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A PRELIMINARY MODEL FOR THE PROTECTIVE ROLE OF THE ENDOCANNABINOID 2-ARACHYDONYLGLYCEROL IN NEUROINFLAMMATION

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14. ABSTRACT
Activation of cannabinoid receptors CB1 (in neurons) and CB2 (in immune cells) by their primary endogenous ligand 2-arachidonylglycerol (2-AG), is both anti-excitatory and anti-inflammatory. Levels of 2-AG are tightly maintained by monoacylglycerol lipase (MAGL), which converts 2-AG into arachidonic acid (AA), and may be the main regulator of AA. Both AA and 2-AG are precursors of inflammatory prostaglandins via cyclooxygenase-2 (COX2) metabolism, which is in turn inhibited by 2-AG binding to neuronal CB1 receptors. Selective MAGL inhibitors have the potential to reduce inflammation. Conversely, excessive MAGL inhibition could lead to immune suppression and an ineffective response to infection. We developed a model of 2-AG metabolism and signaling which describes prostaglandin (PG) production, MAGL activity, 2-AG synthesis rate, COX2 activity, neuronal CB1 receptor densities, and binding of 2-AG to CB1 receptors. The model is used to describe the effect of MAGL inhibitor JZL-184 on 2-AG, AA, and prostaglandin levels as a result of exposure to lipopolysaccharides (LPS) and organophosphates. The model can be extended to include 2-AG binding to CB2 receptors in immune cells (modulating cytokine release), and thereby describe a hypothetical lumped immune response to a biological agent. Model simulations of normal immune responses show removal of the infectious agent with little "overshoot" of immune cells (inflammation). Reducing the immune response by inhibiting MAGL activity may result in ineffective removal of the agent, while increasing the immune response may lead to rapid removal of the agent at the expense of a potentially damaging immune overshoot indicative of chronic inflammation.

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PREFACE

Funding for this project was provided through the Defense Threat Reduction Agency (DTRA) contract 2G806-08-AHB-C. This research was conducted under contract FA8650-10-2-6062 with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF). The program manager for the HJF contract was David R. Mattie, PhD (711 HPW/RHDJ), who was also the technical manager for this project.

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1.0 SUMMARY

Both experimental and clinical studies suggest that inappropriate activation of the inflammatory response participates in the development of diseases characterized by excessive production of cytokines. Inappropriate cytokine synthesis may stimulate excessive inflammatory reactions, causing damage to healthy regional and peripheral tissues and organs. It is therefore not surprising that organisms have several mechanisms regulating the intensity of inflammation. One of these involves the modulation of receptors associated with cannabinoid-binding receptor systems. Activation of cannabinoid receptors, CB1 (in neurons) and CB2 (in immune cells) by their primary endogenous ligands anandamide (AEA) and 2-arachidonylglycerol (2-AG), yield both anti-excitatory and anti-inflammatory properties. Basal levels of AEA and 2-AG are reported to be in the pM and nM ranges, respectively (Blankman *et al.*, 2007). These low levels are tightly maintained by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), which respectively converts AEA and 2-AG into arachidonic acid (AA). The hydrolysis of 2-AG may be the main contributor to AA levels. 2-AG is a precursor of inflammatory prostaglandins via cyclooxygenase-2 (COX2) metabolism, which is in turn inhibited by 2-AG binding to neuronal CB1 receptors. Selective MAGL inhibitors are attracting much attention as they have the potential to reduce inflammation. The net effects from pharmacologically inhibiting MAGL result not only from changes in endocannabinoid levels, but also from the levels of their metabolites and the effect they have on their function, as well as other pathways that may be up- or down-regulated. For example, while AA is typically considered the main precursor of inflammatory prostaglandins via oxidative conversion by COX2, 2-AG has also been found to be a direct substrate for COX2 metabolism. The latter would imply that elevated endocannabinoids could result in an increase in inflammatory prostaglandins. Yet recent evidence suggests that elevated 2-AG suppresses COX2 expression via activation of the CB1 receptor.

In order to understand these complex interactions, we developed a preliminary model of 2-AG metabolism and signaling, with prostoglandin (PG) production as a model output. The model includes:

- MAGL activity, leading to 2-AG degradation to AA;
- 2-AG synthesis rate;
- COX2 activity, leading to prostaglandin E2 (PGE2) production from both AA degradation, and directly from 2-AG;
- CB1 receptor densities on the neurons, modulating COX2 activity; and
- 2-AG binding dynamics to neuronal CB1 receptors.

The model is used to describe experimental data for the effect of the MAGL inhibitor JZL-184 on 2-AG, AA, and prostaglandin levels as a result of exposure to lipopolysaccharides (LPS) and organophosphates (OPs).

The model has been extended to include 2-AG binding to CB2 receptors on immune cells (modulating cytokine release), thereby describing a hypothetical lumped immune response (e.g., to an infectious agent). The extended model includes:

- CB2 receptor densities on the immune cells (macrophages or astrocytes) modulating immune cell recruitment via cytokine release;

- 2-AG binding to CB2 receptors;
- Proliferation of the infectious agent; and
- Removal of the agent by the immune cells.

Model simulations of normal and effective immune responses show removal of the infectious agent with little "overshoot" of immune cells (inflammation). Reducing the immune response (e.g., by inhibiting MAGL activity) may result in the ineffective removal of the agent, while increasing the immune response may lead to rapid removal of the agent, but at the expense of a potentially damaging immune overshoot indicative of chronic inflammation. Both experimental and clinical studies suggest that inappropriate activation of the inflammatory response participates in the development of diseases characterized by excessive production of cytokines. Inappropriate cytokine synthesis may stimulate excessive inflammatory reactions causing damage to peripheral tissues and organs.

Under normal conditions, regulation of the immune response is also achieved by other means. One such mechanism involves the so-called inflammatory reflex, mediated by the vagus nerve, which inhibits the production of cytokines by macrophages via the cholinergic anti-inflammatory pathway. Chronic exposure to cholinesterase inhibitors such as OP pesticides and nerve agents may render this pathway less effective in appropriately terminating the immune response because of down-regulation of nicotinic acetylcholine receptors on the macrophage. Under these conditions, the anti-inflammatory activity of the cannabinoid system may be even more critical to balance overall inflammatory response activities.

2.0 INTRODUCTION

The inflammatory reflex adjusts the intensity and duration of immune system reactions according to actual needs, thus protecting an organism from tissue damage induced by excessive inflammation. Both experimental and clinical studies suggest that inappropriate activation of the inflammatory reflex participates in the development of diseases characterized by excessive production of cytokines. Inappropriate cytokine synthesis may stimulate excessive inflammatory reactions, causing damage to peripheral tissues and organs. It is therefore not surprising that organisms have several balancing mechanisms regulating the temporal intensity of inflammation, including the inflammatory reflex of the vagus nerve (see Section 5.0 below) and the endocacannabinoid system.

