176A and CB_2 selective SR144528 inverse agonists prolong and enhance pain behavior and reverse analgesic effects of cannabinoids[171]. Cannabinoid-induced analgesia involves some combination of non CB targets as well. AEA induced analgesia involves coactivation of TRPV1[172]. PEA itself has antinociceptive properties (in response to inflammatory stimuli), when combined with AEA the effect is 100-fold more potent than each compound alone[17, 121]. AEA also acts synergistically with PPARα agonists[173]. GPR55 knockout mice are insensitive to induced neuropathic or inflammatory pain[174]. 5-HT3[132] and TRPM8[175] may also be implicated in receptor-independent AEA-induced analgesia. Neuronal sensitization can be a consequence of the many aspects of inflammatory response[176].

Opioid Interactions

Even early research compared the ECS to the endogenous opioid system. Indeed these two systems are synergistic and somewhat redundant. Endogenous opioids act as retrograde messengers; released postsynaptically they inhibit excitiatory synaptic transmission on glutaminergic nerve terminals[177]. Cannabinoid and opioid systems use the same effector systems for NO release[178].

Both opioids and cannabinoids are powerful analgesics, but coadministration produces stronger and longer lasting effects[179]. CB_2 activation stimulates local β-endorphin release in rats. Peripheral CB_2 mediated antinociception can be prevented by the opioid antagonist naloxone or β-endorphin antiserum, indicating that the hyperalgesic properties of cannabinoids are in part opioid driven[180].

Both systems play a central role in all forms of addiction. Stimulation of CB or opioid receptors reinforces the hedonistic value of food, increasing appetite and consumption, conversely Rimonabant and naloxone are anorexigenic. Experiments measuring the motivational and reinforcing effects of THC and morphine on rat feeding behavior found that the actions of either agonist could be blocked by either SR141716A or naloxone[181]. Coadministration of an opioid competitive antagonist and THC significantly reduces abuse (frequency of self administration) in squirrel monkeys[182]. CB_1 knockout mice experience the acute effects of morphine but are largely exempt from addiction and severe withdrawal[183].

Inflammation

Inflammation is a complex process by which the body responds to infections, allergic or injurious agents. Exogenously applied cannabinoids including THC[184], AEA[185], PEA and SEA[186] generally have an immunosuppressive, anti-inflammatory and hypothermic effect. Pharmacological[187] or genetic elevation[188] of FAAH produces similar positive outcomes, not additive with PEA or THC. NAAA inhibition has like results[189]. Regulation of both FAAH and NAAA expression is likely of great importance to the control of inflammatory responses.

PEA has substantial anti-inflammatory and anti-oedema properties and is the active ingredient in medications marketed as Normast and Pelvilen[190]. Administration of PEA before and after induction of spinal cord injury (10mg/kg) reduces neutrophil infiltration, nitrotyrosine formation, cytokine expression, NO synthase expression, and apoptosis, culminating in an overall lower degree of inflammation and tissue injury[191]. Mast-cell degranulation (a release of antimicrobial and cytotoxic compounds associated with anaphylaxis) is inhibited by a PEA analog[192]. Though less investigated than PEA, SEA appears to share analogous anti-inflammatory properties[186]. SEA accelerates wound healing[193] and shows therapeutic promise for treatment of anaphylaxis[78, 194].

Both CB_1 and CB_2 receptor expression have been reported in immune cells, but CB_2 plays a greater role in the immune response. CB_2 receptors are activated and upregulated after infection or immune stimulation. CB receptors are undetectable in resting T-lymphocytes, but CB_2 expression becomes evident after superantigen activation[40]. CB_2 expression increases in tissues after a wounding event[41, 195]. Inflammatory responses are attenuated in mice overexpressing CB_2 while CB_2 KO mice exhibit excessive inflammatory reaction to injury or viral challenge combined with an impaired chemotactic response[184, 196].

ECS dysregulation is implicated in some pathological inflammatory conditions. AEA, PEA and CB_1 expression are elevated in patients with active celiac disease as opposed to non celiac patients[187]. Intestinal PEA concentrations are enhanced in patients with ulcerative colitis[13]. High levels of AEA are associated with multiple sclerosis[197].

Concentration of NAEs and 2-AG in inflamed tissues of murines differ by the model used and may be linked to the time course or severity of the response. AEA, PEA or 2-AG were not

significantly changed one hour after formalin induced or two hours after turpentine induced inflammatory response[198]. PEA, SEA and OEA levels did increase in rat testes 9 hours after $CdCl_2$ injection with a dramatic increase in PEA[199]. AEA, PEA and 2-AG rise in rat intestine of rat after induced inflammation, peaking at 3 days post and return to basal levels after 7 days[187].

Several studies report cannabinoids inhibit chemotaxis (directed cell migration). Many synthetic agonists, but not AEA, are observed to block the migration of neutrophils to the affected area after inflammatory stimuli[200, 201]. Cannabinoids including 2-AG inhibit production[202] and function[40] of the chemokine CXCL12. In contrast cannabinoids appear to propagate neuroinflammation. AEA and PEA act synergistically to enhance microglial motility[203]; 2-AG also triggers cell migration[204]. These and other studies (reviewed by Stella[205]) implicate a non-CB GPCR in the regulation of microglial migration and cytokine release.

An early work by Derocq et al. may somewhat illuminate these contrary observations. They cataloged genes upregulated by CB_2 activation including 3 chemokines, 1 cytokine and showed that CB_2 exposure to CP55940 leads to activation of a MAPK cascade followed by receptor desensitization[206]. The anti-inflammatory properties of CB agonists may well depend on the constitutive activity and expression level of the receptor.

Other receptor interactions contribute to the anti-inflammatory properties of cannabinoids. 2-AG suppression of T-cell growth factor interleukin (IL)-2 is PPARγ receptor mediated[128]. PEA activates PPARα; inhibiting transcription of inflammatory response

genes[207], and attenuates inflammation in wild type mice but not PPARα null mice[208]. The anti-inflammatory properties of SEA are counteracted by a TRPV1 antagonist[186].

Excitotoxicity

A subset of CB_1 receptors are present on glutamatergic nerve terminals, their activation results in inhibition of glutamate release in response to depolarization. Overstimulation of glutamate receptors results in excessive intracellular calcium leading to neuronal cell death, termed excitotoxicity. Excessive neuronal stimulation also leads to overproduction of nitric oxide synthase (iNOS). NO, when present in excess can react with superoxide anions to produce the highly reactive and cytotoxic oxidant peroxynitrite[209]. Cell death activates and attracts neighboring microglia, which generate more NO and superoxides that potentiate excitotoxicity[210]. CB_1 receptor activation blocks excitotoxic Ca^{2+} spiking[211]. Win 55212,2[212], CP55940[213] and THC[214] are protective against excitotoxic insults. Reports of AEA actions against glutamate toxicity are mixed[215]. It should be remembered that AEA acts directly at NMDA receptors (a glutamate receptor important for memory aquisition) and could therefore contribute to overstimulation. CB_1 deficient mice tolerate inflammatory or excitotoxic insults poorly[211].

Excitotoxicity is at least in part responsible for neuronal injury in response to hypoxia, ischemia or trauma. Neurodegenerative disorders such as Parkinson's Alzheimer's and Huntington's are associated with free intracellular calcium and oxidative stress. Cannabinoids along with COX-2 inhibitors, NO synthase inhibitors and PPARγ agonists have therapeutic promise in the treatment of these conditions[210].

Cannabinoid Induced Cell Death

AEA treatment has been associated with increased NO production[216]. Acute NO exposure potently increases ceramide synthesis[217]. Ceramides are *N*-acyl fatty acid sphingolipid derivatives. Ceramides mediate an assortment of cellular responses related to growth control or death including the initiation of growth arrest, and apoptic or autophagic cell death[218]. OEA is a potent ceramidase inhibitor[219, 220]. CB_1 activation triggers ceramide generation[221]. Cannabinoids such as THC, CBD, WIN55212,2 and AEA are associated with ceramide accumulation[222, 223]. Ethanolamine produced as a consequence of FAAH hydrolysis is antiapoptotic, via reduction of another class of 'executioner proteins', capsases[224].

Ischemia

An ischemic event describes the disruption of normal blood supply to the target organ. Intracellular ion and metabolite homeostasis is altered by the loss of oxygen and nutrients. The return of blood supply, or reperfusion, initiates a rapid inflammatory response and a respiratory burst of reactive oxygen and nitrogen species that inflict tissue damage.

NAE, particularly PEA, accumulation upon reperfusion of ischemic event are well documented[225-227]. This fact led many to speculate NAEs exert a cytoprotective function. More recently this accumulation is suggested to be a consequence of cell injury or even the cause[19]. Nevertheless several studies demonstrate NAE[228] or cannabinoid[229] application before, during and after ischemic event inhibit apoptosis, decrease inflammation, and improve overall outcomes. Blockade of CB_1 receptors by SR141716A significantly reduces infarct

volumes[227, 230]. NAEs are elevated and cytoprotective under more restricted types of cell stress such as hypoxia[231, 232], nutrient depravation[233] or oxidative stress[234].

Cancer

2-AG and NAE levels are often found elevated in tumor cells[236, 237], yet cannabinoids generally exert antitumoral[235] actions in a number of cancer cell lines by inhibiting tumor growth and metastasis while promoting cell death. CB_1 expression varies by tumor type examined and may be both upregulated[238, 239] or suppressed[240, 241]. However the CB_2 receptor is continually found expressed at higher levels in cancerous cells even correlated with severity of malignancy[42].

In some tumor cell types, cannabinoids inhibit migration[242, 243] and chemokinesis[244]. AEA, 2-AG, THC and CB_2 agonists inhibit tumor promoting growth factors; nerve growth factor in breast cancer cells[245], vascular endothelial growth factor in glioma cell lines[244] and glioblastoma *in vitro* [246], as well as epidermal growth factor receptor expression in prostate cancer cells (EGFR)[247]. AEA and THC decrease tumor growth by inducing cell cycle arrest often followed by apoptotic cell death[235, 241, 248].

AEA, THC and a number of synthetic cannabinoids induce apoptosis in cancer cells[240, 244]. There are many theories regarding how this occurs: mitochondrial-dependent cell death pathways[249, 250], enhanced transcription of several NF-kB regulated apoptotic genes[251], or ceramide accumulation[222]. NAEs may also induce apoptosis via their oxygenated metabolites[252, 253]. Salazar et al demonstrated that THC via CB_1 stimulated ceramide synthesis induced autophagy, upstream from apoptosis, in most of the tested tumor cell

lines[254]. Autophagy-resistant tumors were resisitant to THC growth inhibition[254]. The antiproliferative and proapoptotic actions of AEA may be attributable to lipid rafts. AEA stabilizes lipid rafts. Lipid raft disruption increases expression and activates epithelial growth factor receptors (EGFR)[255], inhibits accumulation of intracellular ceramide[223, 256] and blocks AEA induced cell cycle arrest and apoptosis[257, 258].

Experimental outcomes for cannabinoids in cancer cell lines have not been entirely beneficial. 2-AG actually induces mobilization in CB_2 expressing myeloid leukemia cells paired with significantly elevated CB_2 expression. This effect may blocked by CP55940, THC and WIN55212,2[259]. 2-AG enhances cholangiocarcinoma cell growth, presumably by lipid raft destabilization[223]. THC, at μM concentrations, has been effective in decreasing proliferation in CB_2 expressing human breast cancer tumor samples[241], in contrast THC stimulates growth of human breast carcinoma cells where $CB_{1/2}$ expression is undetectable[260] . Hart et al found that nM concentrations of THC accelerated proliferation in glioblastoma and lung cancer cells[261].

The antitumoral actions of cannabinoids are not entirely credited to $CB_{1/2}$ receptors. AEA induces apoptosis via aberrantly expressed TRPV1 receptors in cervical cancer[262]. AEA and 2-AG inhibition of c6 glioma proliferation may be blocked by simultaneous but not individual use of CB_1, CB_2 and vanilloid antagonists[263]. Cannabidiol induces apoptosis independently of CB_1 CB_2 or Trp channels[264]. PPARγ is also expressed in many kinds of cancer cells. Much like $CB_{1/2}$, PPARγ activation inhibits tumor growth by induction of apoptosis[38, 265] and autophagy[266], yet its inhibition decreases cancer cell motility[267].

Data concerning the effects of entourage compounds on cancerous cells is limited but positive. SEA limits Lewis carcinoma tumor growth and decreases the degree of metastasis[268]. PEA enhances the anti-proliferative effect of AEA in human breast cancer[21]. Tissues expressing high levels of FAAH are not susceptible to AEA induced cell death[269]. FAAH inhibition increases AEA levels and hinders tumor growth[270].

Stem Cells

The hippocampal region of the brain is able to generate new neurons (neurogenesis) throughout the lifespan of mammals[271]. CB_1 and CB_2 are expressed in both embryonic and adult neural progenitor cells. CB_2 expression diminishes as cells differentiate[272] . AEA and 2-AG are produced by neural progenitors[273] and promote their proliferation and neurosphere generation[273-275]. Cannabinoids inhibit deadly glioma tumor formation by inducing apoptosis of affected cells, inhibiting vascularization and by promoting differentiation of glial stem cells[276].

Neural proliferation can by inhibited by stressful stimuli[277]. The anxioloytic and antidepressant effects of CB agonists may be a consequence of ECS regulation of neurogenesis[149]. Neural progenitor cell proliferation and differentiation is down-regulated in conditions associated with brain-inflammation[278]. Endocannabinoids and their congeners generated upon brain injury may result in a return to a basal state.

CB_2 receptors are expressed at low levels in osteoblast progenitors (bone forming cells) and osteoclasts (bone resorbing cells). Cannabinoid stimulation of CB_2 induces osteoblast

proliferation and inhibits osteoclastogenesis[15], reflected in the low bone mass phenotype observed in CB_2 knockout mice[35].

In summary, the biological responses of CB receptors vary by metabolic condition, location and cell type. The action of their primary ligands, AEA and 2-AG, can be shaped by related lipids via common catabolic enzymes. In addition to CB receptors, endocannabinoids, their metabolites and entourage compounds share a suite of molecular targets. This complexity has been described as an 'endocannabinoid soup'[82]. Early researchers assumed use of supposed selective agonists/antagonists conferred mechanistic specificity to observed outcomes. This is not always the case, and untangling the specific roles of any given receptor, ligand, or enzyme has proven to be considerably more difficult than previously assumed.

Given the pathological implications of ECS dysregulation and the broad therapeutic potential of cannabinoid-based drugs, development of a simplistic animal model for the study of these complex interactions would seem extremely beneficial. There is however a dearth of information regarding the ECS and its functions in non-mammalian organisms.

Natural History of the ECS

Endocannabinoids, entourage ligands and their catabolizing enzymes are phylogenetically ancient. While endocannabinoid ligands or enzymes are found in every form of life for which they have been examined, cannabinoid receptors are not. CB receptors are highly conserved across vertebrate species. Human, rat and mouse CB_1 receptors share 97-99% amino acid sequence identity. All vertebrates appear to share the $CB_{1/2}$ doublet[279] though CB_2 homology is more divergent among species (a fact that complicates specific ligand

affinity)[280]. The limited number of non-mammalian vertebrate studies show that cannabinoids serve much the same function as their murine cousins. Cannabinoids effect memory[281] and cognition[282] in birds, motor activity[282, 283] in birds and newt as well as modulate lipid metabolism, growth[284], and appetite[285] in fish.

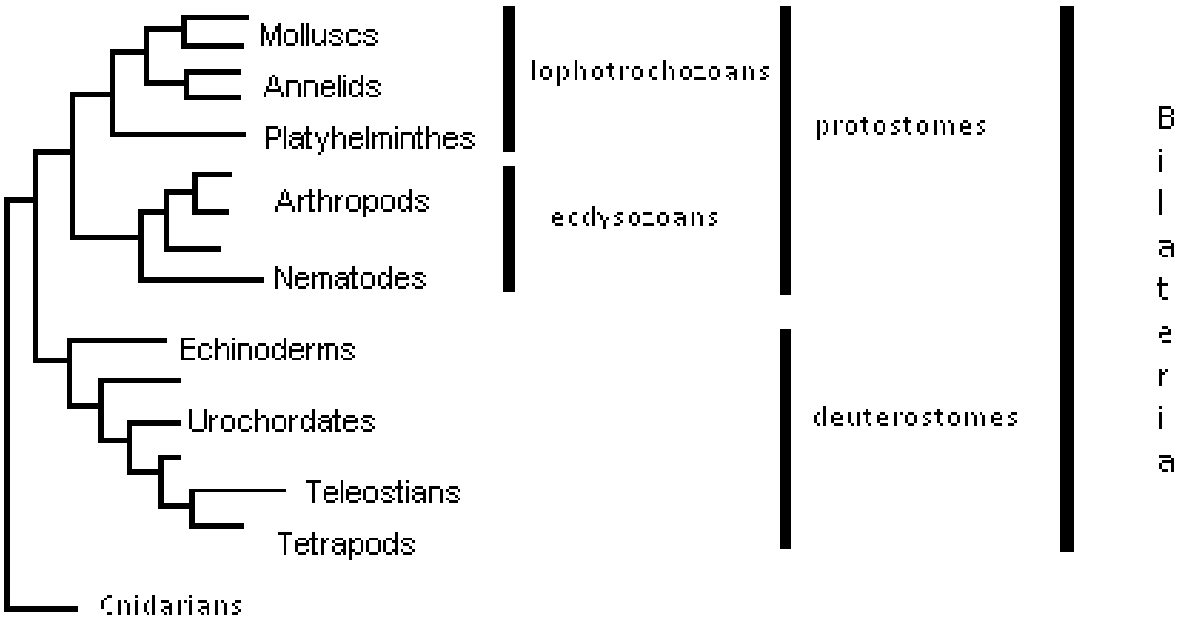

Figure 1 Phylogenetic organization of the various taxa mentioned in this paper

The first cannabinoid-type receptor identified in an invertebrate was found in the genome of a urochordate, *Ciona intestinalis* or sea squirt (CiCNR). CiCNR represents an ancestral-type receptor predating the gene duplication event that gave rise to the vertebrate paralogs CB_1 and CB_2[286].

Recognizable CB receptor homologs are absent below phylum chordata but a primordial cannabinoid receptor may have evolved prior to the divergence of the protostomes and deuterostomes. Radioligand binding assays (shown in Appendix B) demonstrate weak

cannabinoid binding to an as yet uncharacterized receptor across the bilateria, with secondary loss in insects. A partial sequence of cDNA from *H. medicinalis* amplified by RT-PCR was found to encode a 153 amino-acid sequence that displays a 61% identity to the human CB_1 receptor[287]. The putative leech CBR was later investigated by Elphick, using bioinformatic techniques, who suggests it was in fact a PCR artifact arising from DNA contamination[288].

A few works on the ECS in invertebrates have attracted the interest of the broader academic community. In a study which predates the finding of CB_2 receptors in human microglia[38, 204], Stefano et al. found specific AEA binding in microglia from *M. edulis*[289]. This group also observed morphological changes that decrease motility of glial immunocytes after treatment with AEA. The sea urchin model first showed that cannabinoids inhibit the fertilizing capacity of sperm by preventing induction of the acrosome reaction upon encountering a specific ligand in egg jelly[290]. Sea urchin embryos are now being studied to discern ECS involvement in early development[291].

DePetrocellis et al. reported the presence and function of cannabinoid receptors in the evolutionarily ancient *Hydra vulgaris*. AEA inhibited glutathione induced feeding response and was antagonized by SR141716A. Specific binding sites for [H^{3+}] SR141716A were detected in membrane preparations as well as FAAH-like enzymatic activity[292]. Due to the unconventional nature of the binding assay these findings were later questioned by colleagues[288]. In a subsequent study, no [^{3}H]CP55940 specific binding was found for the cnidarian *A*. *albocincta*[293].

