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Review

The diacylglycerol lipases: structure, regulation and roles in and beyond endocannabinoid signalling

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The diacylglycerol lipases (DAGLs) hydrolyse diacylglycerol to generate 2-arachidonoylglycerol (2-AG), the most abundant ligand for the CB_1 and CB_2 cannabinoid receptors in the body. DAGL-dependent endocannabinoid signalling regulates axonal growth and guidance during development, and is required for the generation and migration of new neurons in the adult brain. At developed synapses, 2-AG released from postsynaptic terminals acts back on presynaptic CB_1 receptors to inhibit the secretion of both excitatory and inhibitory neurotransmitters, with this DAGL-dependent synaptic plasticity operating throughout the nervous system. Importantly, the DAGLs have functions that do not involve cannabinoid receptors. For example, 2-AG is the precursor of arachidonic acid in a pathway that maintains the level of this essential lipid in the brain and other organs. This pathway also drives the cyclooxygenase-dependent generation of inflammatory prostaglandins in the brain, which has recently been implicated in the degeneration of dopaminergic neurons in Parkinson's disease. Remarkably, we still know very little about the mechanisms that regulate DAGL activity—however, key insights can be gleaned by homology modelling against other α/β hydrolases and from a detailed examination of published proteomic studies and other databases. These identify a regulatory loop with a highly conserved signature motif, as well as phosphorylation and palmitoylation as post-translational mechanisms likely to regulate function.

Keywords: diacylglycerol lipase; arachidonic acid; synaptic plasticity; phosphorylation; palmitoylation; 2-arachidonoylglycerol

1. THE DIACYLGLYCEROL LIPASES: ELUSIVE ENZYMES RESPONSIBLE FOR THE SYNTHESIS OF 2-ARACHIDONOYLGLYCEROL

Diacylglycerol (DAG) is one of the most studied second messengers in cells; it is generated by the hydrolysis of phosphatidylinositol 4,5-bisphospate in response to the activation of surface receptors that include G-protein-coupled receptors and receptor tyrosine kinases [1]. DAG has many functions in cells, and in addition to directly activating effector molecules, it can serve as a substrate for enzymes that generate alternative signalling lipids [2]. One such pathway involves the hydrolysis of DAG by a DAG lipase (DAGL) activity, the initial step in a pathway that was first studied in the context of arachidonic acid (AA) release in platelets and mast cells [3–5]. This involves the hydrolysis of DAG at the *sn*-1 position to generate 2-arachidonolyglycerol (2-AG) followed by a monoacylglycerol lipase (MAGL)-dependent hydrolysis of 2-AG to generate AA. A tool compound (RHC 80267) that inhibits DAGL activity was identified [6] and was very useful for implicating DAGL activity in a wide range of responses including, in the early studies, thyrotropin-stimulated prostaglandin release in the thyroid [7], prolactin release from pituitary cells [8] and thrombin induced AA release in platelets [9]. RHC 80267 was also used to provide the first evidence for DAGL activity playing a role in the control of axonal growth and guidance in the developing brain [10–12]. However, in these early studies 2-AG was generally considered as the intermediate in a pathway releasing AA in cells, rather than as a biologically active lipid in its own right. This was also thought to be a minor pathway in terms of AA turnover, with the bulk levels of AA in the brain as well as the signalling pools of AA for the cyclooxygenase (COX)-dependent generation of the inflammatory prostaglandins deemed to be regulated by the phospholipase A2 (PLA2) family of enzymes [13]. Also, although DAGL activity could be readily measured, the enzyme(s) responsible for the synthesis of 2-AG eluded identification and characterization for many years. The identification of 2-AG as a candidate endocannabinoid (eCB), the cloning of the *sn*-1 specific DAGLα and DAGLβ and the subsequent demonstration that they are responsible for the synthesis of essentially all of the 2-AG and AA in the brain has

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given a new found impetus to the study of DAGL structure, regulation and function.