The endocannabinoid system plays a pivotal role in stabilizing brain excitability and reducing inflammation (Wallace *et al.*, 2003). This system consist of two known heterotrimeric $G_{i/o}$ coupled G-protein receptors (GCPR), neuronal and immune cell cannabinoid receptors (CB1 and CB2, respectively), their endogenous lipid ligands or "endocannabinoids", and the enzymes responsible for their synthesis and degradation (Howlett, 2005; Pertwee, 2005). CB1 is predominantly expressed in the nervous system and CB2 is primarily found in immune cells throughout the body (Herkenham *et al.*, 1991). Arachidonylethanolamide (anandamide; AEA) and 2-arachidonoyl glycerol (2-AG) are the most biologically active currently investigated endocannabinoids. Unlike classical neurotransmitters, which are stored in vesicles, AEA and 2-AG are synthesized by the action of N-acylphosphatidyl specific phospholipase D (PLD) and diacylglycerol (DAG) lipase, respectively, on membrane phospholipids upon intense synaptic activity at the post-synaptic membrane (Basavarajappa, 2007). They then migrate in a retrograde fashion to the presynaptic membrane where they bind and activate the CB1 receptor, which is predominantly localized on presynaptic axon terminals (Figure 1).

CB1 activation leads to decreased cAMP production, inhibition of N, P/Q N-Type voltage-gated Ca^{2+} channels (VGCCs), as well as activation of inward rectifier potassium currents, and thereby modulating the release of various neurotransmitters and stabilizing excitability. Both AEA and 2-AG bind to and activate CB1 and CB2; however, AEA is a weaker agonist than 2-AG at CB1 and is only a partial agonist at CB2. As a result, 2-AG has been proposed as the true endogenous ligand for both subtypes of CB receptor (Mackie, 2006).

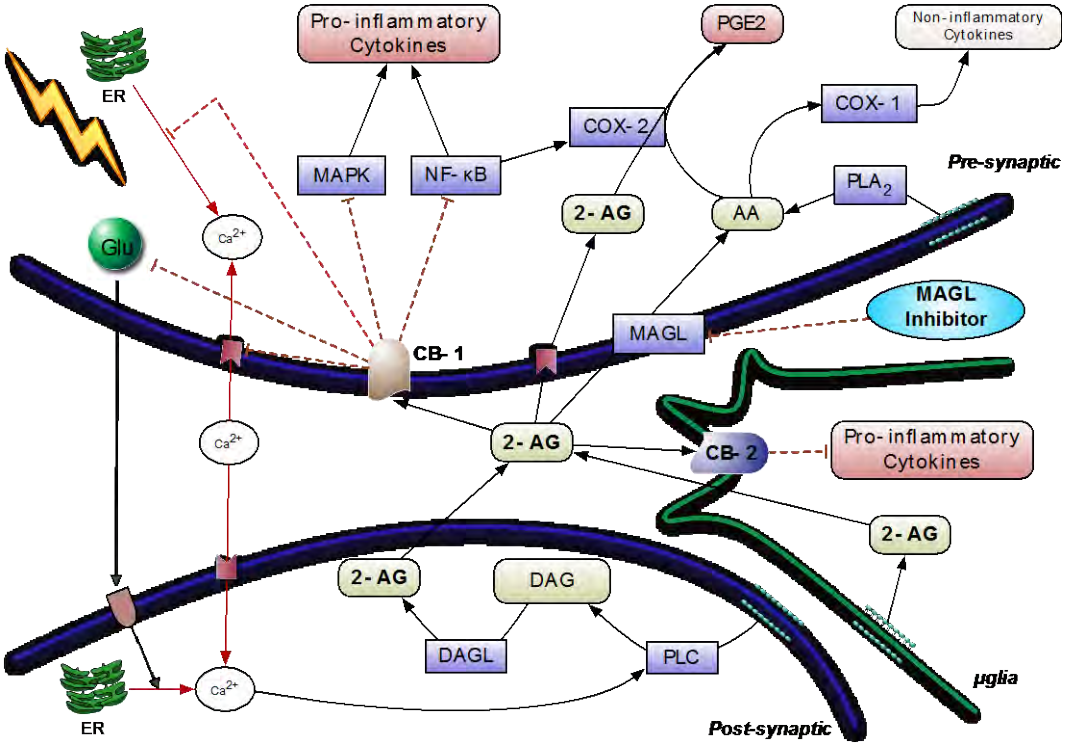

Figure 1. Illustration of Retrograde Signaling by 2-AG to the CB1 Receptor. Rapid Ca^{2+} influx from stressors causes an increased release of 2-AG (and AEA, not shown), which are produced from membrane phospholipids via diacylglyerol lipase (DAGL) (and phospholipase C (PLC)). 2-AG (and AEA) activate the CB receptors CB1 and CB2, resulting in reduced release of neurotransmitters, thereby reducing membrane excitation. In addition, CB receptor activation inhibits mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which are drivers in the production of inflammatory cytokines. NF-kB inhibition also results in decreased COX2 activity and production of inflammatory prostaglandins. 2-AG and phospholipids themselves are degraded by MAGL into AA and glycerol, respectively. 2-AG is also a direct substrate for COX2. Elevated levels of 2-AG suppress NF-kB phosphorylation and COX2 expression through the activation of the CB1 receptor.

The activities of 2-AG and AEA are terminated by their removal from the extracellular space. Little is known about the membrane transport of either endocannabinoid. A putative bidirectional transporter has been suggested by Chicca *et al.* (2012) through the identification of two inhibitors which block both the release and uptake of 2-AG and AEA. Uptake of these lipid compounds may involve different mechanism, including rate-limited diffusion, a putative membrane transporter, or lipid raft endocytosis depending on the cell type. But, once across the membrane, AEA is degraded into arachidonic acid (AA) and ethanolamine by fatty acid amide hydrolase (FAAH), which is bound intracellularly to the membrane. Monoacylglycerol lipase (MAGL), which is cytosolic, degrades 2-AG into AA and glycerol (Hashimotodani *et al.*, 2007) (Figure 2). However, alternative endocannabinoid metabolic pathways which result in AA degradation have been found to exist. For example, Blankman *et al.* (2007) used a functional

proteomic approach to comprehensively map 2-AG hydrolases in the mouse brain. Their data reveal that 85 percent of brain 2-AG hydrolase activity can be ascribed to MAGL, and that the remaining 15 percent is mostly catalyzed by two uncharacterized enzymes, abhydrolase domain containing 6 (ABHD6) and abhydrolase domain containing 12 (ABHD12). The production of AA from these pathways is physiologically important, as AA is the main substrate for cyclooxygenase-1 and cyclooxygenase-2 (COX1and COX2), which catalyze oxidative conversion of AA to prostaglandins, such as PGE2 (Figure 2). COX1 is constitutively expressed in most tissues and is considered a "house-keeping enzyme", shown involved in the maintenance of normal stomach lining and platelet function. COX2 is inducible by various factors including growth factors, cytokines, and tumor promoters and is seen in response to stress and pathological processes.

