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REVIEW AND BIOINFORMATICS RESEARCH

Expression of the endocannabinoid system in fibroblasts and myofascial tissues

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Summary The endocannabinoid (eCB) system, like the better-known endorphin system, consists of cell membrane receptors, endogenous ligands and ligand-metabolizing enzymes. Two cannabinoid receptors are known: CB_1 is principally located in the nervous system, whereas CB_2 is primarily associated with the immune system. Two eCB ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are mimicked by cannabis plant compounds. The first purpose of this paper was to review the eCB system in detail, highlighting aspects of interest to bodyworkers, especially eCB modulation of pain and inflammation. Evidence suggests the eCB system may help resolve myofascial trigger points and relieve symptoms of fibromyalgia. However, expression of the eCB system in fascia-related tissues has not been established. The second purpose of this paper was to investigate the eCB system in fibroblasts and other fascia-related cells. The investigation used a bioinformatics approach, obtaining microarray data via the GEO database (www.ncbi.nlm.nih.gov/geo/). GEO data mining revealed that fibroblasts, myofibroblasts, chondrocytes and synoviocytes expressed CB_1, CB_2 and eCB ligand-metabolizing enzymes. Fibroblast CB_1 levels nearly equalled levels expressed by adipocytes. CB_1 levels upregulated after exposure to the inflammatory cytokines and equiaxial stretching of fibroblasts. The eCB system affects fibroblast remodeling through lipid rafts associated with focal adhesions and dampens cartilage destruction by decreasing fibroblast-secreted metalloproteinase enzymes. In conclusion, the eCB system helps shape biodynamic embryological development, diminishes nociception and pain, reduces inflammation in myofascial tissues and plays a role in fascial reorganization. Practitioners wield several tools that upregulate eCB activity, including myofascial manipulation, diet and lifestyle modifications, and pharmaceutical approaches.

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Introduction

The introduction comprises a broad review of the endogenous cannabinoid (eCB) system, in three sections: 1. cannabinoid receptors, 2. eCB ligands, 3. clinical aspects of receptors and ligands. Ligands bind to receptors may activate receptors (''agonists'') or deactivate receptors (''inverse agonists''). The chemical concepts underlying eCB research may raise anxiety in clinicians. However, the realization that *chemistry is structure* (Ingber, 1998) makes many of these concepts readily understood by bodyworkers. For example, the pharmacological principle of structure–activity relationships (SARs) is analogous to the anatomical concept of structure–function relationships.

After the introduction, this paper investigates the eCB system in fibroblasts, utilizing a bioinformatics approach. Bioinformatics uses networks of computers, software algorithms and internet-accessible databanks to organize, analyze, and predict biological structure and function. This approach poses a new challenge to clinicians, impelling them to grasp the utility and ease of ''GEO,'' the bioinformatics tool used in this study. Bioinformatics democratizes the research process; all one needs is computer access and imaginative questions. Several pre-publication reviewers of this paper immediately grasped GEO to answer questions of their own. The paper finishes with a discussion of clinical applications. The discussion delivers a unique perspective not heretofore presented in the literature—that our task as clinicians who treat pain and myofascial dysfunction is to enhance endogenous eCB activity in our patients.

Cannabinoid receptors

Cannabinoid receptors (CBRs) take their name from the *Cannabis* plant. The *Cannabis* plant is a source of *exogenous* ligands. The ligands are lipophilic (i.e., water-insoluble), thus difficult to study, and took 150 yr to elucidate. Finally, in 1964, Raphael Mechoulam isolated Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Since then, Raphael Mechoulam, Roger Pertwee and many others have identified more than 70 unique *Cannabis* compounds, collectively called the cannabinoids (reviewed in Pertwee, 2005). Candice Pert's co-discovery of the μ-opioid receptor in 1973 launched a quest for CBRs. But CBR discovery awaited the development of water-soluble synthetic THC analogs, such as CP55, 940. In 1988 Allyn Howlett and Bill Devane showed that [^{3}H]CP55, 940 bound to a receptor located in the cell membrane of the neuronal (brain) cells. Two years later, Lisa Matsuda cloned the gene for the CBR and decoded its DNA sequence. The cDNA sequence translates into a chain of 472 amino acids that weave back and forth across the cell membrane. This topology is characteristic of a G-protein-coupled receptor (GPCR). GPCRs are named after their G-proteins, short for guanine nucleotide binding proteins, which function as intracellular ''molecular switches.'' GPCRs include opioid receptors, dopamine receptors, serotonin receptors and many others (reviewed in Howlett et al., 2002).

A second CBR was discovered in 1993, so the receptors became known as CB_1 and CB_2. The two receptors express slightly different structures and slightly different functions: CB_1 principally functions in the nervous system, whereas CB_2 is primarily associated with cells governing immune function, such as white blood cells. Taken together, CB_1 and CB_2 bridge the constituent parts of psychoneuroimmunology and represent a microcosm of mind-body medicine. CB_1 and CB_2 are tensegrity structures that span the cell membrane. A ligand that loads the receptor's *extracellular* surface will distort the shape of its *transmembrane* weave of amino acids, thereby altering the *intracellular* side of the receptor and its interface with the G-protein. This shape-altering ''conformational change'' in the receptor activates the G-protein, which disconnects from the receptor, splits into subunits, and the subunits move around the inside of the cell. The activated G-protein subunits further transduce signal by reorganizing other tensegrity structures (e.g., enzymes and ion channels), causing a ''cascade'' that ultimately governs gene expression and cell behavior. Steve Ingber characterized tensegrity structures as the hardware behind living systems, and signal transduction machinery as the software (Ingber, 1998).

CB_1 is the most common GPCR neuroreceptor in the human brain, but it is distributed unevenly. Highest densities of CB_1 are found in the hippocampus (affecting short-term memory) and parts of the basal ganglia (e.g., the substantia nigra, globus pallidus and the striatum (caudate and putamen)). CB_1 in these nuclei coordinate movement, as does CB_1 in the cerebellum. High densities in the cerebral cortex, amygdala and dorsal horn of the spinal cord affect cognition, mood and emotion, and pain perception. Very low densities are found in the brainstem cardiorespiratory centers, which probably accounts for the lack of lethal effects from cannabis overdose (reviewed in Howlett et al., 2002).