Cannabinoids induce long term depression (LTD) in snail[294], leech[295] and crayfish[296] at synaptic contacts associated with experience-dependant reactive behavior,

similar to LTD in mammals[297]. Exogenous AEA (10^{-6} M) results in elevated NO production when applied to *H. medicinalis* (leech) and *M. edulis* (mussel*)*[178] ganglia, associated with cannabinoid binding sites[287]. The FAAH inhibitor methyl arachidonyl flourophonate (MAFP) proved effective in amplifying and prolonging this effect. These findings have been interpreted as evidence that AEA and 2-AG also act as neuromessengers in the invertebrate nervous system.

Cannabinoid activity appears to be absent in insects. THC produces no observable behavioral responses in either ant or honeybee[293]. 2-AG and NAEs are present in some insects but most insects examined exhibit no incidence of cannabinoid binding (see Appendix B). A specific binding site for [^{3}H]CP55940 was found in *Drosophila* which could not be displaced by SR141716A or SR144528 or WIN55212,2 indicating it is structurally quite different[288]. [^{3}H]CP55940 specific binding in locust was later dismissed by the author since it was not a membrane-associated protein[288].

The hemp plant is largely free of nematode root infestations and hemp extracts have been used as botanical nematicides and animal vermifuges[298], yet for some time all mentions in the literature consistently stated that *C. elegans* did not have cannabinoid-like receptors. These conclusions were based entirely on the use of BLAST algorithms. Cannabinoid ligand binding has since been demonstrated in the nematode *P. redivivus*[293]*,* followed by identification of a low-identity putative ortholog in the *C. elegans* genome[299].

Echoes of an endocannabinoid system are present even outside the metazoa. The protozoan *Tetrahymena pyriformis* is capable of hydrolyzing AEA, OEA and PEA *in vivo*. This FAAH-like activity is associated with a 66kDA protein[300]. These researchers also recognized

the presence of a MAGL-like enzyme in *T. pyriformis*[301]. NAEs are present in yeast Saccharomyces cerevisiae[302] and hydrolyze PEA by way of a non-FAAH like enzyme[303]. Heterologous expression (in yeast) of proteins involved in mammalian neurodegenerative diseases altered PEA production and inactivation[303].

AEA and 2-AG are conspicuously absent in plants. NAEs found in plants are predominantly short chain (12-18C); LEA (NAE 18:2) and PEA (16:0) are the most abundant. NAEs levels are far higher in seeds (μg/g range) relative to adult vegetative tissue (ng/g), first declining upon imbibition[304].

NAE metabolism in plants is analogous to that of animals. NAEs in plants are formed by NAPE hydrolysis, hydrolyzed by a FAAH-like amidohydrolase[305] and oxidized by lipoxygenases[306]. A functional FAAH homolog (At5g64440, atFAAH) was first identified in *Arabidopsis thaliana*[305]. Additional homologs were subsequently found for *Oryza sativa* and *Medicago trunculata*[307]. Although doubted as a true homolog[308], AtFAAH hydrolyzes NAEs in vitro including AEA. AtFAAH deletion in *A. thaliana* results in hypersensitivity to exogenously applied NAEs[309]. AtFAAH overexpressors are particularly susceptible to bacterial pathogens[310] and exhibit accelerated growth[309].

NAEs are suggested to participate in the plant defense response. NAPE synthesis[311, 312] and total NAE levels[313] increase in *Nicotiana tabacum* leaves treated with xylanase (a pathogen response elicitor). Addition of exogenous NAE 14:0 serves to both activate PAL gene transcription (long term defense response) and inhibit alkalinization (short term response). Both effects were reversed by AM281 and SR144528[313, 314]. Tritiated ligand binding studies in *N. tabacum* leaves and microsomes detected specific binding of [^{3}H]NAE 14:0 fully eliminated

by SR144528 and AM281 (1.0μ). [^{3}H]NAE 14:0 binding activity was also measured in microsomes from *A. thaliana* and *M. trunculata*. [^{3}H]NAE 24:0 (AEA) had no binding affinity in plant membranes[314].

Interestingly many novel lipids, including an anandamide analog, with affinity for human CB_1 have been found in the marine sponge *Mycale micracanthoxea*, brown algae *Laminaria angustata* and the cyanobacterium *Lyngbya majuscule*[308].

Bioinformatics

Bioinformatics represents a modern method to solve problems in molecular biology including gene discovery, drug discovery, and molecular phylogenomics. The use of computer algorithms is necessary to adequately process the massive and growing quantity of biosequence (DNA, RNA, protein) data from thousands of organisms available in several large databases. The National Center for Biotechnology Information (NCBI), a division of the National Institutes of Health, provides a centralized resource for biosequence data and a standardized model for data storage and retrieval.

Bioinformatic tools are essential to the contemporary study of phylogenetics. Evolutionary relationships may be inferred by examining genetic information, the greater the similarity the more recent their common ancestor. Identification of homologous protein sequences is the first step in phylogenomic analysis. Two proteins with related folds and related sequences are termed homologous. Orthologs are homologous proteins in different species that arose from a common ancestry; they are a result of speciation and tend to have similar functions. Paralogs are created by successive gene duplication events and perform different but

related functions in the same species. Discovery of orthologous proteins uncovers potential evolutionary relationships among organisms; their identification in a model organism expedites experimental design and interpretation.

Sequence alignment is a technique that describes a likely relationship between two biosequences by highlighting their similarities. A software program, such as BLAST (Basic Alignment Search Tool), inserts gaps and designates portions of the sequences that correspond to one another. BLOSUM or PAM scoring matrices look for excusable substitutions and mismatches. The percentage of shared amino acids and conservative substitutions for the pair is expressed as % identity. The aligned sequences are also assigned an E-value which represents the probability that the alignment occurred by chance. Experimentally determined amino acid positions serving critical roles in protein function and specificity can be used along with sequence alignment tools to predict functional orthlogs[315].

BLAST searches are in no way a substitute for *in vivo* experiments, but they can provide needed information in the absence of real biological evidence. Identification of putative orthologs by *in silico* methods often facilitates their isolation and exploitation *in vivo*. Cannabinoid receptors in *Takifugu rubripes*[279] and *Ciona intestinalis*[286] as well as atFAAH in *Arabidopsis thaliana*[305] were first identified using homology screening.

The Planarian Model

Planarian is a common name referring to non-parasitic flatworms, members of the family Planariidae, class Turbellaria, phylum Platyhelminthes. The remarkable regenerative abilities of planaria have piqued the curiosity of biologists for over a century. This ability is

associated with a large population of stem cells referred to as neoblasts. The most common varieties found in teaching laboratories and historical literature are the “brown” *Dugesia tigrina* and “black” *Dugesia dorotocephala*. Recently, however, the species *Schmidtea mediterranea* (also family Dugesia) is emerging as the model organism for modern molecular biological research due to its diploid chromosomes and existence in both asexual and sexual strains[316].

Planaria exhibit bilateral symmetry, dorso-ventral polarity, cephalization and three germ layers. They are acoelmate with no specialized circulatory or respiratory organs. The digestive system has only one opening, a pharynx in the mid section of the body which acts as both mouth and anus. The area referred to as the parenchyma is mesenchyme filling the space between the skin and gut. The excretory system consists of many flame cells connected by ciliated ducts[316]. Reticular cells remove cellular debris by phagocytosis[317]. Longitudinal, diagonal and circular muscle fibers permit twisting, turning and folding of the body[318]. Ventral ciliated cells and mucous secretions aid in locomotion and adhesion. Most are hermaphrodites, reproducing by cross-fertilization or asexually by fission[319].

Absence of a coelom complicates the phylogenetic placement of the platyhelminthes. Once considered in entirety a primitive group at the base of bilateria, some platyhelminthes retain that status[320]. Conflicting theories place the triclads as members of lophotrochozoa[321] (earthworms and mollusks) or as basal protostomes prior to the divergence of echydyzoa and lophotrochozoa[322].

Planaria are said to have the first “brain”. Neurons in planaria are more similar to those of vertebrates than advanced invertebrates; they possess a single axon with extensive dendritic branching and a spontaneous electrical discharge rate far out of the range of the average

invertebrate (ten-fold slower)[323]. The anterior brain is bilobar, extending to a pair of longitudinal nerve cords that fuse at the tail and are interconnected by a series of laterally placed commissural fibers[324]. The photoreceptors are primitive eyes located in the head section and project axons directly into the brain. Special sensory input from the photoreceptors, chemoreceptors (sensory neurons projecting from the side of the head), and rheoreceptors (pressure receptors) integrate to provide motor responses[319]. The planarian "brain" is a matter of contention; some authors simply refer to it as the "cephalic ganglion".

Buttarelli reviewed the presence of several neurotransmitters found in planaria, noting their analogous functions and interactions to mammals including serotonin, catecholamines, nicotinic-acetylcholine, GABA, benzodiazepines, excitatory amino acids, opioids and cannabinoids[325]. Planaria likely represent the most basic model for studying the interactions of these various receptor/ ligand groups in the central nervous system. They share many genes with higher animals. 95% of nervous system related genes found in *Dugesia japonica* are held in common with *H. sapiens*, including at least one GTP-binding protein G_i alpha subunit[326] .

Neoblasts are totipotent stem cells representing 25-30% of all planarian cells. They are the only cell type capable of dividing; their progeny produce all other planarian tissue types including themselves[327] . A fragment as small as $1/279^{th}$ can regenerate into a complete animal[328]. In the intact animals neoblasts are scattered throughout the parenchyma, replacing cells lost due to damage or as part of physiological turnover during growth or degrowth[329, 330]. When the planarian is injured wound healing occurs rapidly. Within 30 minutes the dorsal and ventral epidermises contract completely, the subsequent relaxation covers the wound[331]. Neoblasts will quickly migrate to the area forming a blastema and

initiate differentiation to give rise to the regenerated tissue[332]. Mitotic activity peaks initially at 4-8 hours[333]. The amputated fragments maintain anterior-posterior, dorsal-ventral, and medial-lateral polar identity[334]. Under stressful conditions many species may reduce in size and shed sexual organs only to quickly return to their original state when food is plentiful and the environment is friendly. Degrowth appears to be an autophagic process[335], occurring by a symmetric decrease in overall cell number rather than cell size[332].

Planaria are known to respond to a variety of environmental contaminants. Traditional endpoints include mortality, rate of fission, motility, righting rate and tumorigenesis[336]. For some time *D. dorotocephala* have been known to develop tumors and aberrant growths in response to some human carcinogens[337] . These growths were sometimes observed to be spontaneous but usually manifested as abnormal regeneration after wounding. Recently cancer-associated genes and signaling pathways have been found in planaria[338]. Abnormal and or accelerated neoblast proliferation can be induced by manipulation of these cancer-associated signaling pathways[336]. Planaria may offer a simple model to study chemically induced tumorigenesis and provide a model for studying links between regeneration and cancer.

Planaria demonstrate negative phototaxis which stimulates their rate of movement. Most behavioral studies measure motility by the number of crosses over gridlines placed beneath a Petri dish. Their published velocity ranges from 26 – 38 cm/min[339-341] in a well lit room and 9 cm/min in semi-dark conditions[342]. Their velocity is relatively independent of size[343]. Loss of their eyes removes their ability to alter movement in response to light[340].

Planaria exhibit specific motor patterns in response to drugs acting on neural

transmission, either in altered rates of activity or stereotyped behaviors. Short term exposure to methamphetamine increases locomotor activity[344] as duration increases, locomotor speed decreases substantively in comparison to controls[341]. Low doses of cocaine induce motionlessness; higher doses increase motility and may be inhibited by a dopamine receptor 2 selective blocking agent[345]. Exposure to a k-opioid agonist increases motor activity and stereotyped behaviors[346]. Reduced motility is the norm for drug withdrawal[347, 348]. Multiple drug withdrawal has a superadditive effect on motility[349].

Planaria also exhibit behavioral and cellular responses to cannabinoids. THC and cannabidiol slow regeneration in *D. tigrina* and are toxic at concentrations of 16×10^{-7} M[350]. Low concentrations of capsaicin stimulate cell division in *D. tigrina*[351]. *Dugesia gonocephala* exhibited a significant increase in activity and exploratory behavior when exposed to 32µM WIN55212,2 in ethanol. Snake-like and screw like stereotyped behaviors were apparent at 160µM with increasingly random movement patterns[342]. Cooexposure to antagonist SR141716A reversed these effects. When denied WIN55212,2 after 10µM exposure for 1 hour, locomotor speed decreased[347]. The inactive enantiomer WIN 55212,3 provoked no behavioral changes. Coexposure to the nitrous-oxide synthase inhibitor $_{L}$-NAME[339], agmatine[352], or an NMDA[353] antagonist attenuated withdrawal. Kappa-opioid antagonists counteract the acute effects of WIN55212,2 (stereotyped behaviors, increased motility)[342] yet are unable to alleviate withdrawal[354]. *D. dorotocephala* exhibit no behavioral response to capsaicin, but do respond to icilin, a TRPM8/TRPA1 agonist[355].

The dominant invertebrate models, *Drosophila* and *C. elegans*, bias all invertebrate studies in favor of echysozoans. These models are undoubtedly important and have contributed

massively to the understanding of life itself, yet are not without their flaws. For example estrogen receptors were previously thought to be absent in invertebrates, but were later found to be secondarily lost in echysozoans and urochordates[356]. The stripped down genome of *C. elegans* is an asset in many respects yet may limit its usefulness in many lines of research. Some portions of the Dipteran genome have experienced an extremely accelerated rate of evolutionary change. Some receptor types found in other echysozoans are more similar to human than those found in their nearer cousins *Drosophila* [357]. This exclusive club is sorely in need of diversity, particularly the addition of a lophotrochozoan representative. Currently, planaria are most attractive for their potential in the study of stem cell biology. Planaria have many added traits worthy of development as a model organism.

The simple yet vertebrate like nature of the planarian CNS, ability to develop tumor-like growths, and demonstrated cannabinoid sensitivity suggest that planaria may be a relevant model to study the primitive ECS.

The purpose of this research is to explore the endocannabinoid system in planaria. Basal levels of 2-AG, AEA and selected NAEs are measured in healthy intact *Dugesia dorotocephala*. Changes in these compounds are compared under conditions of cellular stress and over the first twelve hours following a wounding event. Locomotor velocity is assessed in response to exogenous AEA and 2-AG as well as FAAH/MAGL inhibition. The *Schmidtea mediterranea* genome is searched, using the University of Utah *S. mediterranea* genome database, for orthologous receptors and metabolic enzymes implicated in human ECS regulation.

Hypothesis Statements

- H_0: Background tissue concentrations of 2-AG, AEA, PEA, OEA and SEA in *D. dorotocephala* will not lie outside the range of those reported for other animal organisms.
- H_0: The direction of change in endogenous levels of 2-AG, AEA, PEA, OEA and SEA in response to simulated ischemia in *D. dorotocephala* will not differ from those reported in murine models.
- H_0: 2-AG, AEA, PEA, OEA and SEA concentrations in *D. dorotocephela* will not differ from controls in response to a wounding event, or over the course of the subsequent regeneration period.
- H_0: Behavioral responses in *D. dorotocephela* to exogenous application of endocannabinoids or their pharmacological manipulation will not differ from controls.
- H_0: Homologous receptors and metabolic enzymes implicated in human ECS regulation will not be found in the *Schmidtea mediterranea* genome.

CHAPTER 2

MATERIALS AND METHODS

General Information

Black planarians (*Dugesia dorotocephala*) were purchased from Carolina Biological Supply (Burlington, NC). The animals were kept in a neutral dilute salt solution (1.6mM NaCl, 1.0mM $CaCl_2$, 1.0mM $MgSO_4$, 0.1mM $MgCl_2$, 0.1mM KCl, 1.2mM $NaHCO_3$ in MQ water adjusted to pH7) and when necessary fed fresh commercially available beef liver.

Animals used for the bulk of GC/MS analyses were delivered January 5′ fed January 11, treated, frozen and stored January 15. Those used for set A, (silica cleanup method), arrived October 7, were fed weekly (more than four days before analysis), frozen and stored October 23. Animals used for AEA 1 hour withdrawn/continued exposures and successive 2-AG were delivered January 5, fed weekly, (a minimum four days prior to filming) and filmed over the period of February 4 to February 7. Animals for five minute exposure series were delivered February 25 acclimated at least 24 hours before use, and fed March 5 and 9.

NAE 12:0 and NAE 14:0 were provided by the Kent Chapman laboratory. All other deuterated and reference standards, o-arachidonoyl glycidol and oleoyl ethyl amide were purchased from Cayman Chemical (Ann Arbor, Michigan). Dimethylsulfoxide (DMSO) was used as a carrier for AEA, 2-AG and the enzyme inhibitors. DMSO is analgesic in vertebrates[358] associated with decreased nerve fiber conductance[359]. Pagan found that DMSO reduced motility in *Dugesia dorotocephala* in a concentration and time dependent manner and those concentrations below 0.1% did not display toxic or behavioral effects[360].

All data were analyzed by 2-tailed t-test. An Ftest for variance was conducted prior to each t-test and adjusted accordingly. Skewness and kurtosis were used as measures for normality. Significance was defined as $P < 0.05$.

Sample Procedures for GC/MS Analysis

All animals were allowed to acclimate in their kill vessel without direct handling for at least one hour (excepting the 0hr sectioned samples) before sacrifice in liquid nitrogen. Samples were stored at -80°C until extraction. Wet weights were not taken; prior experience has shown that any molestation may lead to altered NAE and 2-AG concentrations. Four 16 animal replicates were set aside to determine average wet/dry weight.

Stressed Sample Set

In order to simulate an ischemic episode, animals were subjected to hypoxic and overcrowded conditions by containment in small sealed tubes for a prolonged period (0.033 animals per µL for 5 hours). A pilot study showed that after one hour under these conditions oxygen levels were < 0.1ppm and that 2.5 hours stressed was insufficient to case changes in the analytes (PEA, SEA, OEA, AEA, 2-AG).

Sectioned Sample Set

Sixteen animals were placed in a small Petri dish in minimal volume. Each animal was sectioned transversely into at least 3 pieces (head, midsection and tail) using a sanitized scalpel. Petri dishes were flash frozen in liquid nitrogen at 0(immediately) 3, 6 and 12 hours.

Enzyme Inhibitors

Concentrated solutions of enzyme inhibitors were added sequentially to their respective kill vessels to achieve exposure concentrations of 5ppm o-arachidonoyl glycidol (oAG, dual MAGL FAAH inhibitor) and 2 ppb oleoyl ethyl amide (OEtA, FAAH inhibitor) in 0.06% DMSO. Samples were frozen after ~ 5 minute exposure. Inhibitor exposure concentrations were chosen based on IC_{50}s stated on Cayman Chemical Inserts (OEtA IC_{50} = 5.25nM, OAG IC_{50} = 4.5µM, 12 µM for MAGL and FAAH inhibition respectively). Exposure period was chosen based on the limited information concerning invertebrate inhibitor exposure, where FAAH inhibitors were maximally active in *H. medicinalis* and *M. edulis* at less than 4 minutes[287].

Extraction and Cleanup

Lipids were extracted using Folch method with 4:2:1 ratio cold $CHCl_3$/CH_3OH/PBS. Deuterated internal standards, [^{4}H]arachidonoylethanolamide and [^{4}H]palmitoylethanolamide, were added to the chloroform solvent prior to tissue homogenization. Two different homogenization and cleanup procedures were employed. It became necessary to use fewer than 50 animals per trial, sample sets were reduced to 16 animals in a final volume of only 3µL. The initial cleanup techniques proved ineffective because GC/MS performance was compromised by excessive lipid absorption in the injection port. Use of the freezing-lipid filtration technique improved chromatography performance[361].