Another enzymatic pathway, similar to those responsible for the synthesis of endocannabinoids, is that of phospholipase A2 (PLA2); this pathway is also upregulated upon intense synaptic activity. PLA2 catalyses the first step in the formation of arachidonic acid by lysing AA from the sn2 position of membrane phospholipids and, in a calcium dependent manner, generating AA and lysophospholipids (Figure 2). It was conventionally believed that cytosolic PLA2, acting on membrane phospholipids, was the rate controlling enzyme in AA production. However, while studying targets of various organophosphates, Nomura *et al.* (2008) found that FAAH and MAGL were fully inhibited by isopropyl-dodecylfluorophosphonate (IDFP), an insecticide homolog of nerve agent, sarin. They also discovered found mice exposed to low levels of IDFP displayed large increases in AEA and 2-AG, as well as cannabimetic behavior. More interestingly, AA levels in the mice were diminished proportionately to the increase in 2-AG, suggesting that 2-AG and its hydrolysis by MAGL, in particular, may play a greater role in regulating free AA than PLA2.

AEA and 2-AG also are known to serve directly as substrates for COX2, which oxidizes them as readily as it oxidizes AA, directly into prostaglandin E2 ethanolamide (PGH2-EA) and prostaglandin E2 glycerol (PGH2-G), respectively (Kozak *et al.*, 2004). These prostaglandin quickly rearrange into the inflammatory, PGE2. However, it has been shown that elevated levels of 2-AG, as seen following environmental stressors, suppress COX2, but not COX1 expression via activation of the CB1 receptor (Zhang and Chen, 2008; Du *et al.*, 2011) (Figure 2). This antagonism between 2-AG and COX2 introduces another feedback mechanism, which may shift the AA pathway away from an inflammatory response (namely reduced COX2 activity and PGE2), given 2-AG levels are elevated enough to activate CB1. Suppression of COX2 expression would negatively impact the production of inflammatory prostaglandins from AA.

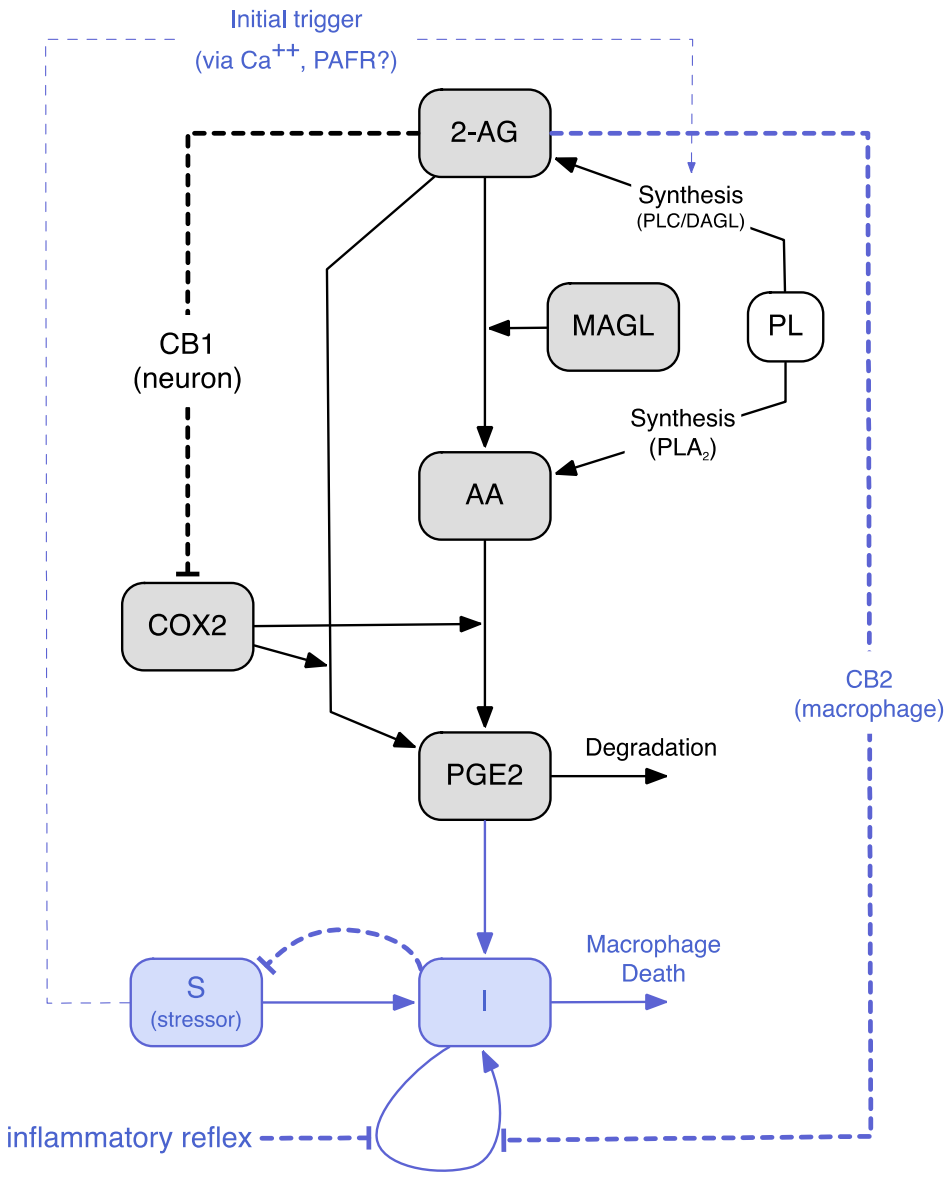

Figure 2. Schematic of the Mathematical Model for the Effect of 2-AG and its Metabolites on Neuroinflammation. 2-AG is metabolized by MAGL to AA, and also inhibits COX2 mediated metabolism of AA and itself to PGE2 via neuronal CB1 receptors. The grayed-out sections represent the gross response of the immune system to a stressor S (which may reproduce, such as a bacterium or virus). The immune response (shown as blue) is envisaged as a population of immune cells I that reduces the stressor population while at the same time self-recruiting (via cytokine secretion). 2-AG modulates the behavior of this immune response by modulating the magnitude of self-recruitment (via the CB2 receptors on immune cells).

The links between excessive AA production and upregulation of COX2 with disruptions of the endocannabinoid system are seen in various neuropathologies. For example, massive levels of FAAH have been found in astrocytes surrounding neuritic plaques in post-mortem brains of

Huntington's disease patients, whereas in healthy control brains, FAAH activity was not detected (Benito *et al.*, 2003). Conceivably the excessive degradation of AEA by the over expression of FAAH in astrocytes could result in a significant production of AA and related pro-inflammatory substances. Marked increases in COX1, COX2, and FAAH have been found in the brains of patients with Alzheimer's Disease (AD); these levels correlate with the number of neuritic plaques, with neuronal atrophy, and with increased levels of PGE2 (Pasinetti and Aisen, 1998; Phillis *et al.*, 2006). MAGL has been suggested as a therapeutic target for AD where Chen *et al.* (2012) showed that inactivation of MAGL, using a highly selective inhibitor, robustly suppressed production and accumulation of β-amyloid associated with reduced expression of β-site amyloid precursor protein cleaving enzyme 1 (BACE1) in a mouse model of AD. The MAGL inhibition also prevented neuroinflammation, decreased neurodegeneration, maintained integrity of hippocampal synaptic structure and function, and improved long-term synaptic plasticity, spatial learning, and memory in the AD animals (Chen *et al.*, 2012). In terms of prevention and disease progression, MAGL inhibition potentially may be considerably more effective for therapy than currently approved drugs for AD, which only mildly improve symptoms for a rather short period.