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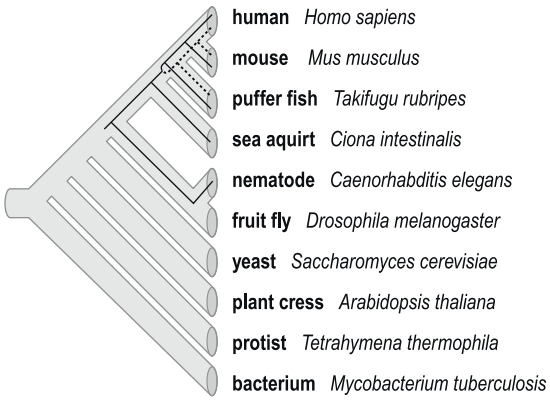

Figure 1 The cannabinoid receptor gene tree within a species tree. The species tree consists of ten organisms whose entire genomes have been sequenced it is represented by thin tubular lines. The gene tree is represented by thicker lines, alternately solid (representing CB_1 gene orthologs), striped (CB_2 gene orthologs), or dashed (representing the ancestral CBR gene).

The genes for CB_1 and CB_2 are paralogs (genes separated by a gene duplication event) with orthologs (genes separated by a speciation event) in all known vertebrate species. A CBR gene tree within a species tree is illustrated in Figure 1. The gene tree was constructed from ten species whose entire genomes have been sequenced, specifically chosen to obtain a balanced species divergence within the evolutionary "tree of life." The human, mouse and puffer fish genomes all express CB_1 and CB_2 genes, whereas the sea squirt and nematode genomes expressed only one gene, which we called the ancestral CBR gene (McPartland et al., 2006). No CBR genes were found in the "lower" organisms with deeper evolutionary roots. These findings suggest that the gene duplication event that gave rise to CB_1 and CB_2 occurred in the ancestor of vertebrates. The ancestral CBR gene that preceded the duplication event may have evolved in the last common ancestor of nematodes and sea squirts—600 million years ago (McPartland et al., 2007).

eCBs and their enzymes

Animals likely did not evolve neuroreceptors for a plant ligand. Indeed the CBR gene evolved eons before the appearance of *Cannabis*, which is not more than 34 million years old (McPartland and Guy, 2004). The first *endogenous* cannabinoid, anandamide (AEA), was discovered in 1992 by a team including Devane (co-discoverer of CB_1) and Mechoulam (28 yr after discovering THC). Soon 2-arachidonoylglycerol (2-AG) was described along with several less-understood eCBs (reviewed in Mechoulam et al., 1998). AEA and 2-AG are metabolites of arachidonic acid and do not resemble THC. However, AEA and 2-AG are lipophilic, like THC, and fit the binding pocket in CB_1 and CB_2. Thus, the effects of THC, AEA and 2-AG substantially overlap, because they all activate CB_1 and CB_2.

AEA and 2-AG are not stored in vesicles like classic neurotransmitters. Rather they are synthesized "on demand" from precursor phospholipids in the neuron cell membrane and immediately released into the neural synapse (Pertwee, 2005). AEA is cleaved from its precursor, *N*-arachidonoyl phosphatidylethanolamine (NAPE) by the enzymes NAPE-PLD and ABHD4; 2-AG is cleaved from diacylglycerol (DAG) by two DAG lipase enzymes, DAGLα and DAGLβ. After the release into the synapse, AEA and 2-AG activate CB_1, and then other enzymes break down AEA (FAAH, FAAH2 and NAAA) and 2-AG (MAGL and COX2). For a full description of these acronyms, see Table 1. Recently a FAAH-blocking agent was described, which prolonged AEA activity in the synapse, analogous to a serotonin uptake inhibitor (reviewed in Pertwee, 2005).

In the CNS, the eCB system serves as a negative feedback mechanism and dampens excessive synaptic release of other neurotransmitters. For example, persistent activation of a nociceptor causes excessive glutamate release in the dorsal horn synapses (see Figure 2). This maladaptively upregulates glutamate receptors in the post-synaptic cell (in this case a wide dynamic neuron that ascends to the brain). However, DAGLα enzymes are located in the post-synaptic cell, and influx of Ca^{2+} from upregulated glutamate receptors causes DAGLα to synthesize 2-AG (Figure 2A). The 2-AG moves *retrograde* (opposite the direction of glutamate) across the synapse to CB_1 located on the presynaptic neuron (Figure 2B). Activated CB_1 closes presynaptic Ca^{2+} channels, which halts glutamate vesicle release. This newly discovered phenomenon is called "depolarization-induced suppression of excitation" (Mátyás et al., 2007). The eCB system "mellows the synapse," and requires neuroscientists to rewrite textbooks that describe the synapse as a "one-way street."

At CB_2 in white blood cells, AEA and 2-AG act as autocrine, paracrine or endocrine modulators and circulate in the blood stream for short periods of time. The eCBs (and THC) are *immunomodulators* and not simply *immunosuppressors* as characterized in the 1970s (Klein, 2005). They do indeed suppress production of Th1 (T-helper1, cellular immunity) cytokines such as interleukin (IL)-2 and interferon gamma (INFγ), as well as tumor necrosis

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Table 1 Fibroblast expression of cannabinoid receptors or endocannabinoid ligand enzyme documented by charts deposited in the GEO profiles database.