For the stressed sample set, samples containing 50 animals/30µl final volume were homogenized using glass tissue grinder followed by cold sonication. After homogenization and centrifugation the organic phase was removed then dried under N_2. Silica SPE columns (1 g bed

wt 6mL volume Supelco Bellefonte, Pa. Discovery DSC) were conditioned with chloroform, methanol, chloroform rinses. Samples were eluted with 50/50 ethyl acetate/acetone after chloroform rinse as described by Muccioli and Stella[362].

For the sectioned sample set, samples containing 16 animals/3uL final volume were homogenized in bead filled glass vials with a beadbeater (glass vials). Freezing lipid filtration samples were reconstituted in acetonitrile, transferred to 1mL centrifuge tubes and placed in -80°C freezer for a minimum of two hours to allow for complete precipitation and centrifuged for 5 minutes at -8°C. The supernatant was removed and freeze filtration repeated.

It should be noted that soaps and detergents contain significant quantities of shorter chained NAEs and their use should be avoided. All glassware was cleaned using only brushes and water followed by multiple chloroform rinses to minimize this source of contamination.

GC/MS Procedures

Delipidized extracts were evaporated to near-dryness under N_2 then derivatized using silylating agent BSTFA for 30 minutes at 60°C and reconstituted with 10% BSTFA in hexane for injection to GC/MS (Agilent 6890 gas chromatagraph with 5973 mass selective detector, Palo Alto Ca., operated in selected ion mode 70eV). The injector temperature was 260°C with a pulse pressure of 25psi, carrier gas helium. The column (Alltech EC^{tm}-5capillary column, Grace Scientific, Deerfield, IL; 30m x0.25mm i.d., 0.25µm nominal film thickness) was programmed with a three ramp temperature gradient (50°C /min from 40-220°C, 5°C/min to285°C, 10°C /min to 300°C) at constant pressure of 7.0psi and an initial flow of 1.0mL/min. Standard curves were

prepared by analyte and internal standard preparations ranging from 10-1000 pg/µL. Retention times and confirming ions are detailed in Table 1.

Procedures for Behavior Analysis

Planarian locomotor activity was evaluated using the automated behavioral analysis software DanioTrack by Loligosystems (Denmark). Special Ibidi (Verona Wi.) slides (200µL trough volume) were inverted on top of a white light emitting source in an otherwise dimly lit room. The slide trough was filled with culture water and appropriate treatment solution in 0.06% DMSO. Treatments, pretreatments and cotreatments were administered in such a way as to keep DMSO vehicle concentration constant. Animals acclimated to dark conditions were placed in the prepared slide tough. Time lapse videos were acquired with a webcam using Anderson's AZcendant software Handy-Avi software version 4.3 set to one frame every two seconds for at least five minutes. Recorded data was evaluated using DanioTrack.

Raffa et al developed a metric called pLMV as a quantitative assessment of planarian locomotor activity[343]. pLMV is the number of gridlines crossed in a given period of time, when converted to a rough estimate of average velocity, planarian velocity under their test conditions (well lit room) ranges from 26-38 cm/min[339-341]. Planarian behavior was measured as the total distance travelled within a given time (5-15 minutes). In these experiments, dark-acclimated planarians were placed above a bright light emitting source. Under these conditions planarian velocity for controls ranged from 39 - 58 cm/min. The increased speed is expected; planarians display marked negative phototaxis which fades in intensity the longer they are exposed to light. The use of a bright light emitting source was

intended to be stimulatory. In this method, one may observe a change in locomotor speed as well as an approximation of anxiety-like behavior.

Variability in measureable behavior increased with the length of time the animal was observed. Standard deviation of total distance moved for control data sets was narrowest when measured for 4 – 8 minutes and increasing thereafter (see Figure 2). A five minute filming period was established since effects on WIN55212,2 planarian motility occurred within the 5-minute window[342] and AEA on murine behavior are immediate[363]. Solvent controls were run on every day of analysis. Animals were compared using the metric 'total distance moved' (cm).

Cannabinoid and Enzyme Inhibitor Exposures

Animals were transferred from culture water to slides pretreated with 6ppb OEtA, 5ppm oAG, 0.5, 1.5, 3, 6, 15, or 30 ppm AEA or 0.05, 0.15, 0.3, 0.6, 1.5 or 3.0 ppm 2-AG. Animals were pretreated with appropriate inhibitor (6ppb OEA, 5ppm OAG) for 5 minutes in microcentrifuge tube. After 5 minute exposure the subject animal was transferred to an AEA or 2-AG treated slide not containing the inhibitor for filming (6ppb OEtA w/ 0.5, 1.5, 3 and 15 ppm AEA and 5ppm OAG w/ 0.05, 0.15, 0.3 and 1.5 ppm 2-AG). Cotreatment slides contained both the appropriate cannabinoid and inhibitor at a constant 0.06% DMSO vehicle concentration.

In order to assess AEA withdrawal behavior; animals were exposed to 3ppm AEA for 1 hour, half were then removed to slides in a continued 3ppm AEA exposure, the others to untreated culture water (withdrawn). Animals were observed for a 15 minute period. To better illustrate the depressive effect of 2-AG/oAG coexposure animals were filmed for a 5 minute

period in untreated culture water, the camera was then stopped, 0.3ppm 2-AG added, and then filmed for an additional 10 minutes. This process was repeated for a 5 minute pretreatment with oAG and 0.3ppm 2-AG for the additional 10 minutes.

Table 1 Quantification and confirming Ions (m/z) used for TMSi derivatives of internal standards and native analytes

Analyte		*Retention time (min)*	*Quantification ion*	*Confirming masses*
Internal Standard	*Native*			
	12:0	10.92	300,	225, 272
	14:0	13.18	328	253, 300
16:0 d4		15.67	360	
	16:0	15.71	356	328
	18:0	18.34	384	309, 356
	18:1	18.04	382	
	18:2	18.00	380	395
	18:3	18.12	378	303
20:4 d4		19.74	408	
	20:4	19.76	404	419
	2-AG	20.21	507	419

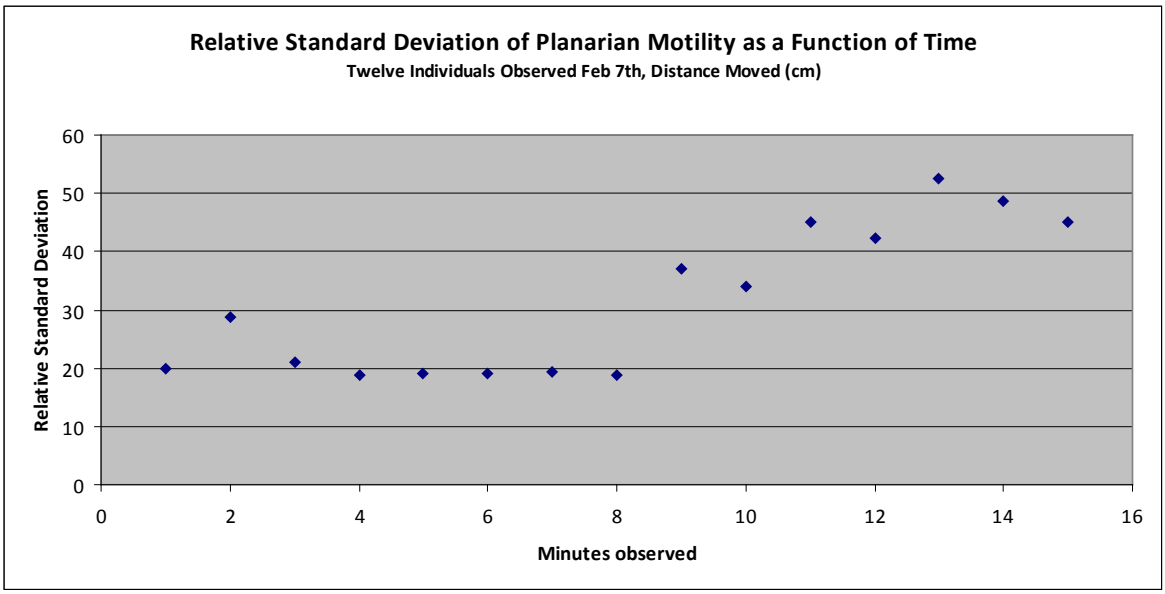

Figure 2 Relative standard deviation of planarian motility as a function of time

BLAST Search Methods

The protein sequences for human CB_1 isoform a (GenInfo identifier number GI:38683844), CB_2 (GI:4502929), TRPV1 (GI:74315354), TRPV2 (GI:20127551), TRPM8 (GI:109689695), TRPA1(GI:116534990), PPARα (GI:50348666), PPARγ isoform1 (GI:116284373), PPARγ isoform2 (GI:20336229), PPARδ isoform 1(GI:284807155), PPARδ isoform 2 (GI:29171750) PPARδ isoform 3 (GI:284807157) PPARδ isoform 4(GI:284807159) GPR55 (GI:21595842), GPR119 (GI:32165516) receptors along with sequences for human FAAH (GI:166795287), MAGL isoform1 (GI:60055786), MAGL isoform2 (GI:51242953), NAAA (GI:58219537), FAAH2 (GI:29477220), NAPE-PLD (GI:170932483), DAGLα (GI:27262632), DAGLβ (GI:218931251), ABDH4 (GI:50658087), PTPN22 (GI:224586929) enzymes were retrieved using the National Center for Biotechnology Information (NCBI) database and screened for pattern recognition to find putative orthologs in the genome of *Schmidtea mediterranea* using the University of Utah S. med Basic Alignment Search Tool (BLAST version 2.2.15) searching proteins (BLASTp) program with MAKER (automated gene prediction) proteins database and nucleotides (tBLASTn) with S.med genome assembly database (v31)[364]. Sequences with the mk4 designation are predicted proteins found within the S.med genome database and can be accessed at http://smedgd.neuro.utah.edu/cgi-bin/gbrowse/smed_mysql/. BLAST searches were performed for other proteins of interest including CB_1 homologs from *Fugu rubripes* (CB1a GI:1545940), and *Ciona intestinalis* (CiCNR GI:74096111), putative CB homologs for *C. elegans* (C02H7 GI:215397672) and *B. floridae* (BfCNR GI:229287403), TRP channels for *C. elegans* OSM-9 (GI:228484886), OCR-1 (GI:193207906), OCR-2 (GI:2911863), OCR-3 (GI:193210941), OCR-4 (GI:193206757), GON-2 (GI:212644978), TRPA-1 (GI:212645948) and

the *D. melanogaster* TRP channels nanchung (GI:45445898) and inactive (GI:24640231) as well as the *D. melanogaster* nuclear receptor E75 isoform a (GI:23093218)

Results with greater than 20% identity and E-values below $1e^{-5}$ were checked for critical residues (accepting conservative substitutions given by BLOSUM62 substitution scoring matrix) that impart ligand recognition. Critical receptor and enzyme motifs required for ligand binding were found in prior reviews[299, 308] and searches using the key words activity profile, molecular docking, critical residue and ligand binding domain. Results were also evaluated using reciprocal BLAST searches against the human genome and those of other model organisms. Results were considered 'confirmed' when the original query was listed among the top hits for the reciprocal BLAST. The percent of query over subject sequence length as well as 'domains of interest' were considered when evaluating results.

CHAPTER 3

RESULTS

Analytical Results

Total analytes, PEA, OEA, SEA, LEA, AEA and 2-AG were present at 89.14±31 nmoles/g dry tissue. The relative composition and concentration of each analyte is listed in Table 2. These data were obtained by silica SPE cartridge delipidization method and calculated based on an average per planarian dry weight of 0.210±0.03 mg. Applicable values (those given as a percentage of whole tissue weight) for NAE and 2-AG concentrations in various organisms were obtained from existing literature (for a more complete listing see Table B.2) and converted to uniform units (pmoles/g) for comparison to values from this study calculated based on an average wet weight per planarian of 3.59±0.15 mg and listed in Table 4. This listing illustrates the wide range and high variability of NAE and 2-AG concentrations found between taxonomic groups and within a given species.

Table 2 Quantification of NAEs and 2-AG in *D. dorotocephala* and relative composition of each analyte

Compound	nmoles/g ± STD Dry Weight Seven Replicates	Relative % Contribution
16:0 (PEA)	12.38 ± 4.5	13.90
18:0 (SEA)	4.061 ± .36	4.56
18:2 (LEA)	4.939 ± .23	5.54
18:1 (OEA)	3.571 ± 2.8	4.01
20:4 (AEA)	3.168 ± 1.2	3.55
2-AG	61.03 ± 25	68.46

Table 3 Quality control results associated with NAE and 2-AG quantification

Compound	% Recovery		%
	Matrix Spike	Blank Spike	Duplicate Deviation
16:0 (PEA)	69.20	61.13	2.78
18:0 (SEA)	66.87	50.44	0.03
18:2 (LEA)	59.63	58.13	22.82
18:1 (OEA)	61.79	60.74	8.15
20:4 (AEA)	72.84	63.92	21.26
2-AG	101.67	169.54	4.27

Absolute values of analyte concentrations for the sectioned sample set cannot be compared to those stated in Table 2, Table 4, or Figure 3 since these data were generated using the freezing lipid extraction method of delipidization. The freezing lipid extraction approach resulted in the same rank order of analytes but was biased toward saturated species, had overall reduced yields and increased variability.

Stressed Sample Sets

Animals in the 'stressed' sample set (see Figure 2) experienced a significant decrease in LEA (NAE 18:2) ($P = 0.011$) and PEA (NAE16:0) ($P = 0.0032$). PEA was decreased over 2.5 times in stressed samples. OEA ($P=0.06$) and AEA ($P = 0.10$) were not significantly lowered, possibly due to high variability in the control sample set. 2-AG levels were essentially unchanged ($P=0.50$). Data sets were compared by analyte using 2-tailed t-test.

Table 4 Comparative listing of NAE and 2-AG concentrations by whole tissue wet weight. Appropriate data compiled and converted to pmoles/g from values listed in Appendix B

Species/TissueType	16:0	18:0	18:1	18:2	20:4	2-AG
pmoles/g wet weight ±SEM or STD						
Vertebrates						
Human brain non-tumor, tumor patient[236]	142±22	63±6.0		5.5±1.0	4.3±0.8	
Mouse brain[226]	289±27.5	60.7±48	246.5±17.7		13.6±3.2	12000±1000
Mouse brain cortex[365]	8728 ±2655	2634± 183	3266± 691	349±54	576± 147	
Rat brain[366]					23±3	4000±1800
Rat brain[367]					2.45±.39	
Pig brain[89]	6844±1636	6929± 1312			172.7 ±28.8	
Sheep brain[89]	2504	2747			nd	
Cow brain[89]	3573	2778			115.1	
Pimephales promelas (fathead minnow) male brain[368]					22.28±0.76	15370±650
Pimephales promelas (fathead minnow) fem. brain[368]					28.88±1.10	19040±610
Invertebrates						
Paracentrodus lividus (sea urchin) ovaries[369]	95±30	10±5			12±4	
Mytilus galloprovincialis (mussel)[370]	70.2±10	32.4±15	8.60±1.5	8.66±1.8	5.18±0.58	
Crassostrea sp (oyster)[370]	54.8±5.3	38.5±4.3	7.99±0.61	nd	Trace	
Tapes decussates (clam) [370]	196±21	108±8.2	4.92±3.07	4.95±3.71	5.76±2.6	
Callista chione (clam)[370]	94.8±15	51.3±12	Trace	Trace	nd	
Venus verrucosa (clam)[370]	131±20	36.0±6.1	08.29±1.8	8.66±2.5	nd	
Aplysia (sea slug) ganglia[371]	Present		Present		10.5±2.7	5700±900
Hirudo medicinalis (leech)[372]	32.3±1.5			5.8	21.5±0.7	147.4±42.7
Theromyzon tessulatum (leech)[373]						112
Drosophila melanogaster (fruit fly) heads[374]	231				< 5.7	337
Drosophila melanogas ter [374]	572				< 3.3	212
Apis mellifera (honeybee)[374]	225				< 12.8	462
Hydra vulgaris polyps[292]	Present		Present		15.6±1.5	11200±1900
Plant Seeds						
Bauhinia congesta[375]	1248±23.7	22.89±2.75	414.7±28.0	204.6±21.9		
Caesalpinia gilliesii[375]	180.3±37.1	80.6±9.16	148.1±18.7	426.6±77.3		
Mimosa borealis[375]	747.9±156	162.7±14.4	402.5±81.4	1107±173		
Lupinus succulentus[375]	1292±77.1	638.0±33.0	752.7±81.7	1422±145		
Lupinis texensis[375]	1235±464	170.3±115	2264±710	2516±1168		
Arachis hypogaea[375]	12454±451	2094±208	24455±1161	14034±1549		
Medicago sativa cv 1701[375]	3839±411	1431±97.7	3164±209	6152±167		
Medicago trunculata cv. A17[375]	604.3±351	198.4±33.6	986.2±227.3	3032±315		
Medicago. trunculata cv. Jemalong[375]	42404±2280	6197±446	29370±2492	37570±2708		
Pisum sativum cv. Early Alaska[375]	2220±103.5	567.8±49.4	3256±160	3586±411		
Pisum sativum[375]	343.9±31.0	115.7±4.58	663.6±28.8	537.9±48.8		
Phaseoulus vulgaris cv. Amarillo del Norte[375]	178.6±37.1	57.69±22.7	74.96±9.52	89.03±17.6		
Vigna unguiculata cv. Tohono O'odham[375]	460.8±43.7	155.7±10.1	218.1±22.4	386.4±21.6		
Glycine max cv.Dare[375]	22437±1783	4945±451	15054±1155	39382±3075		
This Study						
Dugesia dorotocephala	725.5±265	238.1±170	289.6±110	209.4±162	185.7±70	3578±1490

Sectioned Animals

Analytes which demonstrated a significant change in concentration in any of the four observed time intervals (0,3,6 and 12 hours) after sectioning as compared to an unsectioned control or to each other are depicted in Figure 4. Data sets were compared by analyte using 2-tailed t-test. SEA exhibited a significant increase in the 6^{th} hour after sectioning (vs control P = 0.0004, vs 0 hour P = 0.026, vs 3 hour P = 0.010), continuing through the 12^{th} hour (vs control P = 0.003, vs 3 hour P = 0.0037). A decrease in PEA is significant in hour six (vs control P = 0.015), as well as an increase in AEA (vs control P= 0.048, vs 6 hour P = 0.037). No significant changes or trends were observed for OEA, LEA or 2-AG over the time-course of the experiment.

Enzyme Inhibitor Exposures

LEA (P = 0.0056) and 2-AG (P = 0.0057) were reduced in vehicle treated samples (0.06% DMSO, n = 5). The FAAH inhibitor OEtA effected no significant changes in the analytes (n = 7). OEA (P< 0.07) and SEA (P < 0.07) did trend lower. Treatment with the MAGL/FAAH inhibitor oAG did significantly increase LEA (~2x, P = 0.004, n = 5) yet effected no statistically significant change in 2-AG or any other analyte. Data sets were compared by analyte using 2-tailed t-test.