Centonze *et al.* (2007) found that AEA, but not 2-AG, was increased in the cerebrospinal fluid of relapsed multiple sclerosis (MS) patients. AEA concentrations were also higher in peripheral lymphocytes of these patients, indicating an effect associated with increased synthesis and reduced degradation of AEA in part by FAAH. COX2 has also been found to be extensively expressed in MS lesions, suggesting that regulatory variants of the COX2 gene or possible modulation of its expression by the endocannabinoid system (possible via CB1) may be implicated in MS (Mazzola *et al.*, 2007).

3.0 MODEL DEVELOPMENT

Mathematical models can be used to explore the dynamics of systems such as the role of 2-AG in modulating neuroinflammation. In this case, the purpose of such a model is to embody a specific detailed hypothesis for the mechanism involved. Its behaviors under different circumstances then become tests of that hypothesis, and the model (hypothesis) can be modified depending on how well it performs *vis-à-vis* various experimental and observational data.

Such a model will be, of necessity, preliminary; the limited available experimental data are not sufficient to fully parameterize a validated model at this stage. That is not to say, however, that the model is developed in a data vacuum. Quite the contrary, existing experimental data are used in two ways. First, as discussed in Section 2.0, the basic structure of the model, in terms of interacting metabolic and signaling pathways, is distilled from observed data. Second, although initially assigned arbitrarily, parameter values are modified to allow the model to mimic observed data behavior under numerous specific conditions. In this way, although the absolute parameter values remain unverified, their relative values may be effectively constrained.

Figure 2 shows a schematic of our simplified model, based on the considerations outlined in Section 2.0. 2-AG (rather than AEA) is the focus of the model, since it appears to be the primary endogenous ligand for both subtypes of CB receptor (Mackie, 2006). Further, 2-AG and its

hydrolysis by MAGL may be the main regulators of free AA (Nomura *et al.*, 2008). A similar model could be developed for AEA to complement the model described herein.

The preliminary model attempts to incorporate the essential features of the processes described above, and exhibit appropriate behavior under a variety of conditions. The model is semi-quantitative in the sense that it is not parameterized against experimental data. However, the parameters have been optimized against each other in order to elicit appropriate behaviors. The model outputs PGE2 levels, which are assumed to initiate an immune response. The main portion of the model (black areas in Figure 2) includes the following processes and parameters:

- MAGL activity, leading to 2-AG degradation to AA;
- 2-AG synthesis rate;
- COX2 activity, leading to PGE2 production from both AA degradation, and directly from 2-AG;
- CB1 receptor densities on the neurons, modulating COX2 activity; and
- Binding of 2-AG to neuronal CB1 receptors.

The 2-AG concentration is determined by the difference between its zero order basal synthesis rate Syn_{2AG} and saturable hydrolysis by MAGL:

$$\frac{d[2AG]}{dt} = Syn_{2AG} - \frac{k_{cat}^{MAGL}[MAGL]\cdot[2AG]}{[2AG]+K_{MAGL}} - \frac{k_{cat}^{COX2}[COX2]\cdot[2AG]}{[2AG]+K_{COX2}} \qquad \text{(Equation 1)}$$

… where k_{cat}^{MAGL} and K_{MAGL} are the catalytic rates and half-saturation constants for 2-AG hydrolysis. Note that Syn_{2AG} is rapidly triggered on demand, and responds to the stressor, perhaps through abnormal intracellular Ca^{2+} levels. Concentrations of subsequent metabolites AA (by COX2) and PGE2 are determined similarly:

$$\frac{d[AA]}{dt} = \frac{k_{cat}^{MAGL}[MAGL]\cdot[2AG]}{[2AG]+K_{MAGL}} - \frac{k_{cat}^{COX2}\cdot[COX2]\cdot[AA]}{[AA]+K_{COX2}} + Syn_{AA} \qquad \text{(Equation 2)}$$

$$\frac{d[PGE2]}{dt} = \frac{k_{cat}^{COX2}\cdot[COX2]\cdot[AA]}{[AA]+K_{COX2}} + \frac{k_{cat}^{COX2}[COX2]\cdot[2AG]}{[2AG]+K_{COX2}} - Deg_{PGE2}\cdot[PGE2], \qquad \text{(Equation 3)}$$

where Deg_{PGE2} is the first-order degradation rate of PGE2. COX2 is inhibited by 2-AG via the CB1 receptor in neurons. We assume the following simple form for this inhibition:

$$[COX2] = [COX2]_0\left(1-\frac{k_{CB1}[CB1]\cdot[2AG]}{[2AG]+K_{CB1}}\right). \qquad \text{(Equation 4)}$$

Here $[COX2]_0$ is the maximum effective COX2 concentration, which is reduced by the degree of binding of 2-AG to the CB1 receptors. [*CB1*] is the concentration of CB1 receptors, and K_{CB1} is the concentration of 2-AG at which half these receptors are occupied. k_{CB1} determines the impact of 2-AG binding in reducing the "effective" COX2 concentration.

Our model can be extended to simulate a gross immune response to a biological stressor (blue areas in Figure 2). We assume that 2-AG modulates the behavior of the immune response by modulating the magnitude of the macrophage self-recruitment parameter k_3. 2-AG binds to the CB2 receptor on the macrophage, and the rate of decrease of k_3 (from its initial value of k_3^0) is proportional to the number of occupied receptor sites:

$$k_3 = k_3^0\left(1-\frac{k_{14}[CB2]\cdot[2AG]}{[2AG]+K_{CB2}}\right), \qquad \text{(Equation 5)}$$

where K_{CB2} is the AG concentration at which half the sites are occupied, and k_{14} determines the strength of the impact of the CB2 signaling pathway on reducing cytokine production and macrophage recruitment. Note that when all CB2 sites are occupied (i.e., large *[AG]* values), k_3 is reduced to $k_3^0(1-k_{14}.[CB2])$.

The macrophage concentration (degree of inflammation) is given by:

$$\frac{dI}{dt} = k_2 S - k_4 I + k_{13}\cdot[PGE2] + k_3 I \qquad \text{(Equation 6)}$$

A stressor S may be any process that initiates an immune response I, including a bacterial or viral infection (with replication rate k_5). The immune response is envisaged as a population of immune cells that reduces the stressor population at a rate k_1. In addition, this population of cells self-recruits (via cytokine secretion) with a rate constant k_3, and die at a rate k_4:

$$\frac{dS}{dt} = -k_1 I + k_5 S \qquad \frac{dI}{dt} = k_2 S + k_3 I - k_4 I \qquad \text{(Equation 7)}$$

The model is implemented in Berkeley Madonna™, and the code is given in the Appendix.