Protein acronym, *gene symbol*[a]	Protein full name, synonyms (if any), enzyme protein function	Number of GEO profile charts located in search
CB_1, ***CNR1***	Cannabinoid receptor 1	165
CB_2, ***CNR2***	Cannabinoid receptor 2	142
FAAH, *FAAH*	Fatty acid amide hydrolase 1, catabolic enzyme of AEA	82
COX2, *PTGS2*	Cyclooxygenase 2, aka prostaglandin-endoperoxide synthase 2, catabolic enzyme of 2-AG	66
NAAA, *ASAHL*	*N*-acylethanolamine acid amidase), catabolic enzyme of AEA	55
DAGLβ, ***DAGLB***	Diacylglycerol lipase beta, aka *KCCR13L*, synthetic enzyme of 2-AG	36
NAPE-PLD, *NAPE-PLD*	*NAPE-selective phospholipase D, biosynthetic enzyme of AEA*	31
ABHD4, *ABHD4*	Abhydrolase domain-containing protein 4, *aka FLJ12816*, synthetic enzyme of AEA	28
DAGLβ, *DAGLA*	Diacylglycerol lipase alpha, aka ***NSDDR***, *C11ORF11*, synthetic enzyme of 2-AG	21
MAGL, *MGLL*	*Monoacylglycerol lipase, catabolic enzyme of 2-AG*	14
FAAH2, *FAAH2*	Fatty acid amide hydrolase 2, aka *AMDD*, *FLJ31204*, catabolic enzyme of AEA	9

[a]Protein acronym, gene symbol or synonym that received the greatest number of hits in the GEO profiles database is listed in **bold** print.

factor alpha (TNFα). However, they increase secretion of T-helper2 (Th2), humoral immunity) cytokines (IL-4, IL-5, IL-10). Other subsets of lymphocytes including B cells (MZ, B1a) and natural killer NK cells require eCBs and CB_2 to function properly (Ashton, 2007). *Cannabis* has been described as an adaptogen along with *Echinacea* and other plant products that stimulate resistance to disease and stress (Emboden, 1976). The alkylamide compounds in *Echinacea* are potent agonists of CB_2 (not CB_1); *Echinacea* compounds are not psychoactive because CB_2 is rare in the brain (Raduner et al., 2006).

Clinical aspects of the eCB system

CB_1 receptors have been detected as early as gestational day 2 in mouse embryos, so the eCB system is fully functional at every stage of development (Park et al., 2004). Aspects of the eCB system inform the work of Erich Blechschmidt, a biodynamic embryologist studied by many bodyworkers. Blechschmidt (1977) claimed that the embryo is fully functional at every stage of development. The embryo develops in motion, guided by fluid dynamics, and each motion impacts the development of each subsequent development. Fluids moving in channels establish a matrix, a pressure-generated framework, and this directs the formation of connective tissues (Freeman, 2004). Only after the structure is cast by the fluid forces does genetic expression play a role in the embryonic development. The genes do not *act*, but *react* to the external forces, especially hydrostatic pressures (reviewed in McPartland and Skinner, 2005). If chemistry is structure, then gene transcription (at least its initiation) is mechanotransduction.

Axon migration in the embryonic brain is guided initially by the fluid dynamics, a fluid within a fluid (Newman et al., 1985). Subsequent migration is guided by the genetic expression of UNC5 and EPHA1, which are cell membrane receptors found in the tip of axon growth cones. UNC5 and EPHA1 are activated by ligands (netrins and ephrins) found in the extracellular fluids. The activated receptors begin a signal cascade via FAK and Rho. FAK is a focal adhesion-associated enzyme involved in cellular adhesion. Rho is an enzyme (a GTPase) that regulates intracellular actin dynamics. Together, FAK and Rho direct cytoskeletal dynamics, thereby regulating growth cone motility (Dickson, 2002). The eCBs modulate this cascade (by activating Rho), making eCBs vital ingredients in the chemotropic soup that guides neurons to their destinations (Berghuis et al., 2007).

Biodynamic practitioners claim that the fluid forces that organize embryological development are present throughout our life span, ready for our

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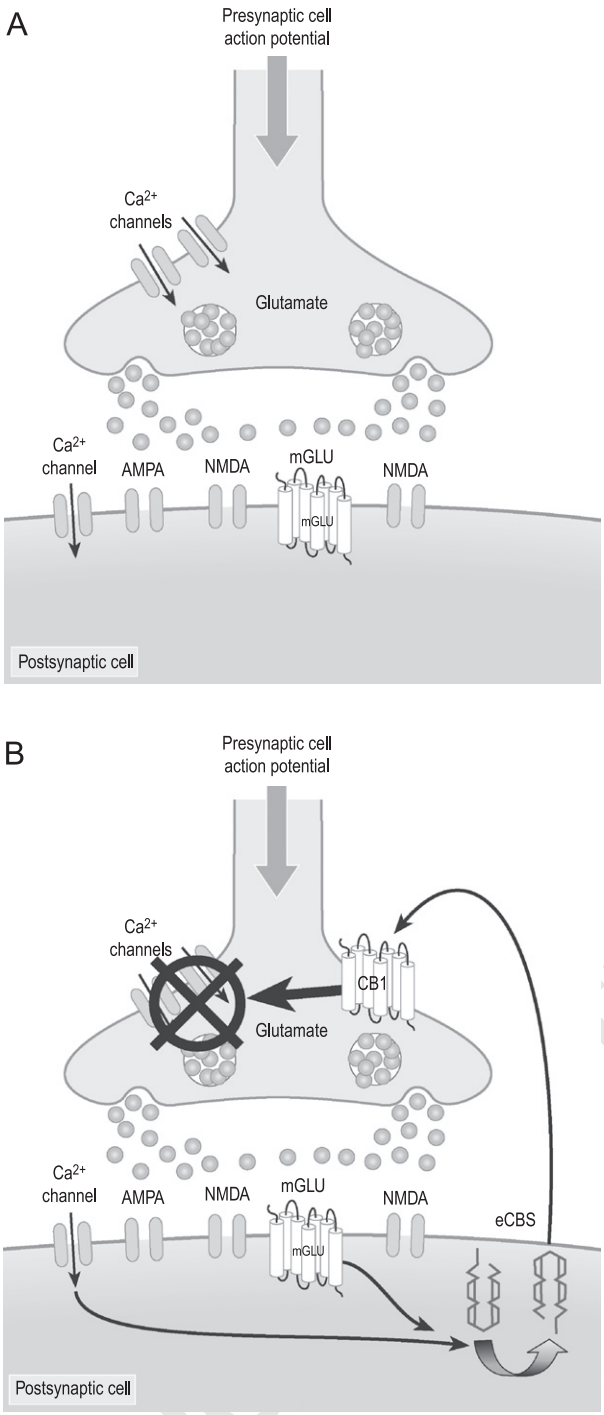