2. THE EMERGENCE OF 2-ARACHIDONOYLGLYCEROL AS AN ENDOCANNABINOID

The CB_1 and CB_2 cannabinoid receptors were identified as the mediators of the effects of tetrahydrocannabinol (THC), the major active component of the cannabis plant [14,15]. The presence of receptors implied the existence of natural ligands in the body, and the hunt was then on to identify the so-called 'endocannabinoids' (eCBs). This effort was largely driven by pharmacological 'grind and bind' assays and/or by testing purified molecules in well characterized CB_1/CB_2-dependent biological assays. These first identified anandamide [16] and then 2-AG as candidate eCBs [17,18]. Similar studies have demonstrated that noladin ether [19,20], virodhamine [21] and N-arachidonoyldopamine [22,23] can elicit CB_1/CB_2-dependent responses. The above are related lipids that clearly share an ability to bind to and activate CB_1 and/or CB_2 receptors in model systems, but it remains to be determined to what extent they actually do so in a physiologically relevant manner in the body. Interestingly, 2-AG is much more abundant than any of the other candidate eCBs, and on the basis of detailed pharmacological studies, it has long been argued by some to be the 'true' eCB [24]. Still, given that it was identified first, anandamide remained in 'pole' position for many years. Despite the fact that RHC 80267 is a non-selective DAGL inhibitor [25,26] and contradictory results have often been reported with this drug [27,28], when used in conjunction with other tools (e.g. cannabinoid receptor agonists and antagonists or additional DAGL inhibitors such as tetrahydrolipstatin—THL), it can generally be used to determine whether an eCB response is more likely to be mediated by 2-AG or anandamide [26,29]. On this basis, the use of RHC 80267 in conjunction with other tools has allowed groups to conclude that DAGL activity is required for a number of established and emerging eCB responses, including synaptic plasticity [30–33], axonal growth and guidance [34] and more recently, adult neurogenesis [35,36]. Nonetheless, absolute proof of the requirement for a DAGL in an eCB response required the identification and characterization of the somewhat elusive enzyme(s).

3. CLONING OF DIACYLGLYCEROL LIPASE α/β AND PROOF OF THEIR IMPORTANCE FOR 2-ARACHIDONOYLGLYCEROL SYNTHESIS

Considerable biochemical efforts made headway into the purification and characterization of a DAGL enzyme from the bovine brain [37–39]. In brief, the studies localized a DAGL activity to a 27 kDa fraction, and demonstrated that the enzyme could be directly stimulated by cAMP-dependent protein kinase, but not by calcium. A second DAGL activity with an estimated molecular weight of 33 kDa was isolated from human platelet microsomes, and this was also not directly stimulated by calcium [40]. However, the enzymes responsible for these activities were not sequenced or cloned. Thus we remain very much in the dark regarding their identity and their relationship to the DAGLs that are described below.

Having implicated a DAGL activity in brain development [10,11], we decided to adopt a speculative bioinformatics approach to try to identify the elusive enzyme(s). A literature search led us to the sequence of a gene in *Penicillium camembertii* (a species of fungus used in the production of Camembert and Brie cheeses) that encodes for a 39 kDa enzyme that can hydrolyse mono- and diacylglycerides [41]. When this sequence was blasted against the draft genome of *Drosophila,* a single related molecule was identified (G. Williams 2002, unpublished data); when blasted against other genomes, two very closely related molecules were identified in a wide range of species, including fish, rodents and man [42]. In terms of overall structure, these molecules are very different from the fungal enzyme. Whereas the fungal enzyme is a single catalytic domain that is normally secreted into culture media [43], the newly identified enzymes have a four transmembrane (4TM) domain followed by a catalytic domain predicted to be intracellular (details given below). The 4TM lipases harbour a consensus serine lipase motif, and they were novel and unrelated to any characterized serine lipase, including MAGL, the enzyme responsible for hydrolysis of 2-AG to AA [44]. On the basis of this, we decided to pursue the idea that they might be the elusive DAGLs. Interestingly, more recent searches reveal the presence of 4TM-containing lipases in some fungi that are the true orthologues of the novel lipases (G. Williams 2012, unpublished observation).