4.0 RESULTS

Figure 3 shows the time-courses of 2-AG, AA, and PGE2 predicted by the model as a result of MAGL activity being reduced by 80 percent. The effect of MAGL inhibition is predicted to increase the rate of 2-AG prediction, while reducing the rate of accumulation of AA as shown in

Figure 3. PGE2 levels rise to a peak, and are then reduced by inhibition of COX2 mediated synthesis from AA by 2-AG acting through the CB1 receptor on the neurons (see Figure 2). The height of this peak is significantly reduced by MAGL inhibition.

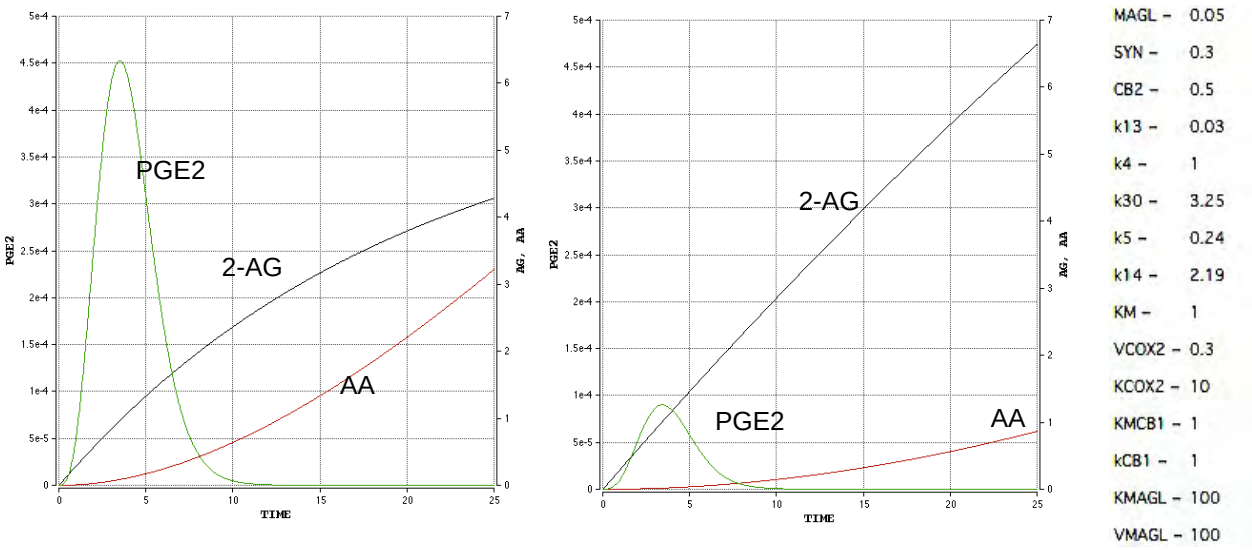

Figure 3. Model Simulations of 2-AG, AA, and PGE2 as MAGL Levels are Reduced by 80 Percent from 0.05 (left) to 0.01 (right) (arbitrary units). Note that as 2-AG levels begin to ramp up, PGE2 levels rise rapidly. Almost immediately, COX2 mediated synthesis from AA is inhibited by 2-AG via CB1 receptors in the neurons. Model parameters used are listed on the right.

Figure 4 shows the dependence on MAGL of the final simulated values of 2-AG and AA (at time 25, arbitrary units), as well as the peak PGE2 values. Nomura *et al.* (2011) have measured changes in 2-AG, AA, and PGE2 levels following induction by the MAGL inhibitor JZL-184 (40 mg/kg via intraperitoneal (ip) injection in mice), both with and without lipopolysaccharide (LPS, 20 mg/kg ip administered 2 to 6 hours prior to measurement). The authors observed that JZL-184 increased brain 2-AG by about 4.3-fold, and decreased AA by about 72 percent of its original value, regardless of LPS administration. LPS increased overall PGE2 levels, and JZL-184 reduced those levels by 76 and 88 percent in the presence and absence of LPS, respectively. Assuming an initial value of MAGL in the model of 0.5 (arbitrary units), these measured changes in 2-AG, AA, and PGE2 levels correspond to inhibition of MAGL by 75 to 95 percent (colored arrows and blue broken lines in Figure 4), which shows that the model is internally consistent with regards to this particular dataset.

Organophosphates themselves have been observed to inhibit MAGL. For example, Liu *et al.* (2013) measured the effect of chlorpyrifos (280 mg/kg via subcutaneous (sc) injection) and parathion (27 mg/kg sc) in rats on 2-AG levels 4 days after dosing. They found increases of about 80 and 16 percent, respectively. If we assume the same model conditions as in Figure 4, these changes correspond to degrees of inhibition of MAGL of about 53 and 20 percent,

respectively (see black triangles in Figure 4). Note that these predictions of MAGL inhibition depend on the specific parameter values in our model, and are subject to change as our model is further parameterized.