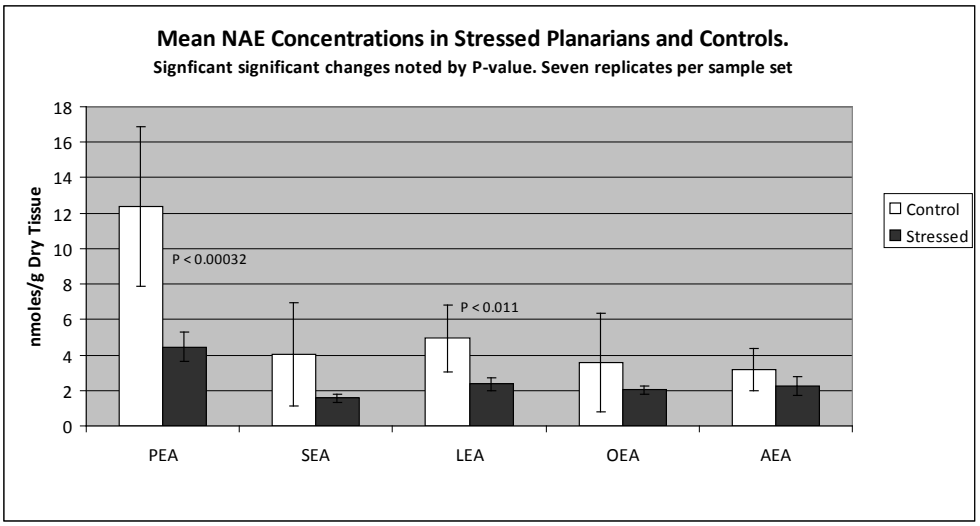

Figure 3 Mean NAE concentrations in stressed planaria and controls (nmole/g dry weight ±STD)

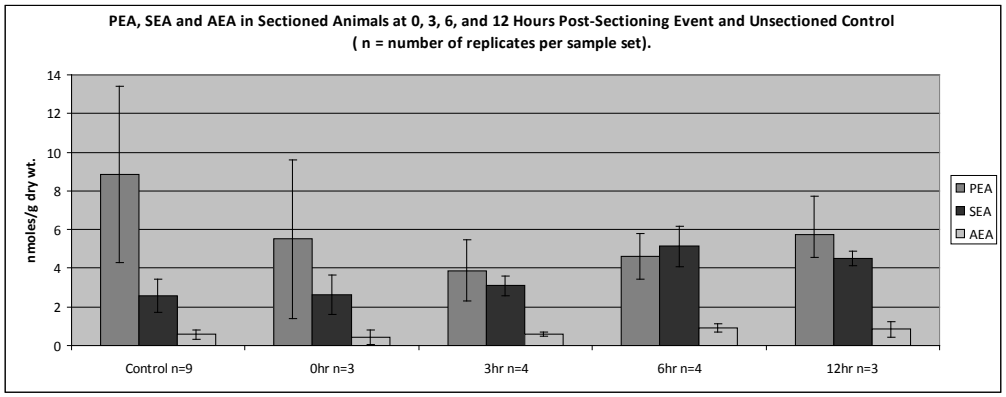

Figure 4 Notable changes of NAE content in the regenerating planarian (nmole/g dry weight ±STD)

Table 5 P-values for significant changes in analyte concentration for sectioned samples

Analyte	Con vs. 3hr	Con vs.6hr	Con vs.12hr	0hr vs.6hr	3hr vs.6hr	3hr vs.12hr
PEA	0.057	0.015				
SEA		0.0004	0.003	0.026	0.010	0.037
AEA		0.048			0.0374	

Con = unsectioned animals
0,3,6,12 hr designations refer to time intervals following sectioning

Behavioral Results

DMSO treatment was not statistically significant when compared with vehicle-free controls (15 min, P = 0.50, Feb 4). Control animals traveled at an average daily range of 39-58 cm/min over a five minute period, vehicle controls ranged from 40-52 cm/min. It was determined that daily planarian activity sometimes varied significantly for culture water controls (1 of 4 days see Figure 5) and frequently for DMSO controls (3 out of 5 days see Figure 6). This variability indicated that results need be compared to their day-specific solvent controls. The average distance moved value for each treatment is represented as a percentage of average daily controls in Tables 6 and 7.

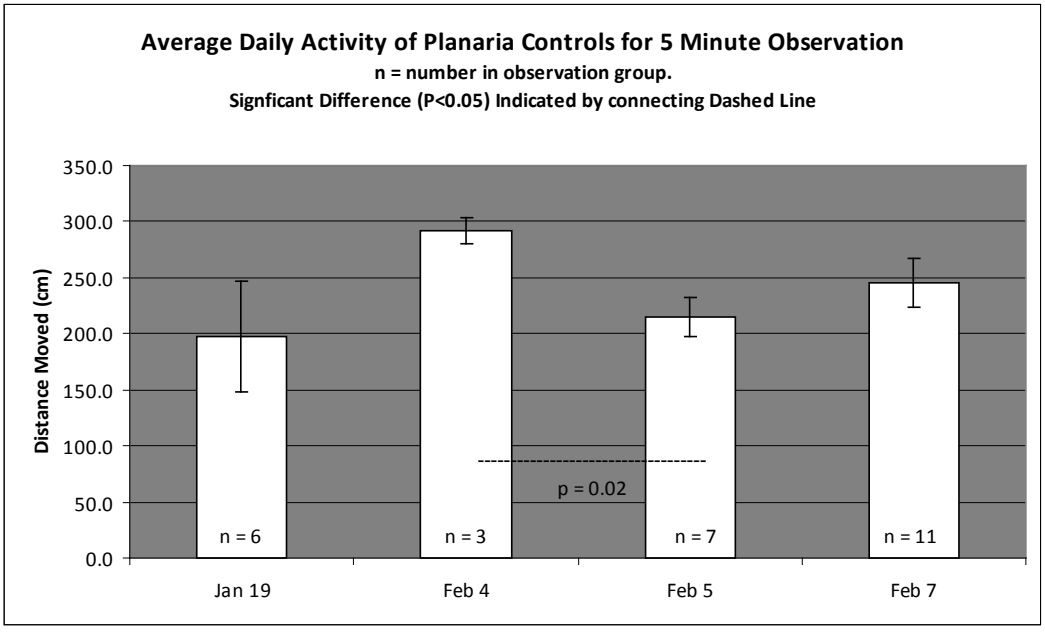

Figure 5 Average daily planarian activity for culture water controls (distance moved ± STD)

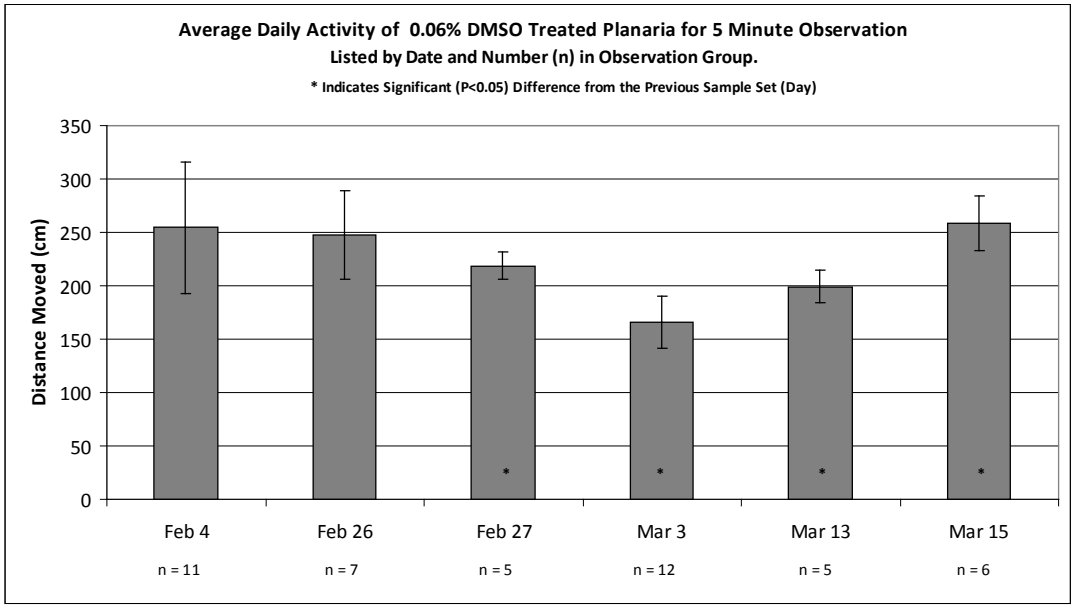

Figure 6 Average daily planarian activity for solvent controls (distance moved ± STD)

Table 6 Planarian motility for AEA and OEtA 5 minute exposures represented as percent of daily solvent controls

	Anandamide									*OEtA*		
Conc. ppm	*0.5*	*1.5*		*3*		*6*		*15*	*30*	*0.006*		
Date	*3/15*	*2/27*	*3/3*	*2/27*	*3/3*	*2/27*	*3/3*	*3/15*	*3/15*	*2/27*	*3/3*	*3/15*
% DM of solvent control	98.2	104	109	91.6	89.2	66.8§	67.1§	108	102	101	76.0‡	104
% rsd	8.6	40	12	28	28	20	31	15	15	24	34	10
N	*10*	*11*	*11*	*4*	*10*	*10*	*9*	*12*	*11*	*9*	*8*	*5*
Pretreated with 6ppb OEtA												
% DM of daily solvent control	95.0 (C)	111 (P)	95.3¤ (P)	109 (P)	85.7 (P)			97.1 (C)				
% rsd	10	22	16	21	39			4				
N	*10*	*9*	*8*	*14*	*12*			*5*				

§ P< 0.0005 compared to daily solvent control. ‡ P< 0.05 compared to daily solvent control. ¤ P< 0.05 compared to no treatment with OEtA. P = pretreated with inhibitor, C= simultaneous exposure and inhibitor pretreated DM = distance moved, N = number of treatment observations associated with average value, % rsd = relative standard deviation of the observation set.

Table 7 Planarian motility for 2-AG and oAG 5 minute exposures represented as percent of daily solvent controls

	2-AG						*oAG*
Conc. ppm	*.05*	*.15*	*0.3*	*0.6*	*1.5*	*3.0*	*5*
Date	*3/13*	*3/3*	*3/3*	*2/26*	*3/13*	*3/13*	*3/3*
% DM of solvent control 5 min	111	98.0	110	90.0	125§	120¤	97.1
% rsd	22	15	20	4.4	15	16	14
N	11	9	6	8	12	13	12
Pretreated 6ppm oAG							
Pretreated with 5ppm oAG							
Date	*3/13*		*3/3*		*3/13*		
% DM of daily solvent control	112		104		89.3†		
% rsd	19		27		19		
N	12		11		6		

§ p<0.002 compared to dmso control. ¤ P < 0.01 as compared to solvent control. † P< 0.002 as compared to no pretreatment with OAG. º P < 0.05 as compared with solvent control. DM = distance moved, N = number of treatment observations associated with average value, % rsd = relative standard deviation of the observation set.

Anandamide and OEtA Exposures

Animals continuously exposed or withdrawn from 3ppm AEA after one hour did not display significantly different activity rates when compared to one another (Figure 7) or their corresponding controls. Distance moved was compared to corresponding daily solvent controls by 2-tailed t-test to determine significance. Data are represented in Table 6 as average distance moved as a percentage of average daily solvent control. Treatment with 6ppm anandamide produced significant ($P < 0.0005$, see Table 6) inhibition of activity on two separate days of testing. 3ppm exposure trended slower than solvent control but was not significant. 15 minute exposures at 3ppm AEA were also conducted and were not significantly different from control. Treatments with 15 and 30ppm anandamide showed no change in activity rate. Treatment with

OEtA alone or in combination with AEA (pretreatment or cotreatment) produced inconsistent results. Locomotor activity was significantly slower for 6ppb OEtA treated animals for experiments conducted on March 3. This effect was not repeated for experiments conducted Febuary 27 or March 15. 1.5ppm AEA exposure pretreated with OEtA did display a statistically significant locomotor depression as compared to 1.5ppm AEA alone for the experiments conducted on March 3, not repeated in the Febuary 27 trial (see Table 6).

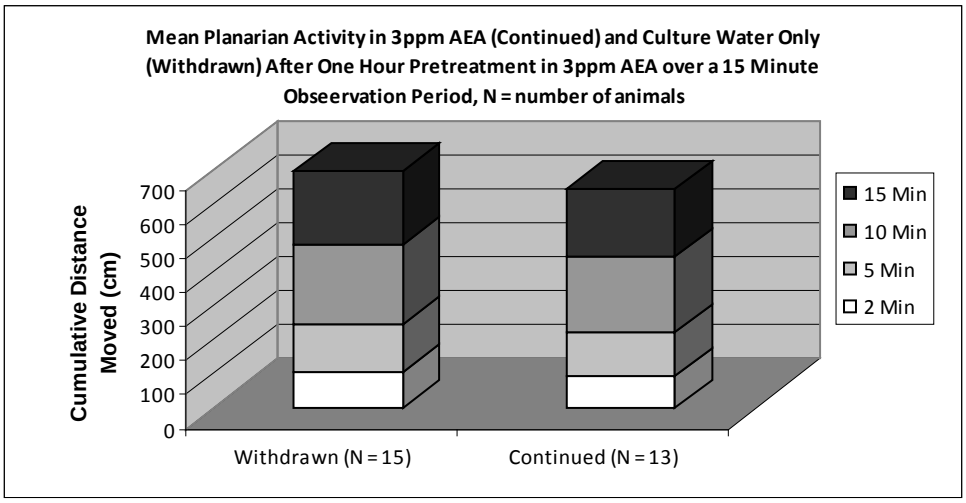

Figure 7 Planarian activity (cumulative distance moved) in 3ppm AEA and culture water only, after one hour pretreatment in 3ppm AEA (N = number of animals per sample set)

2-AG and oAG Exposures

The locomotor speed for animals exposed to concentrations < or equal to 0.6ppm 2-AG was not significantly different from control. Animals exposed to 1.5ppm ($P < 0.002$) and 3.0ppm ($P < 0.01$) 2-AG were significantly faster than control. Pretreatment with oAG before 1.5ppm exposure inhibited this effect ($P < 0.002$). Distance moved was compared to corresponding daily solvent controls by 2-tailed t-test to determine significance. Data are represented in Table 7 as average distance moved as a percentage of average daily solvent control.

No significant absolute or relative change was observed with only 0.3ppm 2-AG exposure for 5 minute (Table 7) or the extended observation period (Figure 8). Addition of 0.3ppm 2-AG to the 5ppm oAG pretreatment significantly reduced planarian locomotor velocity when expressed as a percentage of the individual's speed in the pretreatment period (first 5 minutes) (5-10 min P< 0.046, 10-15 min P< 0.025, 13-15 minutes P < 0.046 as compared to 0.3ppm 2-AG exposure without oAG pretreatment).

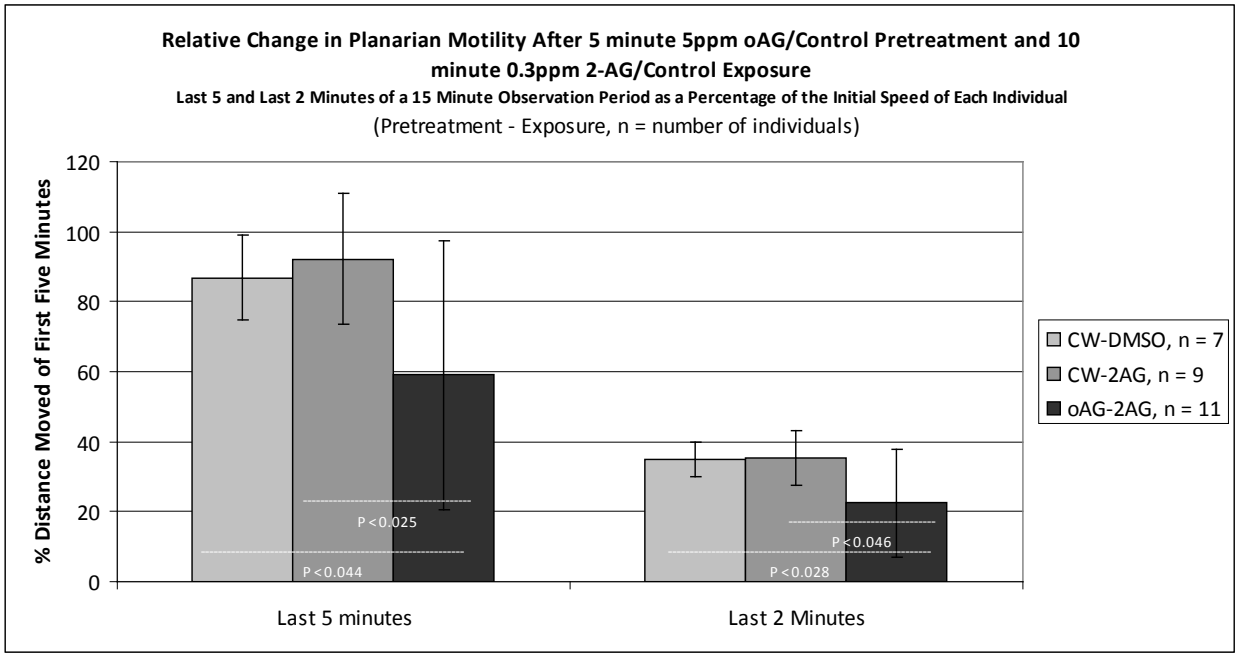

Figure 8 Relative change in planarian motility upon 2-AG exposure after pretreatment with oAG and controls

This conclusion is tenuous at best on its own since the sample set presented with a very high degree of variability, however it does support the observation (see Table 7) that over a 5 minute period 5ppm oAG pretreatment significantly reduced velocity of planarians exposed to 1.5ppm 2-AG. 5ppm oAG, when administered alone, did not significantly change planarian locomotor velocity for either a 5 minute (see Table 7) or a 15 minute observation period (pilot data without daily solvent control). Data collected for the extended observation period of 2-AG

and or oAG exposure were expressed as a percentage of the individuals speed in the pretreatment period. These percentrages were then compared using 2-tailed t-test.

Bioinformatics

Cannabinoid Receptors

38 MAKER proteins were returned for human CB_1 (isoform a) BLASTp search of the *S. med* database. CB_1 isoform b returned an identical list of results, and nearly identical scores. These results were repeated with varying quality for CB_2 and other vertebrate CBr searches (for representative results see Tables C.1 and C.2). Results were checked for conserved residues deemed critical for cannabinoid ligand binding (CB_1: F189, V196, F200, L286[376], K192, W279[377], Y275[378]) and residues specific to CB_2 receptor activity (CB_2:S112, T116, C175, F197). Reciprocal searches for all hits yielded a variety of GPCRs. MK4.00008535.00.01 had the greatest number of conserved residues (CB_1 6/7 CB_2 3/4). Reciprocal searches identify this protein as a possible galanin or opioid receptor. Mk4.007388.02.01 also contained a good number of conserved residues (CB_1 = 5/7 CB_2 = 2/4) but over only ~40% of query. The *S. med* database ontology notation designates mk4.007388.02.01 as an intracellular membrane protein. All other returned results conserved fewer critical residues.

When vertebrate BLAST results were compared to those of *C. intestinalis* CNR, mk4.003002.02.01, mk4.001569.04.01 and mk4.005562.01.01 returned the highest overall scores (see Table C.1). A reciprocal BLAST search did not list CB receptors among the top 50 hits for these proteins, but instead listed D1 dopamine, 5-HT and β-2 andrenergic receptors.

Other GPCRs

BLAST searches for GPR55 yielded five low identity sequences mk4.014127.00.01 (23%, 1e-14, 78%) and mk4.002418.01.01 (24%, 2e-13, 89%) yielded the highest overall scores and were also returned for CBr searches. GPR55 specific critical residues were not found for comparison. Reciprocal searches yielded a variety of GPCRs. BLAST searches for GPR119 yielded many of the same predicted proteins as those for CBr searches (see Table C.3). The top five hits conserved TM 7 (transmembrane 7 region) NPXXY (where X represents any amino acid) and TM 6 CWXP motifs common to fatty acid receptors[379], however the C→S substitution in CWXP motif unique to GPR119 was absent. Reciprocal searches yielded a variety of GPCRs, primarily 5-HT, dopamine and andrenergic.