Figure 2 (A and B) The eCB system dampens excessive nociception at the dorsal horn. (A) Persistent firing of a c-fiber nociceptor opens voltage-gated calcium channels (VGCCs) in the presynaptic axon terminal. Calcium influx causes presynaptic vesicles of glutamate to release into the synaptic cleft. Excessive activation and upregulation of glutamate receptors in the postsynaptic cell opens calcium channels and stimulates DAGLα to synthesize 2-AG. (B) Released 2-AG diffuses across the synapse to the presynaptic cell and activates CB_1, the G-proteins from activated CB_1 close VGCCs, thereby halting release of presynaptic glutamate vesicles.

cooperation in harnessing their therapeutic potency. In other words, the forces of *embryogenesis* become the forces of *healing* after birth (McPartland and Skinner, 2005 iom is evoked by the fact that adult neurogenesis by neural stem cells is guided by the aforementioned "embryonic" axon guidance molecules (Koeberle and Bahr, 2004). Adult neural stem cells express CB_1 (Curtis et al., 2006), and neurogenesis by these cells is governed by the eCB system (Aguado et al., 2007).

Effects on neurogenesis and retrograde transmission illustrated in Figure 2 regulate neural plasticity, thereby affecting adaptive learning, emotional memory and nociception pain. Via these mechanisms and others the eCB system provides neuroprotection in Alzheimer's, Parkinson's, Huntington's, multiple sclerosis, seizure disorders and limits infarct size following cerebral ischemia (reviewed in Pacher et al., 2006). The eCB system balances sympathetic–parasympathetic tone, imparts anti-emetic and antihypertensive benefits, and favorably modulates stress in the HPA axis (reviewed in Pertwee, 2005). AEA and 2-AG (as well as THC) are anti-carcinogenic and inhibit tumor growth in breast, prostate, and lung carcinomas, gliomas, melanomas, lymphomas, and other cancers (Guzman, 2003). Cannabinoids induce apoptosis (programmed cell death) in cancer cells via a CB_1-mediated ceramide–caspase pathway. In non-cancer cells, eCBs actually promote cell survival, via the ERK pathway (Guzman, 2003).

Having inventoried this list of benefits, a dysfunctional eCB system may nevertheless cause harm. The autonomic effects of eCBs have been implicated in hemorrhagic and endotoxic shock, cardiac reperfusion injury, doxorubicin-induced cardiotoxicity and advanced liver cirrhosis (Pacher et al., 2006). Mutations in CB_1 and FAAH genes have been linked with obesity and schizophrenia (Pacher et al., 2006), and the genes for DAGLα and NAPE-PLD share an evolutionary signature associated with genes that harbor mildly deleterious alleles and disease-related phenotypes (McPartland et al., 2007).

Levels of AEA in cerebrospinal fluid are increased in schizophrenics, but the elevated levels are *negatively* correlated with psychotic symptoms (Giuffrida et al., 2004). This suggests that abnormal stimulation of post-synaptic D_2 receptors triggers release of AEA and retrograde signaling via CB_1, thus homeostatically attenuating presynaptic dopamine release. Note that CB_1 and CB_2 exposed to high doses of THC become desensitized (transported to intracellular compartments via endocytosis). High doses of THC may therefore provoke psychiatric illness in susceptible individuals by

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desensitizing CB_1 receptors and diminishing retrograde signaling (Giuffrida et al., 2004). On the other hand, cannabidiol, a nonpsychoactive ingredient in cannabis, shows promise as an antipsychotic agent (Zuardi et al., 2006).

Marijuana famously causes the munchies, and this behavior teleologically begins *in utero,* during blastocyst implantation. The blastocyst makes active contact with the endometrium followed by an uptake of nourishment from the endometrial mucosa, an act characterized as the "earliest suckling function" (Blechschmidt, 1977), and blastocyst implantation requires a functional eCB system (Park et al., 2004). When newborn mice are given rimonabant, a drug that blocks CB_1, they stop suckling and die (Fride, 2004).

Obesity leads to excessive production of eCBs by adipocytes, which drives CB_1 activity in a feed-forward dysregulation via ghrelin, leptin and orexin signaling pathways (Matias and Di Marzo, 2007). Last year a pharmaceutical company sought approval of the CB_1 blocker rimonabant (Accomplia®, Zimulti®) for the treatment of obesity. The US Food and Drug Adminstration rejected the drug because subjects in rimonabant studies suffered depressed mood, anxiety, headache, nausea and diarrhea. Given the myriad benefits of a fully functioning eCB system, it should be no surprise that rimonabant unmasked previously silent multiple sclerosis and seizure disorders and doubled the risk for suicidality (Food and Drug Administration, 2007). Complete blockade of CB_1 might approximate the phenotype expressed by genetically engineered "knockout mice." Mice lacking CB_1 suffer increased morbidity and premature mortality, and show greater aggression, epilepsy, age-related neuron loss, anxiogenic-like behavior, depressive-like behavior, anhedonia and fear of newness (Zimmer et al., 1999; Martin et al., 2002).

From a materialistic viewpoint, the aforementioned moods and emotions represent the rhythmic entrainment of synchronously firing CNS neurons. The eCB system (and *Cannabis*) alters consciousness by modulating these biological oscillators (Crystal et al., 2003; O'Leary et al., 2003; Galarreta et al., 2004). This materialistic perspective has been challenged by the neuroscientists, who argue that consciousness is "nonlocal" and not housed within a specific neural substrate (reviewed by Dossey, 2007). This concept echoes Leonardo Da Vinci, who believed consciousness occupied the "void" of the ventricular system, rather than brain parenchyma (Pevsner, 2002). The ventricular system centers another oscillatory phenomenon known as the primary respiratory mechanism (Sutherland et al., 1967). The ventricular "void" is filled with cerebral spinal fluid (CSF), awash with eCBs (Giuffrida et al., 2004). Cells lining the ventricular system express CB_1 and eCB enzymes (Curtis et al., 2006), which modulate the rhythmic production of CSF (Mancall et al., 1985) and control the eCB levels in the CSF (Ashton et al., 2004).