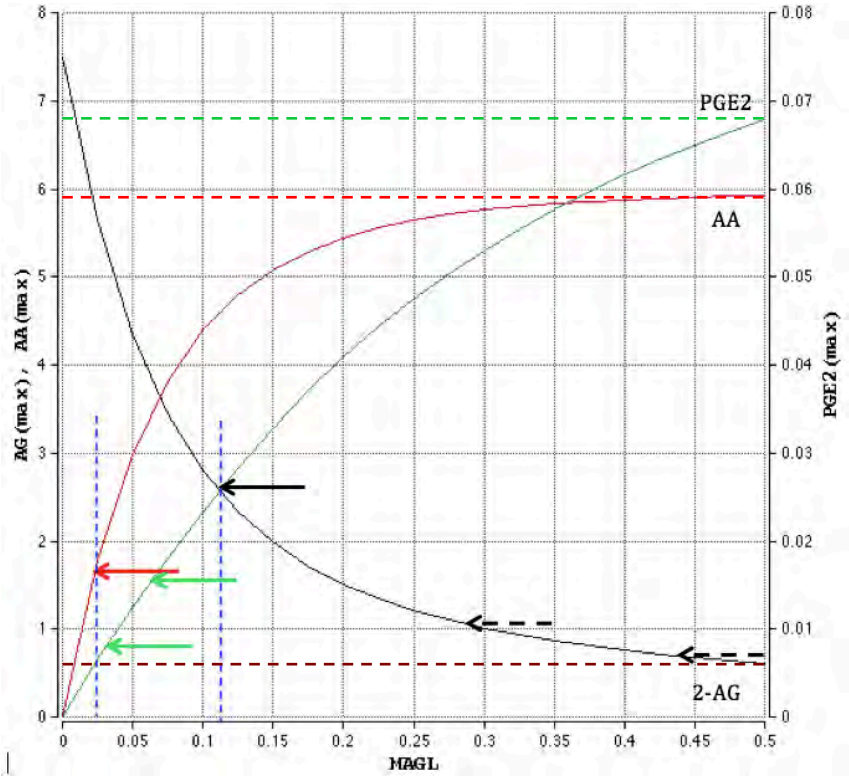

Figure 4. Plot of 2-AG (black) and AA (red) Levels at Time = 25 (arbitrary units) and Peak PGE2 Levels (green) as a Function of MAGL Concentration. Conditions are similar to those in Figure 3. Arrows indicate reductions in MAGL necessary to produce the changes in 2-AG, AA, and PGE2 levels from control values (at MAGL = 0.5, horizontal dotted lines). Solid arrows indicate effects induced by the MAGL inhibitor JZL 184 (40 mg/kg ip) in mouse brain, as observed by Nomura *et al.* (2011, Figure 1B), where 2-AG was observed to increase about 4.3-fold, and AA to decrease by about 70 percent (both in the presence and absence of LPS). JZL 184 caused PGE2 levels to decrease by about 76 and 88 percent, respectively, in the presence and absence of LPS. These results are all consistent with 75 to 95 percent MAGL inhibition by JZL 184 (vertical blue dotted lines) (Nomura *et al.*, 2011). Black dashed arrows represent increases of rat extracellular hippocampal 2-AG (above control, brown broken line) following exposure of rats to the organophosphates (OPs) chlorpyrifos (280 mg/kg, sc) and parathion (27 mg/kg, sc), and correspond to 53 and 20 percent predicted inhibition of MAGL, respectively (data as presented by Pope *et al.*, 2010).

Figure 5 shows the hypothetical model response I (i.e., immune cell number, or extent of inflammation) to stimulation by a reproducing stressor S (e.g., a bacterium or virus) for a particular level of MAGL activity. Similar results are obtained for a non-reproducing stressor

such as a chemical toxin (except the concentration of the stressor always decreases). Note the overshoot of the inflammatory response for times after the stressor is no longer present. The degree of this overshoot can be modulated by altering a number of parameters in the model, each of which corresponds to a specific biological or biochemical process (with corresponding implications for therapeutic intervention). Ideally the immune response should be of sufficient magnitude to remove the stressor in a timely manner, but not be so severe as to lead to excessive overshoot (which corresponds to sub-chronic or chronic inflammation).

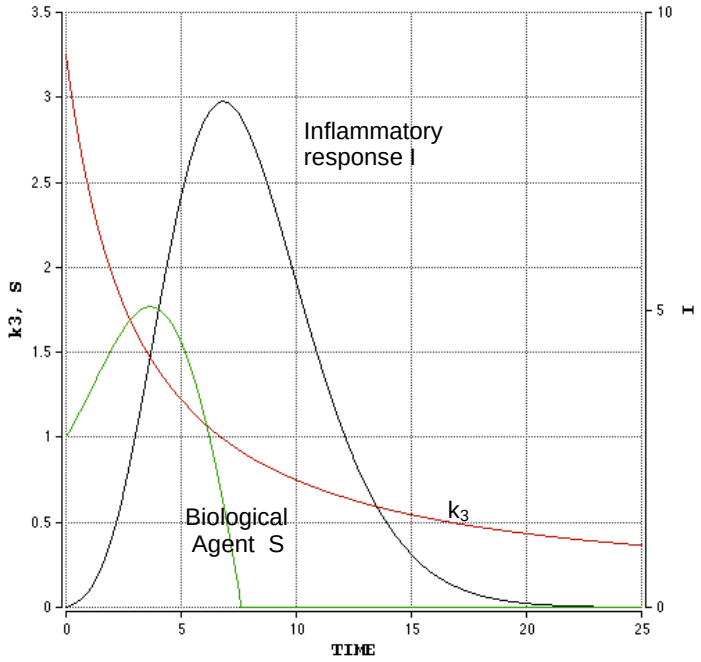

Figure 5. Generalized Model Response I (Immune Cell or Macrophage Number, or Extent of Inflammation) to Stimulation by a Reproducing Stressor S (Bacterium or Virus). The parameter k_3 represents the strength of macrophage self-recruitment, which is downregulated by 2-AG via the CB2 receptor on macrophage. All units are arbitrary.

In assessing the impact of 2-AG metabolism and signaling on the immune response, we consider the case of a biological agent, such as a virus or bacterium. Such an agent is able to reproduce, but is removed by the immune cells (see Section 3 above). Figure 6 shows the effect of increasing the 2-AG synthesis rate, *Syn*, on the overshoot of the inflammatory response, *I*. Note that increasing *Syn* massively reduces inflammation, without much affecting elimination of stressor until a critical value is reached (in this case about 0.34), when infection is no longer controlled.

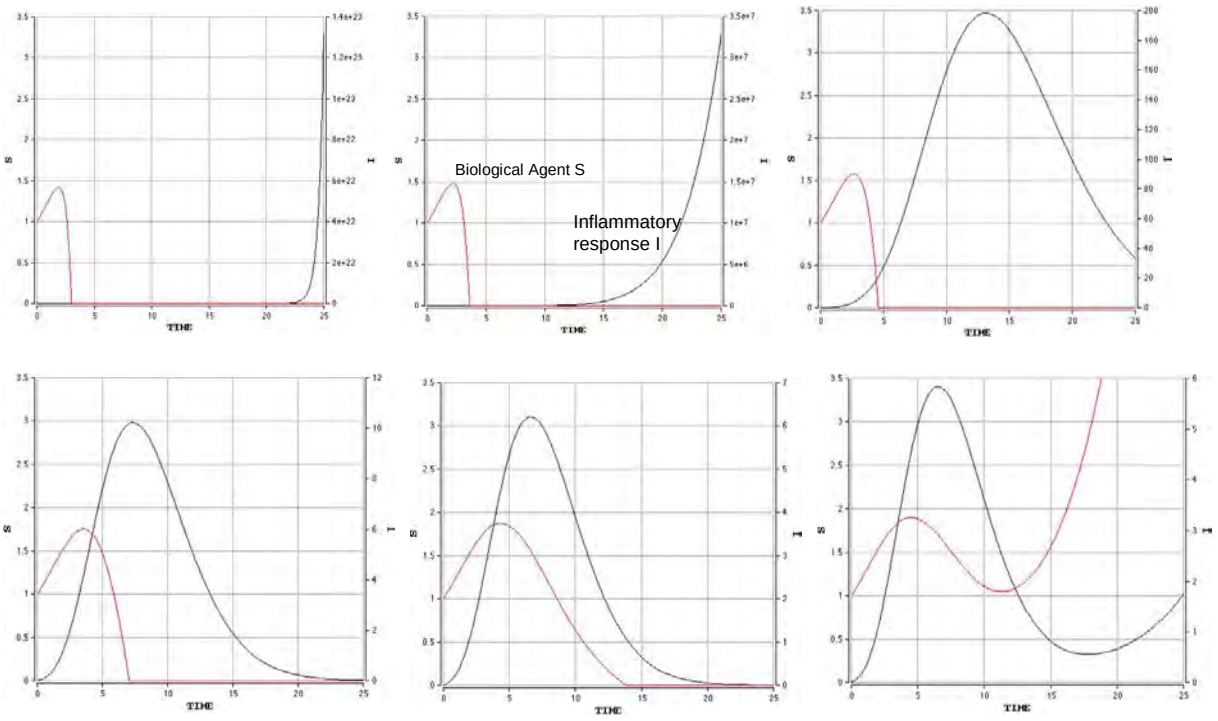

Figure 6. Model Predictions of Effect of Increasing 2-AG Synthesis Rate (Syn) on S and I. Values for Syn are 0, 0.1, 0.2, 0.3, 0.335, and 0.34 (left to right, top to bottom). For zero and small values of Syn, although the infection is controlled, there is massive subsequent runaway inflammation. Note that for values of 0.34 and larger, the infection is no longer controlled.