A careful examination of the intron–exon boundary conservation between the single *Drosophila* DAGL and the two enzymes found in higher species readily identifies the conserved homologue (which we designated as DAGLα), and shows that a gene duplication event has given rise to a second DAGL (designated as DAGLβ; figure 1). DAGLα (containing 1042 amino acids) is larger than DAGLβ (with 672 amino acids). They have a short amino terminal sequence that leads to the 4TM domain, followed by the catalytic domain. DAGLα then differs from DAGLβ in that it has a substantial carboxyl-terminal tail (figure 1). Tagged versions of the enzymes were expressed in COS cells and are found expressed at the plasma membrane, albeit in a punctuate manner that suggests that they are in ordered microdomains in the membrane, and both run on SDS gels at expected molecular weights of approximately 70 kDa (DAGLβ) and approximately 120 kDa (DAGLα) [42]. Detailed enzymology also clearly showed both enzymes to be *sn*-1 specific DAGLs that synthesize 2-AG in cells and that 2-AG produced by DAGLα in cells is released into the media [42]. The enzymes are unique with no obvious close relatives in the genome. A highly correlated spatial and temporal pattern of expression between the enzymes and the CB_1 receptor (discussed below), together with the fact that the enzymes are inhibited by RHC 80267 and a second DAGL inhibitor THL, was consistent with the enzymes being the elusive DAGLs responsible for much of the eCB signalling in the developing and adult brain [42].

There are a number of pathways that can potentially generate 2-AG and given the relatively high levels of

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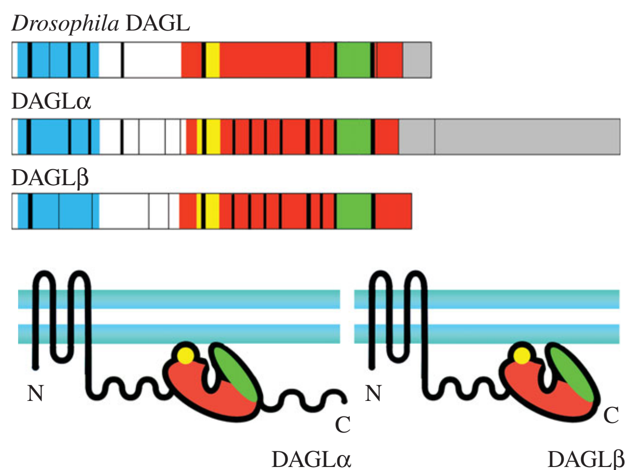

Figure 1. The exon structure of the *Drosophila* DAGL and the vertebrate DAGLα and DAGLβ are illustrated with a linear schematic. The exon boundary locations are shown with vertical lines with the boldest lines indicating their conservation across the three enzymes, and the intermediate thickness lines indicating conservation between two of the enzymes. The region encoding the 4TM domain is shown in blue, the catalytic domain in red, and the tail in grey. Within the catalytic domain a cysteine rich sequence is highlighted in yellow and the regulatory loop encoded by a solitary exon (see figure 2 for details) is coloured green. The overall domain structure of DAGLα and DAGLβ are also shown as schematics with the catalytic domain in red and the cysteine rich insert and regulatory loop again shown in yellow and green, respectively.