Surprisingly little has been published about the eCB system and fascia. Indeed the very presence of CB_1, CB_2, AEA and 2-AG in fascia-related cells has not been established in the peer-reviewed literature. The investigational purpose of this paper was to search for evidence of the eCB system in fascia-related cells and tissues. The investigation used a bioinformatics approach.

Methods

Bioinformatics experiments are described as *in silico,* rather than *in vitro* or *in vivo* experiments. Bioinformatics is particularly adept with genomic and molecular data. For example, the genomic data that created Figure 1 were downloaded from the Entrez "PubMed" server (www.ncbi.nlm.nih.gov/sites/entrez). The upper left corner of the homepage contains a pull-down menu with "PubMed" as the default item. Instead, "Genome" was selected and gene sequences resembling human CB_1 were sought in other species genomes. Then a freeware application, ClustalX, was used to construct the gene tree (Frazer et al., 2003). The results were published (McPartland et al., 2006).

For the investigation of fascia, "GEO Profiles" was selected from the same pull-down menu. GEO is a repository of data derived from microarray experiments. Microarray ("gene chip") technology uses a robot to apply thousands of droplets of different DNA sequences on a grid. Microarray experiments generate far more data than can be published in a scientific paper. For example, a microarray experiment might identify a disease-related gene by comparing gene expression in the normal versus the diseased cells; thousands of other genes were examined in the study and go unreported in the scientific literature but are deposited in the GEO database.

The GEO Profiles database (www.ncbi.nlm.nih.gov/projects/geo/) was queried by combining an eCB name (e.g., CB_1) and a cell type name. "Fibroblast," the predominant cell type in fascia, was chosen for the search. GEO results are presented as charts with red histograms providing a quantitative *signal value* ("how much of this gene is expressed"). However, the signal value is expressed in arbitrary units and may not be reliable due to variability in the quantity of DNA deposited

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in each droplet and the efficiency of cDNA–mRNA hybridization. Therefore, chip data are secondarily analyzed by a qualitative measure (''has this gene been expressed or not?''). This statistically derived *Detection Call* denotes gene transcripts as ''Present'' (i.e., statistically valid), ''Marginal'' or ''Absent'' (i.e., statistically unreliable and displayed in GEO profile as faded red histograms). Unfortunately, the Detection Call may contradict the signal value; genes marked as absent may have higher quantitative signals than genes marked as present. This discrepancy becomes acute in genes with low expression levels. Expression levels are gauged by blue squares appearing in the red histograms. The blue squares represent the percentile ranked signal value of a gene compared to thousands of genes on that chip. In cases of discrepancy genes with measurable signal values nevertheless marked as absent were counted as present if expression levels came close to the limits of detection (percentile rank $<20\%$). GEO results were statistically analyzed with two online calculators, GraphPad (http://graphpad.com/quickcalcs) and VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html).

Results

The query ''CB_1 and fibroblast'' located 165 GEO profile charts, whereas ''CB_2 and fibroblast'' located 142 charts (Table 1). The signal values in these charts were relatively low, judging by the Detection Call results: signal values in 34 of the 165 CB_1 charts (20.6%) were low enough to be statistically unreliable (i.e., Detection Call: absent). Similarly, signal values in 34 of the 142 CB_2 charts (23.9%) contained only ''Detection Call: absent'' results.

Although signal values for CB_1 and CB_2 varied from one chart to the next, some microarray studies compared CB_1 and CB_2 within the same experiment, making their comparisons more reliable. Signal values of fibroblast CB_1 and CB_2 were directly compared in 21 studies amenable to pooling (i.e., studies using identical microarray platforms and protocols). CB_1 signal values (mean 10.4, SEM 1.4) exceeded CB_2 signal values (mean 6.4, SEM 0.85), a significant difference ($p = 0.0002$, paired t test).

Seven studies, identified in Table 2, directly compared fibroblast signal values to signal values in other cell types. Other microarray experiments reported interesting results:

- CB_1 expression in cardiac fibroblasts (mean 3.2, SEM 1.30) doubled following mechanical stretching (mean 6.3, SEM 1.47), but the difference in this small study ($n = 3$) fell short of significance ($p = 0.075$, paired t test) (GEO accession number GDS1035, CB_2 not tested).
- CB_1 and CB_2 expression in human fibroblasts was similar to CB_1 and CB_2 expression in gorilla and bonobo fibroblasts (GDS340).
- CB_1 expression in synovial fibroblasts (''synoviocytes'') equaled 1.5 (GDS386, an $n = 1$ experiment), which increased 5-fold (to 8.3) following exposure to the inflammatory cytokine TNFα. In the same study, CB_2 levels surprisingly decreased (baseline $= 4.1$, TNFα exposure $= 3.5$). These results conflicted with an analogous $n = 1$ study of synovial fibroblasts (GDS1796), where CB_1 decreased following exposure to IL-1β (baseline $= 16.3$, IL-1β exposure $= 7.5$), but CB_2 slightly increased (baseline $= 6.9$, IL-1β exposure $= 8.9$).
- Skeletal muscle CB_1 expression in the vicinity of the neuromuscular junction (mean 325.4, SEM 106.29) was greater than CB_1 expression in skeletal muscle away from the neuromuscular junction (mean 262.7, SEM 48.40), but the difference in this small study (GDS1838, $n = 4$) was not significant ($p = 0.61$, unpaired t test).

Querying GEO with eCB ligand enzymes located fewer GEO Profile charts, because many microarray experiments did not include gene expression of these enzymes, especially the most recently discovered enzymes, such as FAAH2 (Table 1). Nevertheless, GEO profile chart results demonstrated that all nine AEA- and 2AG-metabolic enzymes were expressed in fibroblasts (Table 1).

Discussion

''Data mining'' describes the process of knowledge discovery or retrieval of hidden information from databases. This study used ''top-down'' data mining, where databases were searched to test a hypothesis (''the eCB system is present in fibroblasts''). In contrast, ''bottom-up'' data mining (dredging databases to generate a new hypothesis) may carry pejorative implications amongst experimental scientists. Nevertheless, bottom-up data mining generates worthwhile results, for example the human genome project.