Figure 7 shows the effect of MAGL inhibitor on overshoot at a particular *Syn* rate. The model suggests that inhibiting MAGL can potentially have a large impact on runaway inflammation, without significantly impacting the spread of the infection, until finally at low values the macrophages are unable to recruit reinforcements at a sufficient rate to control the infection. This dependence of the inflammatory response (and stressor levels) on MAGL activity is illustrated in Figure 7. In this particular case, MAGL levels between 0.02 and about 0.12 (arbitrary units) elicit relatively small inflammatory overshoots, while at greater MAGL levels this overshoot increases in a highly non-linear manner.

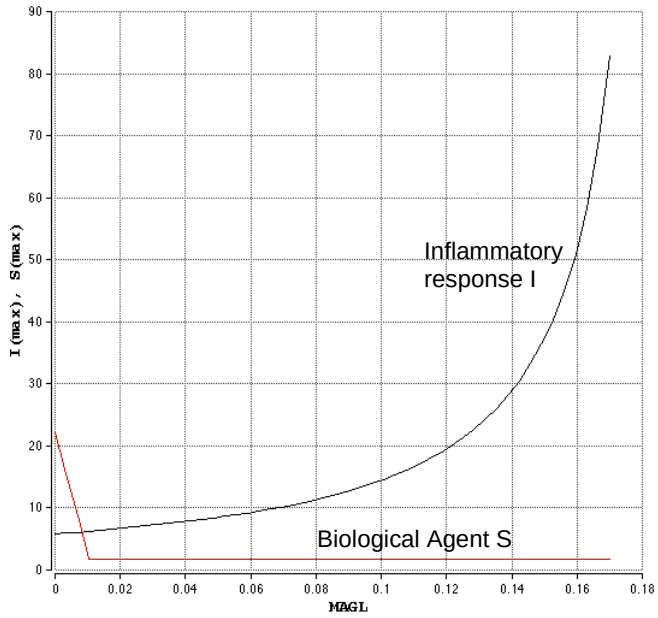

Figure 7. Effect of MAGL Activity on the Peak Values for the Inflammatory Response I, and for the Stressor S. Other parameter values, including the basal 2-AG synthesis rate, are the same as in Figure 3. Note that reducing (inhibiting) MAGL significantly reduces inflammation, while having little effect on the elimination of the stressor until it reaches a low level (of about 0.01 units in the above graph). At this point, the stressor is no longer completely eliminated and will ultimately become a runaway infection.

5.0 DISCUSSION AND CONCLUSIONS

As in so many areas of biology, there are parallel controlling regulatory systems involved in modulating the inflammatory response to an infection or other stressor(s), ensuring the response is regulated, and that it is terminated once it is no longer needed. A major pathway in this regard, the cholinergic inflammatory reflex associated with the vagus nerve is susceptible to chronic OP exposure via downregulation of the alpha-7 nicotinic acetylcholine receptor. Thus, alternative regulatory pathways, such as the cannabinoid system described in the current paper, assume greater responsibility for suppressing inappropriate chronic inflammatory responses under these conditions. This potential weakness of the vagus nerve pathway emphasizes the importance of the endocannabinoid system as a potential therapeutic target for chronic neuroinflammatory diseases associated with chronic OP exposure, such as Alzheimer's (Chen *et al.*, 2012). In particular, it would appear that therapeutic use of MAGL inhibition to reduce inflammation, while maintaining efficacy of the innate immune response to combat infection, would be particularly effective under these conditions.

The theoretical model describes the behavior of the effect of MAGL inhibition on 2-AG and its metabolites, and the subsequent effect on neuroinflammation for a particular choice of parameters. Although parameter values were chosen so as to elicit behavior that is consistent with experimental observations, this set of parameters is not unique, and at this point model

predictions should be considered tentative. The model demonstrates that MAGL inhibition may be an effective means of reducing chronic inflammation from exposures to toxicants. However, MAGL inhibition may be less suitable for treating infectious agents, as the suppression of the immune system may lead to ineffective elimination of the biological agent.

This report describes a "proof of concept" exercise demonstrating how modeling techniques may be used for hypothesis generation and testing. However, many simplifications have been made. For example, PEG2 was used as the major input for the overall inflammatory response. Also, the inherent complexity of the immune response, which depends on a large number of immune cell types and cytokine signaling pathways, has been massively simplified. Current limited experimental data significantly limits further model development. However, available signaling pathway maps (with appropriate pruning), together with experimentally determined rate constants, where available, might in the future be used to provide more biologically relevant detail in the model. As experimental values for specific parameters become available, or the model is refined against more and diverse experimental data sets, the "space" of acceptable parameter values will presumably shrink and predictions will become more reliable. However, it is possible that no unique set of parameter values will be able to describe all observed experimental results; as such, this situation would indicate that the model structure itself would need to be revised to incorporate these results.

6.0 REFERENCES

Basavarajappa BS. 2007. Neuropharmacology of the Endocannabinoid Signaling System-Molecular Mechanisms, Biological Actions and Synaptic Plasticity. Curr Neuropharmacol, 5:81-97.

Blankman JL, Simon GM, Cravatt BF. 2007. A Comprehensive Profile of Brain Enzymes that Hydrolyze the Endocannabinoid 2-Arachidonoylglycerol. Chem Biol, 14(12):1347-56.

Centonze D, Bari M, Rossi S, Prosperetti C, Furlan R, Fezza F, De Chiara V, Battistini L, Bernardi C, Bernardini S, Martino G, Maccarrone M. 2007. The Endocannabinoid System Is Dysregulated in Multiple Sclerosis and in Experimental Autoimmune Encephalomyelitis. Brain. 130(10):2543-2553.

Chen R, Zhang J, Wu Y, Wang D, Feng G, Tang Y-P, Teng Z, *et al.* 2012. Monoacylglycerol Lipase is a Therapeutic Target for Alzheimer's Disease. Cell Reports, 2(5):1329-1339.

Chicca A, Marazzi J, Nicolussi S, Gertsch J. 2012. Evidence for Bidirectional Endocannabinoid Transport across Cell Membranes. J Biological Chem, 287(41):34660-34682.