Metabolizing Enzymes

BLAST searches for FAAH homologs returned 8 proteins fitting basic search criteria. Three paralogous protein results (see Table C.4) conserved the portion of amidase signature sequence unique to FAAH (GGSSGGEGALI[308]), the catalytic triad (S217- S241-K142[380]) and additional residues deemed critical for the enzymatic efficiency of FAAH (I238, G239, G240,S218, R243[308]) .These sequences also maintain many of the prolines (2/3) in a proline-rich motif necessary for enzymatic activity[381]. BLAST searches for FAAH2 returned identical results with lower overall scores and E values than that of FAAH. Reciprocal BLAST searches confirmed the three hypothetical proteins, mk4.001109.06.01, mk4.002051.04.01 and mk4.005541.02.01 as putative FAAH homologs (see Table C.5). Figure C.1 depicts a multiple alignment of mk4.001109.06.01 with FAAH sequences of six other species.

BLASTp searches for MAGL homologs returned 10 proteins meeting the search criteria (see Table C.6). Reciprocal searches confirmed all as potential MAGL homologs. Four of these predicted proteins met the functional criteria for membership in the lipase enzyme family (GXSXG motif and catalytic triad D239, H269 and S122[382]). Only mk4.000137.12.01 conserved the MAGL specific amino acid residue C242[383], essential for enzyme activity. BLAST searches for MAGL isoform 1 and isoform 2 returned identical results. Reciprocal searches of *H. sapiens* and NCBI database at large yielded MAGL as the top result.

BLAST searches for NAAA homologs yielded no results. BLAST searches for COX-2 homologs yielded 1 low scoring match over the domain of interest. A reciprocal BLAST search found mk4.004960.04.01 to be a putative thyroid peroxidase.

BLAST searches for NAPE-PLD yielded two predicted protein results mk4.011893.00.02 (43%, 5e-31, 34%) and mk4.075769.00.01 (32%, 9e-15, 33%), each for ~30% of query length (not overlapping). Reciprocal searches yielded NAPE-PLD as the first result. Mk4.011893.00.02 conserved HXHXDH motif as well as D147 and H253 residues typical to the zinc metallohydrolase family to which NAPE-PLD belongs[384]. BLAST searches for DAGLα/β homologs yielded only one predicted protein, mk4.002913.01.01 (40%, 3e-22, 12%). This 150 amino acid sequence aligned over only a narrow range representing the lipase signature region of DAGLα and DAGLβ. Mk4.002913.01.01 conserves the lipase consensus motif GXSXG[385] and the first serine in the catalytic triad[386]. Reciprocal searches yielded DAGLα as the first result followed by DAGLβ.

BLAST searches for Abh4 homologs yielded six predicted proteins. Mk4.000264.04.01 (47%, 1e-28, 63%) returned the overall highest score. Reciprocal search results yielded Abh5

and Abh4 as top hits. BLAST searches for PTPN22 homologs yielded 32 predicted proteins (all over a narrow range). The reciprocal search for mk4.010153.00.01 (38%, 1e-27, 24%) yielded PTPN22 as a top three result. Reciprocal searches for the other 31 protein sequences yielded either protein tyrosine phosphatase receptors, or other non-receptor type PTPNs. The narrow region of mk4.010153.00.01 and PTPN22 alignment was over the PTPc conserved domain.

TRP Channels

BLAST searches for TRPV1 homologs yielded four predicted proteins, mk4.003971.00.01 (26%, 5e-12, 45%), mk4.000595.09.01 (23%, 1e-19, 43%), mk4.000540.12.01 (32%, 6e-15, 30%) and mk4.000540.13.01 (29%, 3e12, 17% ankyrin repeat region). Mk4.000540.12.01 and mk4.000540.13.01 are found on the same gene, their alignment ranges against TRPV1 did not overlap. mk4.000540.13.01 is essentially an ankyrin repeat and aligns over that region. mk4.000540.12.01 and mk4.000595.09.01 were more similar to *C. elegans* OSM-9, OCR-1-2-3-4 as well as *Drosophila* nanchung and inactive than vertebrate TRPV1 (see Table C.7). Amino acid residues considered critical for capsaicin binding (R114, R491, Y512, S511, T550, E761, C-terminus motif[387])were mostly absent, or outside the domain of these proteins. BLAST search results for TRPV2 were nearly identical to those of TRPV1.

BLAST searches for TRPA1 homologs yielded 173 predicted proteins. Reciprocal searches identified most aligned only over ankyrin repeat regions. Mk4.002685.01.01 (36%, 5e-83, 40%) and mk4.001942.03.01 (25%, 3e-68, 81%) align over a significant portion of the transmembrane region and C-terminus of the receptor, reciprocal searches confirmed both as putative TRPA1 homologs (see Table C.8).

BLAST searches for TRPM8 homologs returned 33 predicted proteins, each also sharing significant alignment with other human, *C. elegans* (GON-2, GTL-1) and *Drosophila* (CG34123) TRPM channels (see Table C.9). Reciprocal searches yielded various TRPM channels as top results. The highest scoring matches, mk4.014768.00.01 and mk4.003628.01.01, aligned over roughly the first 50% of the queries, excluding the ion transport protein conserved domain and C-terminus. mk4.003995.04.01 aligned over roughly the first 30% of the queries, excluding the entire transmembrane region. Lower scoring matches, mk4.003971.00.01 (25%, 8e-29, 34%) and mk4.001014.08.01 (25% 1e-23, 36%) for example, aligned over the TPC and C-terminal kinase conserved domains only.

PPARs

BLAST searches for PPARα, γ, δ homologs returned eleven predicted proteins for all isoforms. Nine of the search results represented only a narrow region analogous to C4-type zinc fingers characteristic of nuclear receptor DNA binding complex. Two predicted sequences, mk4.011673.01.01 and mk4.006000.00.01 aligned over the PPAR ligand binding domain. Reciprocal searches found mk4.011673.01.01 to be a putative thyroid receptor. A reciprocal search of the human genome for mk4.006000.00.01 yielded PPARδ as a top hit behind nuclear receptor subfamily 1. Reciprocal searches of the NCBI database at large generate insect E75 nuclear receptors as best matches (see Table C.10).

Results Summary

In summary NAEs and 2-AG are present in the planarian *Dugesia dorotocephala* at levels

within the wide range thus far documented in other organisms. In general PEA, SEA, OEA and 2-AG concentrations in *Dugesia dorotocephala* are greater than those found in other invertebrates and less than those found in vertebrates, while AEA was present high levels as compared to both vertebrates and invertebrates (Table 4). Significant Changes in SEA, PEA, and AEA levels were observed over the initial twelve hours of active regeneration, most notably a rise in SEA in hours 6 and 12 following the sectioning event. PEA levels dropped 2.5 times in animals subjected to hypoxic overcrowded conditions without reperfusion. Exogenously applied AEA, 2-AG and their catabolic inhibition effected biphasic changes in locomotor velocity, analogous to those observed in murines. Pharmacological FAAH and dual MAGL/FAAH inhibitors, effective in mammals, effected no change in locomotor velocity when administered alone. Dual MAGL/FAAH inhibition in conjunction with exogenously applied 2-AG did result in a significant reduction in locomotor velocity. Cannabinoid receptor homologs were not found in the genome of *Schmidtea mediterranea*. Type-specific homologs for other TRPV1, TRPV2, TRPM8, PPARα, PPARγ, and PPARδ were also absent. Putative TRPA1, TRPV type and TRPM type were found. Putative FAAH and Abh4 homologs as well as a MAGL-like lipase were identified in the *Schmidtea mediterranea* genome.

CHAPTER 4

DISCUSSION

Analytic Results

Background Levels

According to the author's knowledge this is the first report to identify and quantify 2-AG and NAEs in *D. dorotocephala* or any planarian. The absolute values are within the broad range found in other organisms (Tables 4 and B.2). Background levels of AEA in *D. dorotocephala* were at the high end of those found in rodent models, comparable to levels found in porcine brain[89]. Background levels of other NAEs were however below the wide range found in mammals yet well above those found in other invertebrates. 2-AG was present at levels below most recorded vertebrate values and within the wide range found in invertebrates. Absolute and relative concentrations for the analytes of interest differ extensively throughout the recorded literature, but some correlations may be observed by species, tissue type and kill method.

Relative concentrations of PEA/SEA/OEA/AEA/2-AG in *D. dorotocephala* were different than any other individual organism recorded. When employing standard methods of brain harvest, 2-AG concentrations in mammals, are reported to be 100-200 times greater than AEA. Sugiura observed 2-AG in brains of rats killed whole in liquid nitrogen were ~7 fold lower than those killed by decapitation[388]. In this study, the animals were killed whole in liquid nitrogen and 2-AG concentrations were approximately 20 times greater than AEA. PEA concentrations in mammalian tissues are generally 15-20 times those of AEA. OEA and SEA levels are generally lower than PEA, yet considerably higher than AEA. The relative presence of PEA and AEA varies

considerably for invertebrates from nearly 500 times in *Drosophila*[374] to nearly equal in *Hirudo medicinalis*[372]. The relative presence of OEA/SEA/AEA and PEA/OEA/SEA vary as well. In *D. dorotocephala,* the PEA concentration was 3-4 times greater than SEA/OEA/AEA, which were present at nearly equal concentrations.

Whole planaria contain a high proportion of nervous tissue per body weight as compared to other invertebrates[389]. Higher concentrations of AEA and 2-AG are generally found in samples representing nervous tissue. It should also be noted that planarian physiology is in a constant state of flux. The rate and manner of growth and degrowth is without compare among bilaterans. These factors may contribute to a distinctive NAE/2-AG profile in planaria.

Stressed Animals

As discussed above, elevated NAE levels, most notably PEA, are well documented after an ischemic or hypoxic episode. The inflammation and oxidative stress associated with ischemia is likely a result of the reperfusion of oxygen, not ischemia alone[390]. Oxidative stress results from overabundance of destructive reactive oxygen species (ROS) created from metabolic reactions that use oxygen. Without reperfusion oxidative stress does not occur, neither does the associated inflammatory response[391]. Few data are available concerning NAE profiles in hypoxic tissues without reperfusion as it would seem to be moot in a medical context. PEA has been reported to operate as an anti-oxidant when present below normal physiological concentrations and a pro-oxidant at elevated concentrations[392].

In the current study planarians subjected to “stressful” (hypoxic and overcrowded) conditions were not allowed reperfusion. NAE concentrations were not increased but instead

PEA concentrations were decreased. These findings suggest that NAE increases associated with ischemia are not due to hypoxia alone.

Sectioned Animals

AEA, PEA and SEA concentrations were altered in *D. dorotocephela* over the course initial twelve hours of regeneration. AEA was significantly decreased in the hour six after sectioning. PEA also significantly decreased in hour six. PEA is a potent anti-inflammatory and often rises in response to inflammatory stimuli. A more careful examination of available data suggests that PEA changes are linked to the time course and severity of the insult[198].

A significant rise in SEA was observed in hours six and twelve in the sectioned groups. This window coincides with the first of two mitotic peaks observed following wound closure. The biological relevance of SEA has been little studied as compared to its congeners yet appears to have similar anti-inflammatory and anti-anaphylactic properties[186, 194]. SEA treatment may accelerate wound healing[193]. SEA is also an effective anorexic, reducing food intake in already starving mice[164]. A wounded planarian is unable to feed until a new pharynx and functional CNS is formed[393]. The anorexic effect of SEA observed in this single study was different than that of OEA and would be consistent with a foreseen period of nutrient depravation.

Extracellular accumulation of SEA is correlated with high concentrations of NO and peroxynitrite. NO and peroxynitrite inhibit SEA membrane transport and intracellular hydrolysis[394]. NO is an important signaling molecule in vertebrates and invertebrates alike and at high levels induces cell death. NO synthase is prominently upregulated in regenerating

limb of *Ambystoma mexicanum* or axolotl, another popular model for studying regeneration[395].

SEA has been shown to induce cell death via oxygenated metabolites[394]. Gonzalez-Estevez speculates that the mitotic peaks following wound closure may be coupled to some type of cell death that would select the fitter neoblasts by either autophagy or apoptosis[393]. Compensatory proliferation, the induction of proliferation in surrounding tissue by apoptosis of damaged cells, has been observed in irradiated *Drosophila*[396] and in the regenerating *Hydra*[397]. Autophagic-like selective cell deletion has also been described in the regenerating planarian blastema[398]. Healing and regeneration is probably extremely resource demanding, autophagy may provide necessary energy and materials for this process[399]. It would be interesting to determine if elevated SEA in the regenerating planarian is merely coincident with mitotic activity and the regenerative process or plays a more integral role.

Enzyme Inhibitors

The OEA analog and FAAH inhibitor oleoyl ethyl amide effected no significant change in AEA concentration or that of any other analyte. OEA ($P< 0.07$) and SEA ($P< 0.07$) did trend lower in OEtA treated samples. These results would indicate that OEtA did not act as a FAAH inhibitor and that perhaps somehow reduced synthesis of OEA and its saturated congener SEA. LEA was increased in oAG treated samples, but effected no other significant change in analytes. Due to the number of replicates required an increased exposure concentration or duration was not attempted.

Use of FAAH inhibitors in other invertebrate species present with inconsistent results. Methylarachidonoylflourophosphonate (MAFP) potentiated the AEA stimulated NO release *in H. medicinalis* and mussel ganglia[178] yet in a later work MAFP, as well as two other potent FAAH inhibitors (*p*-hydroxymercuribenzoate and arachidonoyltriflouromethyl ketone), had no effect on enzymatic activity in the same species of leech[372]. DePetrocellis found AEA hydrolysis inhibition by arachidonoyltriflouromethyl ketone, MAFP, arachidonoyldiazomethyl ketone and arachidonoylchloromethyl ketone in *Hydra* was statistically significant yet considerably diminished as compared to their activity in mammals[292].

Behavior Results

Planarian locomotor speed varied significantly by day. This could have been due to a number of factors including number of days since shipping or number of days since feeding. The animals were not used for observation till a minimum of 24 hours after arrival or for a minimum of four days after feeding and not starved for more than seven days. Planarian activity has been observed to altar by nutrient status[400] and even change in response to weak magnetic fields or weather disturbance[401]. The nature of the experimental conditions, precipitated activity via bright light, may have amplified these changes.

Anandamide and OEtA Exposures

Anandamide did not induce spontaneous withdrawal in planarians at a dose of 3ppm. This is not surprising since anandamide exposure has never led to spontaneous or precipitated withdrawal in murines. Previously Raffa and Rawls demonstrated *D. dorotocephala* experienced

spontaneous withdrawal after one hour exposure in WIN55212,2[353]. WIN55212,2 treatment and subsequent denial does produce spontaneous withdrawal in murines, THC however cannot.

AEA at a 6ppm exposure reduced locomotor activity; 3ppm exposures trended slower but were not significant. 15 and 30ppm AEA exposures were not different from solvent controls. 1.5 ppm exposures were observed to increase locomotor speed if the observation window were reduced to two minutes. These data are consistent with the finding that AEA at 3ppm inhibits cAMP formation when applied to *H. medicinalis* ganglia[372].

Planarian response to OEtA treatment was unreliable but where significant, had a slowing effect consistent with cannabimimetic activity. Animals exposed to 15ppm AEA after pretreatment with OEtA displayed a significant decrease in locomotor activity when observed in the 2-4 minute window.

Daily solvent controls for March 3 were significantly depressed. On this day planarians were particularly sensitive to OEtA treatment. 6ppb OEtA significantly inhibited locomotor speed, pretreated animals were also significantly slowed for some portion (0-5 and 0-2 minutes) of the observation period upon exposure to 1.5 and 3ppm AEA, these observations were not duplicated on Febuary 27. As of March 3 planarians in our care had been without food for four days, it is unknown how long they had been without food prior to shipping. Nutrient depravation has been noted to decrease planarian activity[400]. AEA as well as OEA vary widely with changes in satiety[123]. OEtA is not only a FAAH inhibitor but an OEA analog. As mentioned above, OEA and SEA trended lower in response to OEtA treatment. These relationships may play some part in the observed behavioral patterns in response to OEtA treatment.

2-AG and oAG Exposures

2-AG, at exposure concentrations of 1.5 and 3.0 ppm, significantly increased locomotor velocity. Changes in planarian activity were not significant when exposed to the dual MAGL/FAAH inhibitor oAG at 5ppm in the absence of exogenous 2-AG. Pretreatment with oAG reversed the stimulatory effects of 2-AG at low concentrations, yielding significant decreases in motility (1.5ppm addition when observed for five minutes and 0.3ppm observed for 10 minutes). 2-AG levels were not increased for animals exposed to 5 ppm oAG for a 5 minute period, nevertheless behavioral observations would suggest that oAG did effect some physiological change in planaria when used in combination with exogenous 2-AG.

Comparison to Murine Models

Exogenous application of endocannabinoids did elicit measurable behavioral responses in *D. dorotocephela*. These results were reminiscent of the observed biphasic relationship of cannabinoid dosage on motor function and anxiety in murines. Romero found that the depressive effects of AEA on motor function in rats were decreased somewhat when the dose was raised from 1 to 3 mg/kg, the effect was recovered at 10mg/kg dosage. The 1mg/kg dose significantly increased rearing and shaking behaviors and was depressed at 3mg/kg and above. Holtzman measured levels of 5-HT and norepinepherine in mouse brain after THC injection. They found that serotonin levels decreased in response to doses < 5mg/kg, increased for doses 5-10 mg/kg, and decreased thereafter. Norepinepherine levels decreased for doses <5mg/kg, remained more or less level for 5-10 mg/kg and increased for doses higher than 10mg/kg. The time course of effects was also observed. The initial response to the lower THC dose (10 mg/kg)

was an increase in serotonin and decrease in norepinepherine. The initial response for a 200 mg/kg dose was a mild increase in serotonin and sharp increase in norepinepherine[134]. This oscillation is reflected in anxiety responses to many cannabinoid drugs, for instance low dosage of HU210 (10μg/kg) is anxiolytic while a higher dose (50μg/kg) is anxiogenic[402]. The anxiolytic and analgesic properties of CP55940 (1000μg/kg)[403] and AEA (100μg/animal)[172] are absent at high doses, at these levels rats emit audible vocalizations which have been interpreted as pain or anxious response. Biphasic concentration-response profiles could be due to non-CB receptor interactions or successive CB_1 activation of G_s and $G_{i/o}$ type G proteins[404]. Either mechanism may well prevent excessive cell excitability by ever increasing concentrations of cannabinoid agonists.

Bioinformatics

Protein homology is considered officially meaningful at 30% identity, 80% sequence length and an E-value of $< 10^{-4}$, but these restrictions do leave out known orthologs[405]. Criterion for E-values is inversely proportional to % query alignment. When allowing a more narrow alignment range, one should elevate the E-value threshold. Sequence alignment algorithms return a number of false positives when the search criteria are too permissive. Use of reciprocal searches and functional analysis may be used to winnow the results[315]. The *S. mediterranea* genome may be sequenced, but proteins encoded by these genes are predicted by algorithms. Sequences aligned over a reduced range may represent a familial protein with a shared conserved domain, or an imperfectly predicted protein.

Cannabinoid Receptors and Associated GPCRs

There are no identifiable CBr homologs in the *S. mediterranea* genome. The most promising selections demonstrated greater homology to opioid, 5-HT, Dopamine and adrenergic receptors. A Putative CBr has been predicted for the cephalochordate *Branchiostoma floridae*[406] (amphioxus), when this sequences was BLASTed against the *S. mediterranea* genome it returned many of the same results as the confirmed vertebrate and Ciona CBrs including mk4.001569.04.01 and mk4.0008535.00.01. A proto-CBR has been proposed in *C. elegans*[299]. When BLASTed against the human genome the putative *C. elegans* proto CBr returns α_1-andrenergic receptor isoforms for the first 15 selections, CB_1 receptors rank 16 and greater. Buznikov also found that the best candidate CBr homologs in sea urchin genomes were more similar to dopamine and α_1-andragenic receptors than $CB_{1/2}$ when BLASTed back against the human genome[291].