Data mining of GEO Profiles provided robust evidence of CB_1 and CB_2 expression in fibroblasts and related cell types, such as myofibroblasts and

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Table 2 GEO charts of fibroblast CB_1 and CB_2 signal values that were directly compared to signal values expressed by other cell types.

CB_1 or CB_2	GEO accession number[a], difference between means[b]	Tissue type[c] (mean signal value, SEM, *n* replicates)
CB_1	GDS951, [d]n.s.d.[e]	F_1 (120.2, 20.75, *6*) = A (104.7, 32.35, *6*)
CB_1	GDS1784, n.s.d.[e]	F_2 (117.3, 4.82, *4*) = A (132.3, 5.64, *4*)
CB_1	GDS1298, n.s.d.[e]	F_3 (82.7, 11.11, *36*) = A (79.5, 10.9, *34*)
CB_1	GDS857, [d]n.s.d.[e]	F_4 (7.3, 1.8, *3*) = M (7.3, 1.1, *3*)
CB_1	GDS2091, $p<0.01$[f]	F_3 (67.0, 4.53, *6*) = C (63.9, 9.63, *6*) > O (53.7, 2.69, *6*)
CB_1	GDS1505, $p=0.0026$[e]	F_5 (8.0, 0.31, *12*) >K (6.0, 0.85, *12*)
CB_1	GDS1402, $p<0.01$[f]	S (0.6494, 0.211, *6*) >F_6 (0.20, 0.009, *7*) = SmM (0.19, 0.023, *26*) = Ep (0.19, 0.042, *6*) = End (0.15, 0.044, *16*)
CB_2	GDS951, [d]n.s.d.[e]	F_1 (292.3, 146.66, *6*) = A (263.4, 101.30, *6*)
CB_2	GDS1784, n.s.d.[e]	F_2 (23.8, 0.57, *4*) = A (26.0, 1.61, *4*)
CB_2	GDS1298, n.s.d.[e]	F_3 (119.4, 19.80, *36*) = A (133.7, 20.40, *34*)
CB_2	GDS857, [d]$p=0.0027$[e]	F_4 (5.5, 0.33, *3*) >M (1.3, 0.95, *3*)
CB_2	GDS2091, n.s.d.[f]	F_3 (3.2, 0.79 *3*) = C (4.11, *3*) >O (9.7, 8.63, *3*),
CB_2	GDS1505, n.s.d.[e]	F_5 (7.9, 0.05, *4*) = K (7.8, 0.11, *4*)
CB_2	GDS1402, n.s.d.[f]	SmM (1.67, 0.261, *26*) = S (1.65, 0.541, *6*) = F_6 (1.50, 0.087, *7*) = Ep (1.32, 0.071, *6*) = End (1.31, 0.042, *16*)

[a]GEO profiles accession number.
[b]Means testing: n.s.d., no significant difference.
[c]Tissue types: F_1, mouse embryo fibroblast (C3H10T1/2 preadipocyte); F_2, mouse embryo fibroblast; F_3, mouse embryo fibroblast (NIH-3T3); F_4, mouse corneal fibroblast (NIH-3T3); F_5, human skin fibroblast; F_6, human skin, liver, and lung fibroblasts; C, chondrocyte; End, endothelial cell; Ep, epithelial cell; O, osteoblast; K, keratinocyte; M, myofibroblast; S, stroma cell (connective tissue from heart, breast, prostate, and skeletal muscle); SmM, smooth muscle cell.
[d]A GEO data set whose numerical signal values were statistically unreliable (Detection Call "Absent").
[e]Unpaired *t* test.
[f]One-way ANOVA with *post-hoc* Turkey test.

synoviocytes. This evidence agreed with Bensaid et al. (2003), who detected CB_1 in mouse embryo 3T3-F442A cells, although Bensaid and colleagues described 3T3-F442A cells as "undifferentiated adipocytes," instead of fibroblasts.

Three microarray studies in Table 2 compared fibroblasts and adipocytes, and collectively reported a 1:1 ratio in CB_1 expression between the two cell types. In contrast, Bensaid et al. (2003) reported CB_1 levels in differentiated adipocytes were 4.35-fold greater than those in undifferentiated 3T3-F442A cells. Similarly, Matias et al. (2006) described a 4-fold increase in CB_1 levels after 3T3-F442A cells ("preadipocytes") underwent 4 days of insulin-induced differentiation into adipocytes. However, 8 and 12 days later, there was only a 2-fold difference between the cell types. Most recently (while this paper was in review), a study by Gasperi et al. (2007) reported that 3T3-L1 cells have the machinery to bind, synthesize and degrade AEA, and that their differentiation into adipocytes increases by approximately 2-fold and 3-fold, respectively, the binding efficiency of CB_1 and the catalytic efficiency of FAAH. Engeli et al. (2005) compared spinal cord tissue and adipose tissue, and reported a 1:1 ratio in CB_1 expression (at levels significantly greater than those in other non-neural tissues). We surmise that fibroblast CB_1 levels are significant as well, if the fibroblast-to-adipose CB_1 ratio is 1:1 (Table 2) or 1:2–4 (Bensaid et al., 2003; Matias et al., 2006; Gasperi et al., 2007).

Results with GEO indicated that fibroblasts also expressed AEA- and 2-AG-metabolic enzymes (Table 1). Thus, fibroblast CB_1 and CB_2 may be signaled by eCBs in an autocrine fashion. This evidence was supported by Matias et al. (2006), who extracted AEA and 2-AG from undifferentiated 3T3-F442A cells. Additionally, fibroblast CB_1 and CB_2 may be signaled in a paracrine fashion, by eCBs secreted from neighboring leukocytes, a rich source of AEA and 2-AG (reviewed in Ashton, 2007).

The importance of eCB signaling in fibroblasts can be deduced by studies of other cell types. Fibroblasts share many signal transduction mechanisms with neuronal cells, and the effects of eCBs upon neuronal cells are well known (see FAK and Rho described in the Introduction section). For

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example, fibroblast growth factor (FGF) obviously stimulates fibroblast growth (hence its name). However, FGF also stimulates neuronal growth, and it does so via a CB_1-dependent mechanism (Williams et al., 2003; Aguado et al., 2007). Fibroblasts also share signal transduction mechanisms with cells that migrate or exert traction, such as macrophages (including monocytes and microglia), B- and T-lymphocytes, eosinophils, astrocytes, interneurons, human embryonic kidney 293 cells, HL60 cells, and trabecular meshwork cells. The effects of eCBs upon migration and cytoskeleton activity in these cells are also well known (reviewed in He and Song, 2007). In this case, CB_2 may play a larger role than CB_1 (Gokoh et al., 2005; Scutt et al., 2007).