Du H, Chen X, Zhang J, Chen C. 2011. Inhibition of COX2 Expression by Endocannabinoid 2-Arachidonoylglycerol is Mediated via PPAR-γ. Brit J Pharmacol, 163(7):1533-1549.

Hashimotodani Y, Ohno-Shosaku T, Kano M. 2007. Presynaptic Monoacylglycerol Lipase Activity determines Basal Endocannabinoid Tone and Terminates Retrograde Endocannabinoid Signaling in the Hippocampus. J Neurosci, 27(5):1211–1219.

Herkenham M, Lynn AL, Johnson R, Melvin LS, de Costa BR, Rice KC. 1991. Characterization and Localization of Cannabinoid Receptors in Rat Brain: A quantitative In Vitro Autoradiographic Study. J Neurosci, 7(2):563-563.

Howlett AC. 2005. Cannabinoid receptor signaling. Handbook Exp Pharmacol, 168:53-79.
Kozak KR, Prusakiewicz JL, Marnett LJ. 2004. Oxidative metabolism of endocannabinoids by COX2. Curr Pharmaceut Design, 10:659–667.
Liu J, Parsons L, Pope C. 2013. Comparative Effects of Parathion and Chlorpyrifos on Extracellular Endocannabinoid Levels in Rat Hippocampus: Influence on Cholinergic Toxicity. Toxicol Appl Pharmacol, 272(3):608-15.
Mackie K. 2006. Mechanisms of CB1 Receptor Signaling: Endocannabinoid Modulation of Synaptic Strength. Int J Obes, 30(Suppl 1):S19-23.
Mazzola SI, Gomez LM, Benedetti MD, Salviati A, Ottaviani S, Malerba G, Ortombina M, Pignatti PF. 2007. COX2 Promoter Region Polymorphisms in Multiple Sclerosis: Lack of Association of −765G>C with Disease Risk. Int J Immunogenet, 34(2):71-74.
Nomura DK, Blankman JL, Simon GM, Fujioka K, Issa RA, Ward AM. 2008. Activation of The Endocannabinoid System by Organophosphorus Nerve Agents. Nat Chem Biol, 4:373-378.
Nomura DK, Morrison BE, Blankman JL, Long JZ, Kinsey SG, Marcondes MCG, Ward AM, *et al*. 2011. Endocannabinoid Hydrolysis Generates Brain Prostaglandins that Promote Neuroinflammation. Science, 334(6057):809-813.
Pasinetti GM, Aisen PA. 1998. Cyclooxygenase-2 Expression is Increased in Frontal Cortex of Alzheimer's Disease Brain. Neurosci, 87:319-24.
Pertwee RG. 2005. Pharmacological Actions of Cannabinoids. Handbook Exp Pharmacol. 168:1-51.
Phillis JW, Horrocks LA, Farooqui AA. 2006. Cyclooxygenases, Lipoxygenases, and Epoxygenases in CNS: Their Role and Involvement in Neurological Disorders. Brain Res Rev, 52(2):201-43.
Pope C, Mechoulam R, Parsons L. 2010. Endocannabinoid Signaling in Neurotoxicity and Neuroprotection. Neurotoxicology, 31(5):562-571.
Wallace M, Blair R, Falenski K, Martin B, and Delorenzo R. 2003. The Endogenous Cannabinoid System Regulates Seizure Frequency and Duration in a Model of Temporal Lobe Epilepsy. J Pharmacol Exp Ther, 307:129-137.
Zhang M, Chen C. 2008. Endocannabinoid 2-Arachidonylglycerol Protects Neurons by Limiting COX2 Elevation. J Biol Chem, 283:22601-11.

APPENDIX: IMPLEMENTATION OF MODEL IN BERKELEY MADONNA™

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METHOD RK4

STARTTIME = 0
STOPTIME = 25
DT = 0.02

AG'=SYNAG-MAGL*AG*kcatMAGL/(AG+KMAGL)-AG*COX2*kcatCOX2AG/(AG+KCOX2AG)
init AG=0 ; 2-AG initial concentration
SYNAG=1 ; 2-AG basal synthesis rate
MAGL=1
kcatMAGL=1 ; MM metabolism of 2-AG to AA by MAGL, kcat = catalytic
rate
KMAGL=1 ; Km value
kcatCOX2AG=0 ; "efficiency" of COX2 to convert 2AG to PGE2
KCOX2AG=1

AA'=MAGL*AG*kcatMAGL/(AG+KMAGL)-AA*COX2*kcatCOX2AA/(AA+KCOX2AA)+SYNAA
init AA=0
kcatCOX2AA=1
KCOX2AA=1
SYNAA=0 ; AA synthesis from phospolipids via PLA2

COX2=COX20*(1-kCB1*CB1*AG/(AG+KMCB1)) ; COX2 is inhibited by 2-AG via
CB1R in neuron
COX20=1
kCB1=1 ; note - increasing this slightly reduces COX2 and COX2
mediated synthesis from AA
KMCB1=1
CB1=1

PGE2'=AA*COX2*kcatCOX2AA/(AA+KCOX2AA)+AG*COX2*kcatCOX2AG/(AG+KCOX2AG)-
deg*PGE2
init PGE2=0
deg=1

I'=k2*S-k4*I+k13*PGE2+k3*I; mac concentration (inflammation), induced
by stressor, self-recruitment (via cytokine release) AND PGE2, reduced
by death rate k4
k13=1

k1=0.1 ; killing of stimulus by macs
k2=0.1 ; recruitment of macs by stimulus

k3=k30*(1-k14*CB2*AG/(AG+KM)) ; mac self-recruitment (cytokine
release) reduced by 2-AG via CB2R on macs
k30=1
k14=1
KM=1
```

```
k4=0.1 ; mac death rate
CB2=1
init I=0

S'=-k1*I+k5*S ; stimulus concentration
Init S=1
k5=0.1

limit S >=0
limit I >=0
limit AG >=0
limit AA >=0
limit PGE2 >=0
limit COX2 >=0
limit k3 >=0
```

LIST OF ACRONYMS

2-AG	2-arachidonylglycerol
AA	arachidonic acid
ABHD6	abhydrolase domain containing 6
ABHD12	abhydrolase domain containing 12
AD	Alzheimer's Disease
AEA	arachidonylethanolamide/anandamide
BACE1	β -site amyloid precursor protein cleaving enzyme 1
CB1	neuronal cannabinoid receptor
CB2	immune cell cannabinoid receptor
COX1	cyclooxygenase-1
COX2	cyclooxygenase-2
DAG	diacylglycerol
DAGL	diacylglyerol lipase
FAAH	fatty acid amide hydrolase
GCPR	$G_{i/o}$ coupled G-protein receptors
IDFP	isopropyl-dodecylfluorophosphonate
LPS	lipopolysaccharide
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MS	multiple sclerosis
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
OP	organophosphate
PG	prostaglandin
PGE2	prostaglandin E2
PGH2-EA	prostaglandin E2 ethanolamide
PGH2-G	prostaglandin E2 glycerol
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
VGCC	voltage-gated Ca^{2+} channel