Cannabinoid induced behavioral effects in mammals are ascribed to their influence on neurotransmitter release. Neurotransmitters analogous to those found in mammals are present in planarians[325], and they exhibit altered rates of activity or stereotyped behaviors in response to drugs acting on neural transmission[339, 342]. In this study, behavioral changes in *D. dorotocephala* were observed in response to exogenously applied endocannabinoids and to drugs known to alter endocannabinoid levels. Previously behavioral changes in this species have been reported in response to the cannabinoid agonist WIN55212,2, effects that could be reversed by NMDA[353] antagonist. Instances of cannabinoid induced LTD have been reported in several invertebrates, and well studied in leech. These observations taken together would suggest that endocannabinoid ligands also serve as neuromessengers in the invertebrate CNS.

Neuromessenging need not be mediated by a CB-like GPCR. Endocannabinoids are known to directly interact with a neurotransmitter gated ion channels[130]. Stimulation or antagonism of neurotransmitter associated GPCRs such as Dopamine, 5-HT or acetocholine receptors could effect a similar response. These receptor types are present in the *S. mediterranea* genome.

BLAST searches for GPR119 returned many of the same and most interesting results as CB receptor searches. This may be simple coincidence since both are GPCRs. BLAST searches for GPR55 did not return identical results. Little is yet known about GPR119 except that it is a free fatty acid and free fatty acid amide receptor[407] and OEA, PEA and SEA bind with reasonably high affinity[119].

The MAKER protein mk4.0008535.00.01 shared the greatest number of amino acid residues critical for cannabinoid binding. It was however more similar to human μ-opioid receptors than to CBrs. Endogenous opioid ligands are peptides and represent a different ligand/receptor evolutionary relationship than seen in the ECS. It is unlikely that endocannabinoids or NAEs would have any affinity for an opioid receptor as we know it. Amphibian opioid receptors do present a somewhat more forgiving affinity profile for exogenous ligands, far less is known of invertebrate opioid-type receptors[408].

Metabolizing Enzymes

Three putative FAAH homologs can be identified in the genome of *S. mediterranea*. FAAH-like activity has been reported in species as diverse as leech, sea urchin, *Arabidopsis*, and *Tetrahymena*. FAAH belongs to the well conserved ‘amidase signature sequence’ (AS) family that share a common amino acid motif. AS enzymes possess a Serine-serine-lysine catalytic

triad which promotes amide bond hydrolysis[409]. AS family enzymes are ubiquitous. A database search will find orthologs most numerous among the bacteria and fungi. A portion of the amidase signature sequence, GGSSGGEGALI (215-225) is well conserved in vertebrates and considered to be unique to FAAH. Its conservation does not however appear to be necessary for hydrolysis of NAEs. AtFAAH and the *Tetrahymena* FAAH-like enzyme (SB210, GI:146146721) do not conserve this portion yet effectively hydrolyze AEA as well as other NAEs[300, 307]. The three putative FAAH homologs found in the *S. mediterranea* genome conserve this sequence in addition to the catalytic triad.

Deletion of the 'polypropyline region', PTVPPLPFR (307-315), renders FAAH enzymatically inactive[381]; effects of the loss of any singular residue in this region have not been ascertained. Prolines generally confer flexibility and any substitution is assumed to limit enzyme binding options. The proposed putative FAAH homologs found in *S. mediterranea* exhibits a P(313)→C substitution. AtFAAH as well *Tetrahymena* SB210 express a singular proline substitution in this region.

One putative MAGL homolog was identified in the genome of *S. mediterranea*. MAGL appears to be highly conserved in a wide range of organisms. Potential MAGL homologs can be bioinformatically identified in vertebrates, invertebrates, fungi, protozoa, plants, archaea, bacteria and viruses[308]. MAGL belongs to the lipase enzyme family. Lipase enzymes possess a serine-asparagine-histidine catalytic core; the serine is contained in a GXSXG consensus motif. Substitution of C242 inhibits MAGL enzymatic efficiency ~80%[383], otherwise little functional data exist to differentiate MAGL from other members of the lipase family. Some putative MAGL homologs identified may well be other varieties of lipase enzymes.

BLAST searches for NAPE-PLD and DAGL, the primary synthetic enzymes for NAEs and 2-AG respectively, were inconclusive. Sequences representing only fragments of these enzymes were found. DAGL is of particular interest since DAGL expression is upregulated in the blastema of regenerating axolotl[395]. DAGL-like enzymes have been identified in insects, and have been predicted in *C. intestinalis* and *C. elegans*[299]. Stereoinspecific DAGL-like enzymes can be found in fungi and green plants. A DAGL enzyme and its polyunsaturated fatty acid metabolites are necessary in the activation of TRP channels which in turn initiate the photo-excitation of rhodopsin in the *Drosophila* eye[410]. Non-vertebrate NAPE-PLD homologs have been both predicted (*C. elegans*) and confirmed (copepods) yet appear to have suffered secondary loss in hexapoda and tunicates[411].

Alternative synthetic pathways have been recognized. NAEs may also be formed through the double acylation of NAPE by α/β-hydrolase 4 (Abh4), and another less understood pathway involving protein tyrosine phosphatase, non-receptor type 22 (PTPN22)[65]. 2-AG may also be synthesized by the combined actions of phospholipase A_1 and phospholipase C, or may be formed from arachidonic acid containing lysophosphatidic acid through the action of a phosphatase[66]. BLAST searches revealed a putative Abh4 homolog in the *S. mediterranea* genome, yet no likely PTPN22 counterpart.

The 2R hypothesis suggests that at least two whole genome duplication events (WGD) occurred in vertebrate evolution, the first taking place the in the early vertebrate ancestor followed by another in teleost fishes[412]. The paralogs CB_1 and CB_2 have been attributed to the first vertebrate WGD as well the enzyme pairs DAGLα-DAGLβ, Abh4-Abh5, COX1-COX2 and NAAA-ASAH1. Abh5, COX1 and ASAH1 are not known to participate in endocannabinoid

signaling[413]. No sequences bearing significant homology to NAAA or ASAH1 were found in the *S. mediterranea* genome. No putative COX-1 or COX-2 homologs were found in the *S. mediterranea* genome, though other cyclooxygenase homologs are present.

Using mirrored cladograms Mcpartland demonstrated the evolutionary correlation between biosynthetic and catabolic enzymes that share the same substrate, (FAAH–Abh4 and NAAA-NAPE-PLD). More importantly he showed a strong cladistic connection between the incidence of CB receptors and the DAGL enzyme. Phylogenetic profiles for PTPN22 and GPR55 are mirrored as well[413]. This hypothesis assumes that any gene encoding a protein unable to carry out its function in the absence of a partner would be rendered physiologically futile, and that such pairings would undergo parallel evolution. The finding of putative FAAH and Abh4 homologs in the *S. mediterranea* genome does support this hypothesis.

Transient Receptor Potential Channels

TRP channels were first characterized in *Drosophila*, and later found to be well conserved in multicellular animals. Seven types of TRP channels have been characterized in flies and nematodes, six of which are found in mammals[414]. All TRP channels play a role in sensory perception including hearing, olfaction, photoresponse, thermosenstation, osmosensation, chemosensation and mechanosensation[414]. Many putative TRPV-, TRPA- and TRPM-type channels can be identified in the *S. mediterranea* genome, but none could be identified as specific TRPV1, TRPV2 or TRPM8 homologs. These putative TRP-type channels are more similar to those found in nematode and insect than human orthologs.

TRPA1 is the only member of the TRPA family encoded in the human genome; it was previously named ANKTM1 due to the presence of many N-terminal ankyrin repeats. Two TRPA channels may be found in *C. elegans*, and four in *Drosophila*[414]. TRPAs regulate touch sensation in *C. elegans*[415]. All insects contain TRPA orthologs and modulate hygrosensation[416] and high temperature thermosensation[417].

Gon-2, gtl-1 and 2 and ced-11 are TRPM channels found in *C. elegans*. Gtl-1 governs Mg^{2+} homeostasis in nematode intestine, deletion disrupts intestinal rhythmic activity and defecation[415]. A single TRPM is present in the genome of *D. melanogaster*, its physiological role has not been described[418]. The mechanisms for thermal activation of TRPV and TRPM channels (hot/cold) appear to be similar. Icilin, a TRPM8/TRPA1 agonist, induces increases locomotor activity in D. dorotocephala[355].

Nematode OSM-9 was among the first TRPVs identified, followed by the OSM-9 like channels OCR-1, OCR-2, OCR-3 and OCR-4[419]. OCR-1 is involved in regulating chemosensation, egg-laying and social feeding, The specific functions of OCR-3 and 4 are unknown[415]. OSM-9 and OCR-2 deleted nematodes exhibit impaired responses to odor, touch and osmolarity changes[419]. OSM-9 and OCR-2 are activated by a number of polyunsaturated fatty acids[415]. Arachidonic acid induces avoidance behavior in nematodes, a reaction absent in OSM-9 mutants [420]. Two TRPV-type channels are found in Drosophila; nanchung and inactive. Deletion of genes encoding nanchung results in a deaf mutant, the inactive mutant has locomotor defects. Nanchung and inactive respond to changes in osmolarity but not capsaicin, menthol or temperature[421].

TRPV1 affinity for capsaicin may be a recent evolutionary development. Avians and reptiles are insensitive to capsaicin[99]. Insects in general are indifferent to capsaicin, and no specific behavioral response has been observed in *C. elegans*[419]. In contrast capsaicin sensitivity has been demonstrated in both the American cockroach[421] and the land snail[422]. AEA binds to TRPV1 on the same intracellular binding site as capsaicin. Capsaicin insensitive avian TRPV receptors are not activated by anandamide[99]. It would therefore seem unlikely that AEA could directly activate any TRPV found in planaria if they are capsaicin insensitive. Capsaicin effects no acute behavioral response in planaria[355]. Baguna did however observe another physiological effect. At low to medium concentrations (10^{-4} to 10^{-6} M) capsaicin greatly stimulated cell division in planarians. Higher concentrations proved lethal[327].

TRPV, TRPA and TRPM channels are modulated by lipids and lipid derived molecules[420] and may represent a means by which NAEs and 2-AG can effect a number of physiological outcomes. TRPV4, the mammalian equivalent of OSM-9[423], for instance is stimulated by downstream cytochrome P450 AEA metabolites[106]. Members of the TRP channel family are expressed throughout the bilateria; most integrate sensory input and facilitate responses to outside stimuli. In mammals, TRPV1 channel activation alone is sufficient to trigger long-term synaptic depression (LTD) in hippocampal neurons[424]. It would seem sensible that an encounter with some noxious agent would lead to an experience-dependant behavioral change.

Peroxisome Proliferator-Activated Receptors

One nuclear receptor with significant alignment and homology over the PPAR ligand binding domain was identified in the *S. mediterranea* genome. PPARs are members of the thyroid subgroup of nuclear receptors[425]. PPARs are considered to be fast evolving[426]. When viewed graphically they appear as a distinct out-group from other nuclear receptors, along with their nearest relative insect E75. Appearance of PPAR group corresponds to the emergence of the early vertebrates[427]. The initial divergence of proto-PPAR to PPARγ and PPARα/δ likely occurred during the first whole genome duplication event that produced $CB_{1/2}$ receptors. PPARα-δ split took place later in vertebrate development[412]. Nuclear receptors and nuclear receptor ligands may have evolved prior to the development of affinity for one another[356]. It also may be possible that both OEA and AEA bind to a proto-PPAR. There is evidence that invertebrate nuclear receptors serve functions analogous to PPARs in vertebrates. Deletion of the nematode nuclear receptor nhr-49 results in considerable changes in fatty acid metabolism much like PPARα deletion in mice[428].

Other Observations

Ligands may affect a variety of measurable changes without binding to a receptor. AEA, at physiologically relevant concentration, directly modulates several voltage-gated ion channels[130]. Cannabinoids are extremely lipophilic molecules. Elphick hypothesized that their actions in invertebrates may be due to the effects of non-specific membrane disruption[288]. The relatively new study of lipid rafts has shown that endocannabinoids can effect the stability

of lipid rafts, and that the status of these microdomains can effect intracellular processes[223, 256].

NAEs and 2-AG are present in planaria, as well as other invertebrates. Cannabinoids affect measureable changes in behavior for planarians, and synaptic transmission in other invertebrates. Radioligand binding assays have detected binding sites for CP55940 in membrane fractions of several echinoderms and protostomians (see Table B.1). The binding sites are assumed to be CB-like GPCRs since the CP55940 can be displaced by SR141716A, yet likely CB receptor homologs cannot be found in their genomes. Endocannabinoids are not gene-products but derived bioactive molecules; there is less evolutionary pressure to conserve the ligand/receptor pair. Prior to emergence of CB receptors, endocannabinoids may have found a partner in another central nervous system associated GPCR or a number of other potential physiological targets.

In this study changes in PEA concentrations were observed under conditions of hypoxic stress and in SEA during active regeneration. In plants, NAE levels rise in response to a pathogen threat and initiate transcription of defense response genes[314], in mammals PEA and SEA are potent anti-inflammatory agents. Cytoprotection afforded by PEA in cells suffering oxidative stress is independent of CB and TRPV receptors[228, 234]. The specific mechanisms behind these qualities are still poorly understood, and are often lumped together with the beneficial features of cannabinoid agonists. The non CB-mediated actions of cannabinoids and NAEs could perhaps be better assessed in simple animal model lacking CB receptors. The humble planarian is a worthy and proven subject. It may yet reveal the mysteries of regeneration and much more.

Conclusions

Background tissue concentrations of 2-AG, AEA, PEA, OEA and SEA in *D. dorotocephala* were not outside the range of values reported for other animal organisms. The directional change of NAEs in *D. dorotocephala* subjected to simulated ischemia was different from murine models; this difference may be attributed to the denial of reperfusion. NAE levels in *D. dorotocephala* did change over the course of the initial twelve hours of regeneration, most notably an increase in SEA in hours six and twelve. Exogenous application of endocannabinoids did elicit a behavioral response, of a biphasic nature, in *D. dorotocephala*. The effectiveness of pharmacological manipulation of endocannabinoids and NAEs in *D. dorotocephala* was inconclusive. Cannabinoid receptor homologs were not found in the genome of *Schmidtea mediterranea*. Type-specific homologs for other TRPV1, TRPV2, TRPM8, PPARα, PPARγ, and PPARδ were also absent. Putative TRPA1, TRPV type and TRPM type channels were found. Putative FAAH and Abh4 homologs as well as a MAGL-like lipase were identified in the *Schmidtea mediterranea* genome.

APPENDIX A

ADDITIONAL INFORMATION REGARDING CANNABINOID PHARMACOLOGY

Table A.1 Cannabinoid agonists and antagonists

A selection of K_i values of CB_1 and CB_2 agonists and antagonists (inverse agonists) for the *in vitro* displacement of [^{3}H]CP55940, [^{3}H]*R*-(+)-WIN55212, or [^{3}H]HU-243 from CB_1 and CB_2 specific binding sites.			
	CB_1 K_i (nM)	CB_2 K_i (nM)	Reference
Agonists			
Endogenous			
Anandamide	61-543nM	279-1940nM	[429]
2-arachidonoylglycerol	370nM – 1.9µM	150nM – 1.4µM	[430]
ACPA	2.2	715	[431]
HEA	53.4	ND	[431]
DEA	34.4	ND	[431]
2-AG ether	21.2	>3000	[431]
Phytocannabinoid			
Δ^9-Tetrahydrocannabinol	35-53nM	4.9-75nM	[430]
Δ^8-THC	47.6	39.3	[430]
Cannabidiol	4350	2860	[431]
ABb-cannabidiol	>10000	>10000	[431]
Synthetic			
WIN55212,2	1.9-123nM	0.3-39nM	[430]
CP55940	0.5-5nM	0.7-2.8nM	[430]
Nabilone	1.84	2.19	[429]
HU-210	0.06-0.1	0.2-3.2	[431]
JWH-015	383-ND	14-430	[431]
JWH-133	677	3.4	[431]
ACEA	1.4	>2000	[430]
O-812	3.4	3870	[430]
AM1241	280	3.4	[429]
R-(+)-methandamide	17.9	868	[430]
JWH-051	1.2	0.032	[430]
JWH-139	2290	14	[430]
L-759633	>1000	6.4-20	[430]
L-759656	>4000	12-19	[430]
HU-308	>10000	22.7	[430]
O-1966	5055	23	[432]
O-3853	1509	6	[432]
CB_1 selective antagonists/inverse agonists			
SR141716A	1.8-12	514-13000	[431]
AM251	7.5	2290	[431]
AM281	12	4200	[432]
LY320135	141	14900	[430]
CB_2 selective antagonists/inverse agonists			
SR144528	28-ND	0.04-2.0	[431]
AM630	5152	31.2	[431]
Other			
O-1918 (CB_x antagonist)	>30000	>30000	[431]

Table A.2 Enzyme and transport inhibitors

List of enzyme and transport inhibitors.	
FAAH Inhibitors	
URB-597	oleoylethylamide (OEtA)
URB-532	palmitoylisopropylamide
PF-3845[143]	CAY10400
N-arachidonoyl serotonin	CAY10401
Methyl arachidonoyl flourophosphonate (MAFP)	CAY10435
Diazomethylarachidonoyl ketone (DAK)	CAY10402
Arachidonoylsulfonyl fluoride	OL-135
Arachidonoyltriflouromethyl ketone (ATFMK)	URB524
1-oxazola[4,5-*b*]pyridin-2-yl eicosa-5*Z*,8*Z*,11*Z*,14*Z*-tetraen-1-one	JP83
N-arachidonoylglycine (NAGly)	JP104
N-arachidonoyllisoleucine (NAIle)	Ibuprofen
Oleoyltriflouromethyl ketone	Suprofin
N-2-(acetoxyacetyl) pentadecylamine	Ketorolac
2-methyl-1-oxazolo[2,4-*b*]pyridine-2-yl-9*Z*-octadecen-1-one	Indomethacin
1-oxo-1-[5-(2-pyridyl)oxazol-2-yl]-9(Z)-octadecene	Flurbiprofen
1-[5-(pyridine-2-yl)-1,3,4-oxadiazol-2-yl]-octadec-9-en-1-one	IbuAM5
R-palmitoyl-2-methyl ethanolamide (RP-2ME)	
MAGL Inhibitors	Dual FAAH/MAGL Inhibitors
JZL-184[143]	JZL-194[143]
O-1428	o-arachidonoyl glycidol[433]
O-4081	
NAM	NAAA Inhibitor
IDFP	[(S)OOPP] [189]
MOPF	
EOPF	AEA Uptake Inhibitors
S-nonyl BDPO	AM404
CPO	VDM-11[434]
URB602	
URB754	DAGL inhibitor
	O-3841[67]
Inhibitor information from[80] unless otherwise noted	

Table A.3 Endocannabinoid and NAE receptor interactions

Effects of 2-AG, NAEs and selected synthetic agonists and inverse agonist on the G-protein coupled receptors, transient receptor potential channels and peroxisome proliferator-activated receptors mentioned in this thesis.