Within the neuron cell membrane, CB_1 is localized to a scaffolding microdomain known as the "lipid raft" (Rimmerman et al., 2007). This is true for other cell types (Sarnataro et al., 2005; Bari et al., 2007), and probably true for fibroblasts as well. Fibroblast lipid rafts anchor integrins, which are transmembrane receptors that link extracellular ECM ligands (such as collagen and fibronectin) to the intracellular cytoskeleton (Gaus et al., 2006). Integrin receptors transmit signal via intracellular enzymes discussed previously (e.g., FAK, Rac and Rho), and these in turn regulate the actin–microtubule–cytoskeleton system. The integrin-centered cluster of signaling proteins is known as a "focal adhesion," and it regulates fibroblast growth, remodeling and migration (Gaus et al., 2006). It is easy to speculate that focal adhesions are modulated by a mechanism that is FGF- (Abe et al., 2007) and CB_1-dependent (Aguado et al., 2007). Considering the prominence of FGF during embryogenesis (Williams et al., 2003), FGF- and CB_1-induced fascial reorganization is another example of bringing the "embryonic tool kit" back into action to restore health.

CB_1 may affect other aspects of fibroblast function. Fibroblast-like synovial cells exposed to inflammatory TNFα secrete metalloproteinase enzymes, which facilitate articular cartilage destruction (Johnson et al., 2007). Johnson and colleagues decreased metalloproteinase secretion by treating the cells with ajulemic acid (AjA). These authors hypothesized that AjA worked via PPARγ receptors. Our GEO results, however, showed that synovial cells exposed to TNFα had a 5-fold increase in CB_1 levels (GDS386). AjA binds and activates CB_1 (Vann et al., 2007), and CB_1 activation dampens the effects of TNFα (Ashton et al., 2007). Related research has shown that articular cartilage destruction and nitric oxide-induced proteoglycan degradation and collagen breakdown are decreased by AEA (Mbvundula et al., 2005).

Clinical discussion

Practitioners wield several tools that upregulate eCB activity, including bodywork, diet and lifestyle modifications, and pharmaceutical approaches. Many bodyworkers induce "cannabimimetic" changes in their patients, such as anxiolysis, easement of suffering, increased sense of well-being and even euphoria. We conducted a randomized, blinded, controlled clinical trial (McPartland et al., 2005) that measured AEA levels twice, pre- and post-osteopathic manipulative treatment (OMT). The OMT intervention consisted of myofascial release, muscle energy technique, and thrust techniques. OMT subjects experienced cannabimimetic effects (based upon a questionnaire), which correlated with an increase in post-OMT serum AEA levels (more than double pre-OMT levels). Neither cannabimimetic effects nor changes in AEA levels occurred in control subjects. A smaller OMT trial reported little change in AEA levels, but showed significant post-OMT augmentation of *N*-palmitoylethanolamine (PEA), a short-chain analog of AEA (Degenhardt et al., 2007). PEA is discussed below.

The cellular mechanisms underlying OMT have been modeled by *in vitro* stretching of fibroblasts (Dodd et al., 2006). An aforementioned GEO study (GDS1035) used an identical Flexercell apparatus, and reported a doubling of CB_1 expression in fibroblasts following cyclic equiaxial stretching. Speculatively, the stretching of CB_1 may activate the receptor in the absence of ligand. "Constitutive activity," the activation of G-proteins in the absence of ligand, has been measured in CB_1 (reviewed in Howlett et al., 2002). Correspondingly, hydrostatic pressure applied to smooth muscle cells stretches the angiotensin 1 receptor into an active conformation (Zou et al., 2004). Cells that line the cerebral ventricles express CB_1 (Curtis et al., 2006), and these cells may be compressed by hydrostatic pressure generated during the osteopathic CV4 technique, possibly releasing eCBs or directly activating CB_1 (McPartland and Skinner, 2005). Pert (2000) hypothesized that energy therapists heal patients by inducing a vibrational tone that shifts neuroreceptors into constitutively active states, or the vibrational tone triggers release of endorphins that activate the neuroreceptors. Oschman (2000) described crystalline materials within Q2 biological structures that generate piezoelectric fields when compressed or stretched. Examples of crystalline materials applicable to our study in-

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clude the phospholipids that surround CB_1 within cell membranes, and collagen in the ECM that surrounds fibroblasts.

Fibroblasts react to acupuncture needle rotation, a response modulated by Rho and Rac signaling (Langevin et al., 2006). Langevin and colleagues conducted a microarray study, but have not deposited their results in the GEO databank. The eCB system works through Rho and Rac (e.g. Berghuis et al., 2007; He and Song, 2007), and acupuncture may work through the eCB system (Li et al., 2007), rather than the endorphin system as assumed previously (Harbach et al., 2007). Similarly, the eCB system may be responsible for ''runner's high''—running on a treadmill raises serum AEA levels (Sparling et al., 2003; Dietrich et al., 2004). Chronic stress downregulates CB_1 expression, so stress reduction may enhance the eCB system (Hill et al., 2005). Acute ethanol ingestion decreased AEA and 2-AG in most brain regions (Gonzales et al., 2002), and chronic ethanol downregulated CB_1 expression (Ortiz et al., 2004).

Dietary inclusion of fish oils containing DHA (docosahexaenoate 22:6 w-3) and other polyunsaturated fatty acids increased AEA and 2-AG levels in the brain (Berger et al., 2001; Watanabe et al., 2003). Oral administration of *Lactobacillus* upregulated CB_2 in intestinal epithelial cells, and relieved symptoms of irritable bowel syndrome (Rousseaux et al., 2007).