Target	Assay	Unit	2-AG	AEA	PEA	SEA	OEA	CP55940	WIN	Sr141716
CB_1[111]	GTPγS	EC_{50}nM	519±48	31±6	>30000		>30000	0.2±0.01	18±3	
CB_2[111]	GTPγS	EC_{50}nM	618±45	27±6	>17000		>30000	0.3±0.01	1±0.2	
GPR55										
[111, 115]	[^{33}S]GTPγS	EC_{50}nM	3±1	18±3	4±1		440±145	5±1	NE	600
[112-114, 435]	[Ca^{2+}] mobilization	EC_{50}μM	NE	5	NE			NE	NE	antag
								antag		
			NE	7.3				NE		
[114, 435]	pERK	EC_{50}μM	NE	NE	NE		NE	NE	NE	NE
				10						
GPR119[119]	Reporter gene	EC_{50}μM		SE	E	E	3.2±0.3	NE	NE	
TRPV1										
[105, 436, 437]	[Ca^{2+}] mobilization	EC_{50}μM	NE	1.15±0.15	NE	PE	0.9±0.1	NE	SE	antag
[95]			8.4	0.63±0.2	SE		PE			
[438]	PKC stimul						2±0.3			
TRPV2[105]	[Ca^{2+}] mobilization	EC_{50}μM	NE	NE				SE	NE	
TRPM8	[Ca^{2+}] mobilization HEK-293									
[102]	vs. icilin IC50	IC_{50}μM		0.15±0.08	NE					52.±10
[102]	vs. menthol IC50			3.1±0.6	NE					NE
TRPA1[103, 105]	[Ca^{2+}] mobilization			NE				SE		
				E						
PPARα										
[124, 125]	Binding assay	EC_{50}nM		NE		NE	120±1			
		pEC_{50}		4.61			4.34		4.74	
[121]	Reporter gene	EC_{50}μM			3.1±0.4	NE				
[122]	Oxymetabolite 15-Hete-g		E							
PPARγ										
[125, 127]	Binding assay	EC_{50}μM		7	NE		NE			
[121, 127]	Reporter gene			8	NE					
[128]	Interleukin-2	IC_{50}μM	6.9							
[122]	Oxymetabolite 15-Hete-g		NE							
PPARδ										
[121, 125]	Binding assay	EC_{50}μM			NE		1.1±0.1			
[122]	Oxymetabolite 15-Hete-g		NE							

NE = no effect. SE = slight effect. PE = no effect but potentiated AEA. E = effects but EC50 not reported

APPENDIX B

COMPILATION OF LITERATURE VALUES CONCERNING ENDOCANNABINOID-ASSOCIATED LIGANDS AND ENDPOINTS IN INVERTEBRATES

Table B.1 Evidence of ECS components in non-vertebrate and non-plant organisms

Evidence of ECS components in non-vertebrate, non-plant organisms. A) ligand binding assays measuring displacement by [^{3}H]CP55,940 of bound SR141716A (fmol/mg) or ∞ displacement by AEA of bound [^{3}H]SR141716A AEA, Ω displacement by CP55,940 and SR14176A of bound [^{3}H]anandamide, √ specific [^{3}H]CP55,940 without displacement B) Incidence of AEA or 2-AG hydrolysis in vivo C) incidence of decreased of synaptic potentials in presence of stated ligand D) Incidence of altered cAMP release in response to cannabinoids. Y = recorded incidence N = negative results. Rat included for comparison.

Clade	Species – Tissue Type	Ligand Binding	Amidase Activity	Incidence of Decreased Synaptic Potentials	cAMP
Deuterostomes	*Rat cerebellum*[293]	648±16			
	Ciona intestinalis – sea squirt[439]		Y		
	Ciona intestinalis – neural tissue[293]	134±3			
	Ciona intestinalis – intestine[293]	ND			
	Ciona intestinalis – haemolymph[293]	35±2			
	Ciona intestinalis – testes[293]	30±6			
	Paracentrotus lividus[369]		Y		
	Lytechinus variegates[291]	√			
	Strongylocentrotus purpuratus[440]	√			
Lophotrochozoa	*Aplysia californica* (sea slug)[288, 441]	-		THC 10^{-4}M	
	Mytilus edulis (mussel)[289]	Ω			
	Hirudo medicinalis (leech)[372]	Ω	Y	CP55,940 10µM	Y
				2-AG 60µM[297]	
	Theromyzon tessulatum (leech)[372]	Ω	Y		
	Helix lucoorum L. (snail)[294]	170±13		AEA 10^{-5}M	
	Lumbricus terristus (earthworm)[293]	78±5			
Ecdysozoa	*Apis mellifera* (honey bee)[374]	N			
	Drosophila melanogaster[288, 374]	N, √			
	Gerris marginatus[374]	N			
	Spodoptera frugiperda (moth) [374]	N			
	Zophobas atratus (beetle) [374]	N			
	Schistocerca gregaria (locust)-muscle[288]	√	Y		
	Panagrellus redivivus (nematode)[293]	55±5			
	Jasus edwardi (rock lobster)[293]	104±3			
	Procambarus clarkia (crayfish)[296]			ACPA 10µM	
	Homarus americanus (lobster)[442]			THC	
	Peripatoides novae-zealandiae [293]	90± 11			
Other	*Hydra vulgaris*[292]	∞	Y		
	Actinothoe albocincta (Cnidaran)[293]	ND			
	Tethya aurantium (Poriferan)[293]	ND			
	Tetrahymena pyriformis[300, 443, 444]		Y		Y
	Saccharomyces cerevisae[303]		Y		

Table B.2 Tissue levels of 2-AG and NAEs in various organisms

Species/ TissueType/ Information	Unit	16:0	18:0	18:1	18:2	20:4	2-AG
Human (brain non-tumor, tumor patient)[236]	pmol/g	142±22	63±6.0	62±~4.0*	5.5±1.0	4.3±0.8	
Human [13](blood)	pmol/ml blood	~12-15					
Human (colon)[13]	pmol/mg lipid	~2.5				~0.5	
Human (colon)[187]	pmol/mg lipid					~0.4	~200
Human (cerebral spinal fluid)[197]	pmol/ml					2.4±1.3	~70
Human (plasma)[225]	pmol/mg lipid	2.05±0.31				1.81±1.53	6.80±9.50
Rat (brain)[366]	pmol/g					23±3	4000±1800
Rat (brain) killed by decapitation[388]	nmol/g						1.54
Rat (brain) killed in liquid nitrogen[445]	nmol/g						0.23
Rat brain[446]	nmol/g						3.36
Rat brain[447]	nmol/g						65
Rat (brain)[367]	pmol/g					2.45±.39	
Rat (hindpaw)[198]	pmol/mg lipid	5.6±1.0				0.69±0.14	51.1±9.0
Rat (bladder)[198]	pmol/mg lipid	3.35±1.7				0.21±0.11	36.7±13.6
Mouse (brain)[226]	pmol/g	289±27.5	60.7±48	246.5±17.7		13.6±3.2	12000±1000
Mouse (brain cortex)[365]	pmol/g	8728 ±2655	2634± 183	3266± 691	349 ±54	576± 147	
Mouse (skin)[13]	nmol/g	~0.5				~0.05	
Pig (brain)[89]	µg/g	2.05±0.49	2.27± 0.43	2.1*		0.06 ±0.01	
Sheep (brain)[89]	µg/g	0.75	0.90	1.0*		nd	
Cow (brain)[89]	µg/g	1.07	0.91	1.0*		0.04	
Marmota monax (groundhog) active[448]	pmol/ml plasma	~50		~125		3.67±0.98	nd
Marmota monax (groundhog) torpor[448]		~ 100		~40		4.0±0.98	nd
Taeniopygia guttata (zebra finch) brain[449]	pmol/g					~30-75	~10-15000
Carrasius auratus (goldfish) Telencephalon[285]	pmol/mg lipid					~0.15	~1.5
Carrasius auratus (goldfish) Hypothalamus[285]	pmol/mg lipid					~0.45	~1.2
Carrasius auratus (goldfish) cerebellum[285]	pmol/mg lipid					~0.15	~0.8
Pimephales promelas (fathead minnow) male brain[368]	pmol/g					22.28±0.76	15370±650
Pimephales promelas (fathead minnow) fem. brain[368]	pmol/g					28.88±1.10	19040±610
Ciona intestinalis (sea squirt)[439] range of values over different tissues	pmol/mg lipid	5.2 - 3.7				0.06 - 5.4	2.7 - 50.6
Ciona intestinalis (sea squirt)[439] cerebral ganglion	pmol/mg lipid	17.3±3.5				~3.6	~15
Paracentrodus lividus (sea urchin) ovaries[369]	pmol/g	95±30	10±5			12±4	

Species/ TissueType/ Information	Unit	16:0	18:0	18:1	18:2	20:4	2-AG
Amblyomma americanum (tick) salivary glands[450]	pmol/mg lipid	18.5±2.3	38.8±18.3	9.4±4.9		< 1	268.5±27.5
Amblyomma americanum(tick) Well Fed - salivary glands[450]	pmol/mg lipid	10.2±3.4	33.8±22.4	8.1±2.1		< 0.5	33.9±0.3
Agabus affinis (water beetle) defensive glands[451]							Present
Drosophila melanogaster (fruit fly) heads[374]	pmol/g	231				< 5.7	337
Drosophila melanogas ter [374]	pmol/g	572				< 3.3	212
Apis mellifera (honeybee)[374]	pmol/g	225				< 12.8	462
Theromyzon tessulatum (leech)[373]	pmol/g						112
Aplysia (sea slug) ganglia[371]	pmol/g	Present		Present		10.5±2.7	5700±900
Hirudo medicinalis (leech)[372]	pmol/g	32.3±1.5			5.8	21.5±0.7	147.4±42.7
Mytilus galloprovincialis (mussel)[370]	ng/g	21.0±3.0	10.6±4.8	2.8±0.8	2.8±0.6	1.8±0.2	
Mytilus galloprovincialis 24 hr post mortem [370]	ng/g	53.0±3.9	32.8±2.6	3.6±0.7	3.5±0.6	3.0±0.4	
Crassostrea sp (oyster)[370]	ng/g	16.4±1.6	12.6±1.4	2.6±0.2	nd	Trace	
Crassostrea sp 6h postmortem [370]	ng/g	39.8±5.7	11.0±1.9	3.5±0.5	Trace	2.6±0.9	
Crassostrea sp 24h postmortem [370]	ng/g	45.7±1.0	23.4±8.0	3.9±0.4	Trace	4.9±1.8	
Tapes decussates (clam) [370]	ng/g	58.8±6.2	35.4±2.7	1.6±1.0	1.6±1.2	2.0±0.9	
Tapes decussates 20 min boiling[370]	ng/g	51.6±7.8	23.4±9.6	1.3±1.0	1.1±0.2	3.8±0.8	
Callista chione (clam)[370]	ng/g	28.4±4.5	16.8±3.9	Trace	Trace	nd	
Venus verrucosa (clam)[370]	ng/g	39.2±6.1	11.8±2.0	2.7±0.6	2.8±0.8	nd	
Hydra vulgaris polyps[292]	pmol/g	Present		Present		15.6±1.5	11200±1900
Caenorhabditis elegans (nematode) Briston N2[452]	pmol/mg protein					0.3±0.1	12±6
Caenorhabditis elegans (nematode) AB1[452]	pmol/mg protein					0.5±0.2	79±9
Caenorhabditis elegans (nematode) TR403[452]	pmol/mg protein					0.5±0.7	28±25
Caenorhabditis elegans (nematode) CB4856[452]	pmol/mg protein					0.4±0.5	31±6
Caenorhabditis elegans (nematode) *fat-3*[452]	pmol/mg protein					nd	nd
Caenorhabtitis briggsae (nematode) AF16[452]	pmol/mg protein					2.3±1.8	57±1
Pelodera strongyloides (nematode)[452]	pmol/mg protein					0.17 ±0.04	70±17
Tetrahymena thermophila (Protist) 27ºC[301]	pmol/mg protein	1.8±0.3	0.3 ±0.1	0.6±0.3	2.3±0.8	< loq	< loq
Tetrahymena thermophila (Protist) 33ºC[301]	pmol/mg protein	2.8± 0.60	0.3±0.15	0.6±0.20	3.7±0.70	<loq	< loq
Saccharomyces cerevisiae (yeast) exponential phase[302]	pmol/µm lipid P	21.3	5.6	7.2			
Saccharomyces cerevisiae (yeast) stationaryl phase[302]	pmol/µm lipid P	22.5	7.3	16.1			
Plants							
Bauhinia congesta[375]	ng/g	37.4±7.1	7.5±0.9	135± 9.1	66.2±7.1		
Caesalpinia gilliesii[375]	ng/g	54.0±11.1	26.4±3.0	48.2± 6.1	138±25.0		
Mimosa borealis[375]	ng/g	224±46.7	53.3±4.7	131± 26.5	358±55.9		
Lupinus succulentus[375]	ng/g	387±23.1	209±10.8	245± 26.6	460±47.0		
Lupinis texensis[375]	ng/g	370±139	55.8±37.6	737± 231	814±378		

Species/ TissueType/ Information	Unit	16:0	18:0	18:1	18:2	20:4	2-AG
Arachis hypogaea[375]	ng/g	3730±135	686±68	7960± 378	4540±501		
Medicago sativa cv 1701[375]	ng/g	1150± 123	469±32	1030± 68	1990±54		
Medicago trunculata cv. A17[375]	ng/g	181±105	65±11	321± 74	981±102		
Medicago. trunculata cv. Jemalong[375]	ng/g	12700±683	2030±146	9560±811	12154±876		
Pisum sativum cv. Early Alaska[375]	ng/g	665±31	186±16.2	1060± 52.1	1160±133		
Pisum sativum[375]	ng/g	103±9.3	37.9±1.5	216± 9.4	174±15.8		
Phaseoulus vulgaris cv. Amarillo del Norte[375]	ng/g	53.5±11.1	18.9±7.43	24.4±3.1	28.8± 5.7		
Vigna unguiculata cv. Tohono O'odham[375]	ng/g	138±13.1	51±3.3	71.0±7.3	125± 7.0		
Glycine max cv.Dare[375]	ng/g	6720±534	1620±148	4900±376	12740±995		

The information listed here for invertebrates is, to our knowledge, comprehensive. Information listed for vertebrate and plant species is representative only. < loq = less than limit of quantification, nd = no detect, ~ = inferred from graphical representation, Present = no specific information given, * Sum of reported values for 18:1 n-9 and 18:1 n-7.

APPENDIX C

BLAST RESULTS

Table C.1 Top S.med database BLASTp search results for representative CBRs

S. med predicted protien	Human CB1 (i1)			Human CB2			Fugu CB1a			Ciona CiCNR		
	%i	E	%L	%i	E	%L	%i	E	%L	%i	E	%L
mk4.003002.02.01	28	4e-20	63	24	1e-12	71	25	1e-16	62	23	2e-11	77
mk4.001569.04.01	23	2e-16	58	23	8e-18	84	22	5e-15	58	22	2e-13	71
mk4.013827.00.01	25	3e-13	40	29	2e-11	32	21	1e-11	44	25	2e-7	20
mk4.017826.00.01	30	3e-13	44	30	3e-11	45	30	2e-11	45	25	1e-12	72
mk4.002418.01.01	24	1e-15	59	25	1e-12	84	21	1e-14	72	20	1e-7	49
mk4.004728.01.01	26	1e-11	53	27	8e-14	61	25	3e-11	55	21	5e-7	44
mk4.000526.00.01	23	3e-11	57	23	4e-12	73	24	2e-10	54	27	1e-4	20
mk4.000742.09.01	21	7e-10	61	28	2e-11	73	28	1e-9	60	26	2e-10	60
mk4.012659.00.01	26	2e-11	57	25	8e-6	60	25	5e-9	60	22	1e-8	72
mk4.003634.00.01	21	2e-10	68	24	1e-11	58	20	8e-9	54	21	9e-8	58
mk4.000375.06.01	23	4e-10	65	21	6e-10	81	23	8e-10	69	24	1e-7	74
mk4.003906.01.01	21	1e-13	47	24	1e-11	64	22	2e-13	46	22	3e-11	61
mk4.010158.01.01	28	2e-12	43	27	2e-10	42	28	1e-10	45	27	4e-10	34
mk4.008535.00.01	25	2e-10	59	23	1e-12	84	24	6e-9	71	23	2e-6	55
mk4.005562.01.01	23	7e-13	67	30	6e-10	82	23	6e-14	67	18	3e-6	73
mk4.002569.02.01	24	7e-10	64	19	.019	36	22	2e-8	62	23	.002	65
mk4.003634.00.01	21	2e-10	68	24	1e-11	58	20	8e-9	54	21	9e-8	58
mk4.005939.01.01	25	2e-12	55	27	7e-10	57	24	1e-9	52	23	3e-8	49
mk4.031060.00.01	21	7e-06	59	20	.022	44	27	.0003	16	29	.0002	30
mk4.021573.00.01	22	9e-9	69	20	2e-9	88	25	5e-6	39	21	1e-6	54
mk4.014127.00.01	23	6e-12	61	20	2e-8	87	21	2e-8	59	25	1e-8	62
mk4.011160.01.01	24	3e-10	58	27	3e-9	68	23	2e-9	57	28	3e-11	55
mk4.000992.05.01	24	2e-5	35	26	4e-8	39	29	2e-4	19	32	8e-11	30
mk4.011371.00.01	23	9e-10	56	28	1e-8	65	33	4e-11	55	26	4e-11	65
mk4.005766.00.01	25	3e-9	52	25	5e-7	62	25	8e-9	59	26	2e-10	63
mk4.036740.00.01	28	2e-8	26	22	7e-7	45	26	2e-7	33	27	9e-10	50
mk4.014774.01.01	24	2e-6	51	29	1e-5	40	28	1e-5	41	27	3e-6	64
mk4.007921.00.01	28	5e-8	26	32	2e-4	36	29	4e-7	21	26	3e-5	31
mk4.001291.01.01	21	2e-7	65	21	2e-8	75	23	2e-8	70	23	2e-12	75
mk4.007388.02.01	28	1e-10	43	31	2e-10	31	28	3e-10	43	23	1e-5	32

%i = % identity E= E value and %L = % query length.

S. med Predicted proteins carry the mk4 designation, sequences available from S. med genome database.

Table C.2 Representative S.Med database BLASTp search results and functional analysis for potential CB1/2 homologs

	CB_1 gi:38683844								CB_2 gi:4502929			
S. mediterreana predicted proteins	F189	K192	V196	F200	Y275	W279	L286		S112	T116	C175	F197
Mk4.003002.02.01	+	-	+	-	-	+	+		-	+	+	-
Mk4.001569.04.01	+	-	-	-	+	-	-		-	-	+	+
Mk4.013827.00.01	-	-	-	-	-	+	+		-	-	x	x
Mk4.017826.00.01	+	-	+	-	+	-	-		+	-	x	x
Mk4.002418.01.01	-	+	-	-	+	+	+		-	-	-	+
Mk4.010158.01.01	+	-	+	-	+	-	-		+	-	x	x
Mk4.000526.00.01	-	-	+	-	-	+	+		-	-	-	+
mk4.005939.01.01	-	-	-	-	+	+	+		-	-	x	x
mk4.012659.00.01	-	-	-	-	-	+	+		-	+	x	x
mk4.000742.09.01	+	-	+	+	-	-	-		+	-	x	x
mk4.000375.06.01	-	+	+	-	-	-	+		-	-	-	+
Mk4.007388.02.01	+	-	+	+	+	+	+		-	+	-	+
mk4.008535.00.01	+	+	+	+	-	+	+		+	+	-	+
mk4.005562.01.01	+	-	+	-	-	+	+		+	-	+	+
mk4.001291.01.01	-	-	-	-	-	+	-		-	+	-	-
mk4.014127.00.01	-	+	+	-	-	+	-		-	-	-	-
Ciona intestinalis												
445655 ciad70124	+	+	+	-	+	-	+		-	+	+	-

CiCNR listed for comparison.

Sequences bearing a GenInfo (GI) number are available from NCBI database. S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.

Table C.3 Top S.Med database BLASTp search results for potential GPR119 homologs

Predicted protein	% Identity	E value	% Query
mk4.001569.04.01	26	5e-28	93
mk4.004728.01.01	25	6e-23	62
mk4.003002.02.01	24	6e-23	83
mk4.005562.01.01	22	3e-16	83
mk4.001678.03.01	28	5e-13	60

S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.

Table C.4 Top S.Med database BLASTp search results for FAAH homologs, paired with specific amino acid residues considered critical for enzymatic activity

FAAH gi:166795287	DIGGS 238-241	S217	K142	S218	R243	GGSSGGEGALI 215-225	PPLP 310-313	E	% i	% L
Mk4.001109.06.01	DIGGS	+	+	+	+	GGSSGGEGAIL	PPLC	2e-106	37	95
Mk4.002051.04.01	DIGGS	+	+	+	+	GGSSGGEGAIL	PPLC	6e-91	36	96
MK4.005541.02.01	DIGGS	+	+	+	+	GGSSGGEGAIL	PPLC	2e-89	35	90
S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.										

Table C.5 Representative reciprocal BLASTp search results of NCBI database for S.med putative FAAH homolog mk4.001109.06.01

Mk4.001109.06.01	*X. tropicalis* GI:58332564	*M. musculus* GI:226443015	*C. elegans* GI:71990152	*T. thermophila* GI:146161510	*A. thaliana* GI:186532737
E value	7e-95	7e-88	2e-73	1e-56	3e-17
% identity	39	39	32	29	23
% Query	87	91	94	94	75
Sequences bearing a GenInfo (GI) number are available from NCBI database. S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.					

Table C.6 S.Med database BLASTp search results and functional analysis for potential MAGL homologs

MAGL gi:6005786	GxSxG 120-124	D239	H269	C242	E value	% Identity	% Query
Mk4.000137.12.01	G+S+G	+	+	+	2e-43	33	87
Mk4.012753.00.01	G+S+G	+	+	-	1e-40	30	91
Mk4.020061.00.01	G+S+G	+	+	-	6e-38	30	90
Mk4.000137.11.01	G+S+G	+	+	-	7e-31	31	82
S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.							

Table C.7 Comparison of S. med predicted proteins with known TRPV-type channels

S. med Predicted Protein	Human TRPV1 GI:74315354			OSM-9 GI:228484886			OCR-1 GI:193207906			Nanchung GI:45445898			Inactive GI:24640231		
	%i	E	%L	%i	E	%L	%i	E	%L	%i	E	%L	%i	E	%L
mk4.000540.12.01	32	6e-15	30	35	1e-41	28	30	3e-31	33	43	3e-34	33	34	2e-37	33
mk4.000595.09.01	23	1e-19	43	32	6e-74	51	25	1e-40	53	27	1e-45	42	30	1e-68	37
%i = % identity E= E value and %L = % query length.															
Sequences bearing a GenInfo (GI) number are available from NCBI Database. S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.															

Table C.8 Representative NCBI database BLASTp search results for S.med putative TRPA1 homologs mk4.002685.01.01 and mk4.001942.03.01

Mk4.002685.01	*H. Sapiens* GI:116534990	*X. tropicalis* GI:189230220	*A. gambiae* GI:186694312	*D. mojavensis* GI:296034212
E value	1e-80	4e-87	5e-92	1e-86
% identity	36	38	39	38
Mk4.001942.03.01	*H. Sapiens* GI:116534990	*C. elegans* GI:212645948	*D. rerio* GI:208401165	*P. obsoletus* GI:291191476
E value	1e-56	7e-73	1e-66	8e-53
% identity	25	28	27	27
Sequences bearing a GenInfo (GI) number are available from NCBI database. S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.				

Table C.9 Comparison of S. med predicted proteins against known TRPM-type channels

	mk4.014768.00.01			mk4.003628.01.01			mk4.003995.04.01			mk4.001014.08.01			mk4.003971.00.01		
	%i	E	%L	%i	E	%L	%i	E	%L	%i	E	%L	%i	E	%L
Human TRPM8 GI:109689695	27	7e-61	66	25	2e-41	62	26	5e-36	37	25	1e-23	36	25	8e-29	34
Human TRPM2 GI:109730277	28	2e-75	44	27	2e-60	45	29	4e-28	31	24	1e-27	30	25	3e-28	19
Human TRPM5 GI:219517806	25	2e-50	53	23	8e-41	55	25	2e-34	35	23	8e-19	26	26	3e-30	25
X. laevis TPRM8 GI:239049983	30	8e-56	46	25	1e-37	53	24	2e-24	39	25	5e-22	36	25	9e-28	26
C. elegans Gon-2 GI:212644978	44	4e-96	40	23	1e-28	35	29	4e-44	23	22	3e-18	27	26	8e-16	22
C. elegans GTL-1 GI:52082722	34	4e-123	46	24	4e-34	39	28	4e-48	29	25	7e-17	15	23	3e-15	31
D. melanogaster CG34123 GI:22090229	40	6e-145	37	23	3e-40	32	29	1e-47	30	23	2e-20	16	24	5e-23	14
%i = % identity E= E value and %L = % query length.															
Sequences bearing a GenInfo (GI) number are available from NCBI database. S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.															

Table C.10 Representative NCBI database BLASTp search results for S. med putative nuclear receptor MK4.006000.00.01

Protein	Species	GeneInfo #	% identity	E value	% Query
E75 NR	*T. castaneum*	270014294	29	4e-28	28
PPARδ iso 4	*H. sapiens*	284807159	30	3e-22	48
PPARα	*H. sapiens*	1514595	29	2e-21	42
PPARγ	*H. sapiens*	20336229	28	5e-22	36
PPARδ	*X. laevis*	148233022	29	1e-21	49
PPARα	*X. laevis*	214664	27	7e-22	39
PPARγ	*X. laevis*	148223591	27	2e-21	53
PPARα2	*T. rubripes*	148233501	33	1e-24	34
PPARγ	*T. rubripes*	147904405	27	6e-19	31
Sequences bearing a GenInfo (GI) number are available from NCBI database. S. med predicted proteins carry the mk4 designation, sequences available from S. med genome database.					

```
P  1    MD----KINNVDPKTKNN-------------------ILKISCAVVAVG--AAGYIGYVVFRKIKTANL-----------  44
H  1    MV--------------------QYELWAALPGASGVA--LACCFVAA----AVALRWSGRRTARGAV------------  41
M  1    MV--------------------LSEVWTALSGLSGVC--LACSLLSA----AVVLRWTRSQTARGAV------------  41
F  1    ML--------------------ALNT----NDIVGLCALLGCSAATV----VLLLRWLDRKRIGKQV------------  39
N  1    MW--------------------FFYV-----------AIVIIGFFSP----KLIDKYNRKQKLRTLI-----------  32
T  1    MTIKDIPYINVPPGTNGNGHSESFFEKIVNYKFSVLQLAYLFAAYLAL----KIIWNLIVYIYHKIRN-----------  64
A  1    MG----KYQVMKRASEVDLSTVKYKAETMKAPHLTGLSFKLFVNLLEAPLIGSLIVDYLKKDNGMTKIFRNTVIPEEPMF  76

P  45   ------------IQKKRKELEQKKNDLITKLEDIGSPF--INMD---KITNLSLNDLLKAIENGTLTPVDILQSYQAKA  106
H  42   ------------VRARQRQRAGLENMDRAAQRFRLQNPD--LDSE---ALLALPLPQLVQKLHSRELAPEAVLFTYVGKA  104
M  42   ------------TRARQKQRAGLETMDKAVQRFRLQNPD--LDSE---ALLALPLLQLVQKLQSGELSPEAVLFTYLGKA  104
F  40   ------------RLARARRDAALGRMEEAVRRLSAEHPV--ADPE---GILSLSLPELASKLKDGSLSPEMVLHSYMRKA  102
N  33   ------------KQRAADRKINFEWAKNSFQKL----DE--SRAV---EISAKPFEELRNSLKNGEIGPVETLRAFQRKA  91
T  65   ------------SLLIKKAKLYRKNRDDQLQAFQIPTPS--LDQDTLQKVLNEDVTSIKKLLSKGKVTSEDLVNIFAKRC  130
A  77   RPEFPSQEPEHDVVIVGEDESPIDRLETALKCLPQYDPSRSLHADPVSSFRYWKIRDYAYAYRSKLTTPLQVAKRIISII  156

P  107  LVL---DKEYNFITDLNPN------ADVDVLGGETSKR--GILKGIPVSIKESFMVKGCDSTMGMPDR-MFQPSEKDDMM  174
H  105  WEV---NKGTNCVTSYLAD-CETQLSQAPRQ---------GLLYGVPVSLKECFTYKGQDSTLGLSLN-EGVPAECDSVV  170
M  105  WEV---NKGTNCVTSYLTD-CETQLSQAPRQ---------GLLYGVPVSLKECFSYKGHASTLGLSLN-EGVTSESDCVV  170
F  103  LDV---TSELNCVTDYLPE-CETQLVHLRGQKT------KGLLYGVPISLKDNFNYTGHDSTLGLLNQ-LNRPACEDSVI  171
N  92   YES---TEKTNCVCFFIQE-ALEIAENLEHLATDPNYQ-KPPLFGVPVSIKESIHVKNLDSTAGYAQK-INNPSDANSVS  165
T  131  QQF---NPQLEAITHLKYEEAIMKAKECDKLRKEKSPLVQGLLFGIPISIKEIFDEKGYPSTVGCIQR-LNYVPVEDGFI  206
A  157  EEFGYDKPPTPFLIRFDANEVIKQAEASTRRFEQGNPI--SVLDGIFVTIKDDIDCLPHPTNGGTTWLHEDRSVEKDSAV  234

P  175  VRTLHLQGMIPFVTTNVCQTGYSLESSNPIYGVTLNPFNKSRTCGGSSGGEGAILGCGASLVGLGSDIGGSIRVPAYYCG  254
H  171  VHVLKLQGAVPFVHTNVPQSMFSYDCSNPLFGQTVNPWKSSKSPGGSSGGEGALIGSGGSPLGLGTDIGGSIRFPSSFCG  250
M  171  VQVLKLQGAVPFVHTNVPQSMLSYDCSNPLFGQTMNPWKPSKSPGGSSGGEGALIGSGGSPLGLGTDIGGSIRFPSAFCG  250
F  172  AKVLKVQGALPFMKTNIPQSMLNYDCSNPIYGRTLNPLNHKKTPGGSSGGEGSLIAAGGSILGIGSDIGGSIRFPSAFCG  251
N  166  VDQLIRLGAVPFVHTNIPIALLSYGCSNGVYGTTLNPLDNSRVPGGSSGGEAALVSLGGSVLGIGTDVGGSIRTPASFCG  245
T  207  IQLLRKSGAIPLVRSNVPQCCFTFESVNRIYGRVKNPWDLTKMAGGSSGGEASIIASRLCPIGLGSDQGGSIRIPAAMCG  286
A  235  VSKLRSCGAILLGKANMHELGMGTTGNNSNYGTTRNPHDPKRYTGGSSSGSAAIVAAGLCSAALGTDGGGSVRIPSALCG  314

P  255  VYGLKPTAMRFSRRHGKL---RYPTQNIINVSVGPMARRIDDLVTVFKAMACGDL------YHLDPYCPPLCFNDKIYEK  325
H  251  ICGLKPTGNRLSKSGLK---GCVYGQEAVRLSVGPMARDVESLALCLRALLCEDM------FRLDPTVPPLPFREEVYTS  321
M  251  ICGLKPTGNRLSKSGLK---SCVYGQTAVQLSVGPMARDVDSLALCMKALLCEDL------FRLDSTIPPLPFREEIYRS  321
F  252  ICGFKPTANRLSKLGVK---TSSAGQKSVAAMIGPLARDVDSLVLCMRALLCEEM------FQLDPTIPPLPFNEEIYSS  322
N  246  LAGFKSSSDRSPQLGKT---ASIPGRQLLLSVEGPIAKNIDVCVEYLRLKWNDPLL-----YKKDVYMPPVKFQENLYNS  317
T  287  IYGFKPTSGRCVINGLTHYSEAFDGQTINKACAGPMAKSMDDTILLFKALCDPNILKEFNISQIDPNLTILPIDENALND  366
A  315  ITGLKTTYGRTDMTGSL-------CEGGTVEIIGPLASSLEDAFLVYAAILGSSSADRYN-LKPSPPCFPKLLSHNGSNA  386

P  326  KRPLR-IGYYVHLGGNQITPVPAVARAVEMAKNALES-AGHTLVEFDIPDI-DYAIF-ELYFRCLFT-DGGVGFVEKYIA  400
H  322  SQPLR-VGYYET-D-NYTMPSPAMRRAVLETKQSLEA-AGHTLVPFLPSNI-PHALE--TLSTGGLFSDGGHTFLQNFKG  394
M  322  SRPLR-VGYYET-D-NYTMPTPAMRRAVMETKQSLEA-AGHTLVPFLPNNI-PYALE--VLSAGGLFSDGGCSFLQNFKG  394
F  323  TRPLR-IGYYET-D-DATMATPSMKRAVHETKELLER-AGHKMVPFTPPSVEKSMFE--LIVKGLLA-DGGSTFLDNFKG  395
N  318  EKPLK-IGYYTF-D-GYQTASPAYQRAVRETVAVLKE-LGHELVPFEVPQP-DHMYS--IFCAGATA-DGGLYLMDSLAN  389
T  367  SRKKRRFGYFKTL--EVIDSCLAAQRAVDISIEKLRN-LGHEVIEVEIPKQ-NEIIH--AFLQNSFS-DDMQNLKDILKG  439
A  387  IGSLR-LGKYTKW--FNDVSSSDISDKCEDILKLLSNNHGCKVVEIVVPEL-EEMRAAHVISIGSPTLSSLTPYCEAGKN  462

P  401  DNPVDSSISFTTNVLKVPNFLKRFVSYIIYPFDKNQSHALYAIAGVPSVYGIWELNEKIKDYREKVLTKWQEEKLDGLIC  480
H  395  DF-VDPCLGDLVSILKLPQWLKGLLAFLVKPLLPRLSAFLSNMKS-RSAGKLWELQHEIEVYRKTVIAQWRALDLDVVLT  472
M  395  DF-VDPCLGDLVLVLKLPRWFKKLLSFLLKPLFPRLAAFLNSMCP-RSAEKLWELQHEIEMYRQSVIAQWKAMNLDVVLT  472
F  396  DQ-VDPNLKTQVSTYGLPYWLKIFISFIVKPVFPRLSKLLQNIKGIRSVKDLWKHHLEVEAYRQEFISQWKKLEIDAVLC  474
N  390  DI-IPPEADIGFPVAKLPHFIQRLLRKYWHHRRERQIIQELPHDT----EEMRQMHEKIEDYRHEFVLAMRAKKLDALVC  464
T  440  ES-FLDEYQMLDFLASIPNSMKKVLAFLMGALGEKRLKDHILADMNIDSHDYKAVVYQILQLRKEVLKVFDENKIEAIIC  518
A  463  SK----------------------LSYDTRTSFAIFRSFSAS--------DYIAAQCLRRRLMEYHLNIFKD--VDVIVT  510

P  481  PVLPICAPPLNTSGYIMDIISYTAIYNLLDYPAGSSPVTTVNQNDIDLLLK-SYPRNTRTHRKIIEYQKDSIGMPVGVQS  559
H  473  PMLA-PALDLNAPGRATGAVSYTMLYNCLDFPAGVVPVTTVTAEDEAQMEHYRGYFGDIWDKMLQKGMKKSVGLPVAVQC  551
M  473  PMLG-PALDLNTPGRATGAISYTVLYNCLDFPAGVVPVTTVTAEDDAQMEHYKGYFGDMWDNILKKGMKKGIGLPVAVQC  551
F  475  PILS-PALTIGYPGKLSTAVSYTILYNLVDFPVGVVPVTTVTKDDEEGLKAYKGHKKDYWDKLLKEALTDSIGLPVSVQC  553
N  465  PAFGCPPPHHGMPNKILGANSYPALYNLIDFAAGTVPVTVQKQEDEVELR--KMKTEDSWDRRVVTESKNCIGLPVSVQI  542
T  519  PANATPALPHGSSADVADIVAYQFMWNILDFTCGVIPVTRVEEGEQHYENA--RVKDSISKKIDKYMRMKTEGLPIAVQV  596
A  511  PTTGMTAPVIPPDALK---NGETNIQVTTDLMRFVLAANLLGFPAISVPVG----------------YDKEGLPIGLQI  570

P  560  VSMLWREETCLRIMRDISRNIK----------------------  581
H  552  VALPWQEELCLRFMREVERLMTPEKQSS----------------  579
M  552  VALPWQEELCLRFMREVERLMTPEKRPS----------------  579
F  554  VALPWQEEQCLRLMKEVETL-TQERRTSNF---------------  582
N  543  ATPPYREEMCLRLLKQVEAKIGLYSRTH----------------  570
T  597  VAPPFKEEVCLNVMKIIDNGVQFYKQIEFPSLEGEEKELEIVEKD  641
A  571  MGRPWAEATVLGLAAAVEELAPVTKKPAIF--------YDILNTN  607
```

Dugesia dorotocephala putative FAAH mk4.001109.06.01 (P) aligned with FAAH *Homo sapiens* GI:166795287 (H), *Mus musculus* GI:226443015, (M), *Xenopus tropicalis* GI:58332564 (F), *Tetrahymena thermophila* GI:146161510 (T), *Arabidopsis thaliana* GI:186532737 (A) and *Caenorhabditis elegans* GI:71990152 (N) using COBALT multiple alignment tool from NCBI. highlighted alignments represent four or more matched amino acids or conservative substitutions as described by the BLOSUM substitution matrix. The catalytic triad residues S217, S241 and K142 are underlined. Other residues important for FAAH activity I238, G239, G240, S218 and R243 are indicated by italics.

Figure C.1 Multiple alignment of *Dugesia dorotocephala* putative FAAH, mk4.001109.06.01, with known FAAH homologs

APPENDIX D

LEGEND OF ABBREVIATIONS

Abbreviation	Term
AEA	*N*-arachidonoylethanolamide, Anandamide
2-AG	2-Arachidonoylgylcerol
5-HT	5-Hydroxytryptamine
Abh4/5	α/β-hydrolase 4/5
Abn-CBD	Abnormal cannabidiol
AS family	Amidase signature sequence family
AtFAAH	*Arabidposis thaliana* FAAH
BLAST	Basic alignment search tool
BLASTp	Basic alignment search tool for proteins using a protein query
BLASTx	Basic alignment search tool for proteins using a nucleotide query
BLOSUM	Blocks of amino acid substitution matrix
BSTFA	bis-Trimethyl silyl trifluoroacetamide
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CBr/CNR	Cannabinoid receptor
CiCBR	*Ciona intestinalis* CBr
CNS	Central nervous system
COX1/2	Cyclooxygenase 1/2
DAGL	Diacylglycerol lipase
DMSO	Dimethyl sulfoxide
ECS	Endocannabinoid system
FAAH	Fatty acid amide hydrolase
GABA	γ-Aminobutyric acid
GC/MS	Gas chromatography-mass spectrometry
GPCR	G-protein coupled receptor
KO	Knock-out
LEA	Linoleoylethanolamide
LTD	Long-term depression
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
NAAA	N-acylethanolamine-hydrolyzing acid amidase
NAE	*N*-acylethanolamines
NAPE	*N*-acylphosphatidylethanolamine
NAPE-PLD	*N*-acylphosphatidylethanolamine phospholipase D
NCBI	The national center for biotechnology information
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
oAG	o-Arachidonoyl glycidol
OEA	Oleoylethanolamide
OEtA	Oleoyl ethyl amide
PAM	Point accepted mutation

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEA	Palmitoylethanolamide
PPAR	Peroxisome proliferator-activated receptors
PTPN22	Protein tyrosine phosphatase non- receptor type 22
S. med	*Schmidtea mediterranea*
SEA	Stearoylethanolamide
tBLASTn	Basic alignment search tool for nucleotides using a protein query
THC	Δ^9-Tetrahydrocannabinol
TRP	Transient receptor potential
TRPA	Transient receptor potential ankyrin
TRPM	Transient receptor potential melastatin
TRPV	Transient receptor potential vanilloid

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