Turning to pharmaceuticals, acetaminophen (paracetamol) is converted into *N*-arachidonoylphenolamine by the liver, a compound that activates CB_1 (Högestatt et al., 2005). Ibuprofen and other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX2, an enzyme that breaks down 2-AG. So NSAIDs may prolong 2-AG activity. NSAIDs also inhibit FAAH and therefore enhance AEA activity (Fowler, 2004). The tricyclic antidepressant desipramine increased CB_1 levels in the brain (Hill et al., 2006), whilst fluoxetine decreased CB_1 expression (Oliva et al., 2005). THC and cannabidiol may widen their own therapeutic windows by increasing AEA levels, and THC surprisingly upregulated CB_1 expression when administered acutely (reviewed in McPartland and Guy, 2004). Adelmidrol, a synthetic analog of PEA, has been topically applied to improve wound healing in animals (Panagiotis et al., 2007). Ultrasound sonification of adelmidrol gel also showed efficacy in the treatment of lateral epicondylitis (Sioutis et al., 2004).

Fibromyalgia, a disorder involving diffuse myofascial pain, may be a syndrome of eCB deficiency (Russo, 2004). During the menstrual cycle, AEA decreases during the luteal phase (circa day 21) and rises during the follicular phase (circa day 10), due to the progesterone-induced upregulation of FAAH (enzyme that breaks down AEA) in the luteal phase. In a study of healthy women with normal menstrual cycles, the decrease in AEA corre-

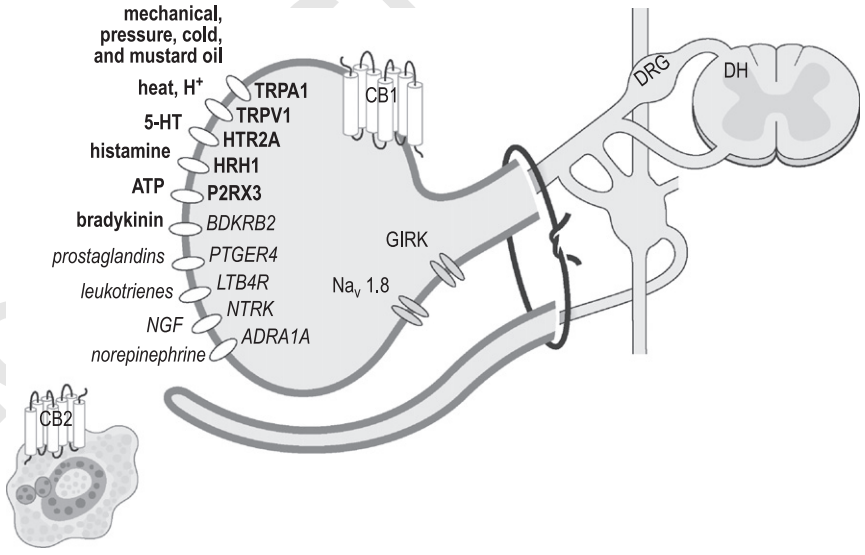

Figure 3 Schematic illustration of a polymodal c-fiber nociceptor, with its proximal terminal in the dorsal horn (DH), cell body in the dorsal root ganglion (DRG), and an enlarged view of the distal terminal. A suture loop separates the enlarged view from the rest of the nociceptor. Below the nociceptor is a peripheral sympathetic postganglionic neuron. Within the distal terminal are five receptors for activators (regular font) and five receptors for *sensitizers* (in *italics*), named by their gene symbols. Also embedded in the distal terminal are two ion channels ($Na_v1.8$ and GIRK) and CB_1. A lymphocyte expressing CB_2 is nearby.

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sponded with hypersensitivity to algometer-induced pressure pain during the luteal phase. Several subjects "changed" fibromyalgia diagnosis during the course of a menstrual cycle, fulfilling the tender point criterion (tenderness $\leqslant$4 kg at $\geqslant$11 points) during the AEA-deficient luteal phase or menstrual phase, but never during the AEA-rich follicular phase (Dunnett et al., 2007).

Myofascial pain is a common reason why patients self-medicate with cannabis (Ware et al., 2005). This fact led us to hypothesize that myofascial trigger points (MFTrPs) were endowed with CB_1 receptors (McPartland and Simons, 2007). The etiology of MFTrPs has been attributed to abnormal acetylcholine-related depolarization of motor endplates (i.e., the neuromuscular junction), followed by release of inflammatory cytokines (Mense et al., 2003; Shah et al., 2005). Our hypothesis that MFTrPs were endowed with CB_1 receptors was supported by a GEO study (GDS1838) that showed greater CB_1 levels in skeletal muscle near the neuromuscular junction. Two new papers confirmed our results (Newman et al., 2007; Sánchez-Pastor et al., 2007), showing that CB_1 activation in motor endplates dampened acetylcholine release.

Myofascial dysfunction may recursively loop into eCB system dysfunction: CB_1 receptors in a nociceptor are synthesized in the dorsal root ganglion and carried by axoplasmic flow to insertion sites in the distal terminal of the nerve (Figure 3). In the distal terminal, CB_1 activity dampens the activity of activators and sensitizers. CB_1 activity closes Na^+ channels and opens K^+ channels, hyperpolarizing the nociceptor (keeping it from firing), and preventing peripheral sensitization and hyperalgesia (Agarwal et al., 2007). However, mechanical barriers that restrict axoplasmic flow will prevent CB_1 receptors from reaching the distal terminal (Hohmann and Herkenham, 1999). The ligation loop in Figure 3 represents carpal tunnel syndrome, thoracic outlet restriction, piriformis syndrome, or any other mechanical barrier that bodyworkers treat and eliminate. This restores axoplasmic flow, facilitating CB_1 transport to its peripheral site of action.

Conclusions

The eCB system exemplifies the osteopathic concept that we possess self-regulatory mechanisms that are self-healing in nature. The overall role of the eCB system can be summarized as "resilience to allostatic load," a phrase synonymous with health. The eCB system dampens nociception and pain, decreases inflammation in myofascial tissues and plays a role in fibroblast reorganization. Understanding of the modulation of CB_1, CB_2 and eCBs represents new approaches for practitioners to treat a variety of structural and functional disorders.

Uncited references

Gary-Bobo et al., 2006; Robbe et al., 2006.

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