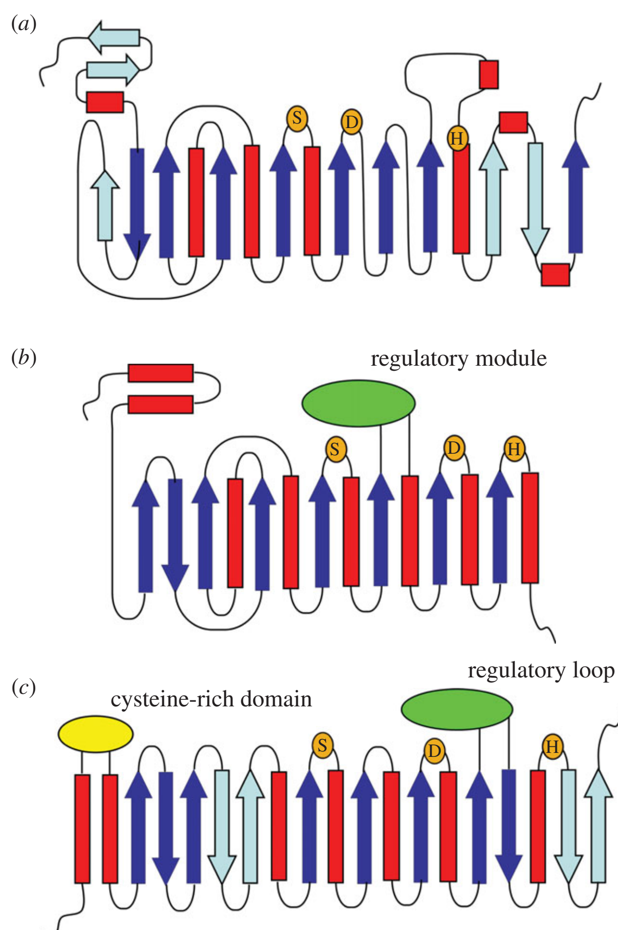

Figure 2. The fold topologies of the catalytic domains of three α/β hydrolase lipases—namely (*a*) the pancreatic lipase, (*b*) hormone sensitive lipase and (*c*) DAGL. The fold consists of a core eight β-sheets (shown in dark blue) linked in various ways via α-helices (red), β-sheets (light blue) and loops. The relative location of the catalytic triad (serine, aspartic acid and histidine) is also shown. The hormone sensitive lipase has a large regulatory module inserted between the serine and aspartic acid (highlighted in green). The DAGLs have two conspicuous inserts, a cysteine rich insert show in yellow and a putative regulatory loop shown in green.

this lipid in tissues, it would be reasonable to assume that there might be a large bulk pool and a smaller more dynamic signalling pool. On the basis of this, it was difficult to predict the impact that the loss of DAGLα or DAGLβ might have on the total level of 2-AG in tissues. With this in mind, the dramatic loss of 2-AG from various tissues in DAGLα and DAGLβ knockout mice reported in two independent studies came as a big surprise. In DAGLα knockout mice, there is an 80 per cent reduction in 2-AG levels in the brain and spinal cord, and an approximately 60 per cent reduction in the liver. In DAGLβ knockout mice, there is a 50 per cent reduction in the brain, no appreciable reduction in the spinal cord, but a remarkable 90 per cent reduction in the liver [45]. 2-AG levels are reduced by approximately 80 per cent in the cerebellum, hippocampus and striatum in an independently generated line of DAGLα knockout mice, with no appreciable change in these brain regions in the DAGLβ knockout mice. Importantly, this study also showed that stimulus-induced increases in 2-AG are also lost in the brains of the DAGLα knockout mice [46]. Thus, the DAGLs are responsible for most of the 2-AG in the brain, spinal cord, liver and other tissues. But importantly, they make different contributions within each tissue, and although there is little doubt that both enzymes synthesize 2-AG in the brain, DAGLα appears to be much more important in synapse-rich regions. The results from the knockout animals also show that they cannot compensate for each other's loss—but nonetheless, there might be some co-operation between the enzymes.

4. DIACYLGLYCEROL LIPASE-DEPENDENT ENDOCANNABINOID SIGNALLING IN THE DEVELOPING AND ADULT BRAIN

The above studies unequivocally identified DAGLα and DAGLβ as the enzymes responsible for 2-AG synthesis in the brain and other tissues. There are numerous eCB responses that the DAGLs might or might not drive, but we will limit our comments to three aspects of the 'wiring and firing' of the brain. These have recently been reviewed in considerable detail [29,47]; so we simply focus on the 'take home' messages.

During development, neurons extend axons in a precise manner to reach and innervate their targets. Receptors on the axonal growth cone can either promote directed growth when engaged by a positive guidance cue, or inhibit growth when engaged by an inhibitory guidance cue [48]. Fasciculated axonal growth involves axons extending together in an ordered manner to form an axonal tract, and this can be driven in part by cell adhesion molecules (CAMs) including NCAM,

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N-cadherin and L1 interacting with the same CAM on other axons within the tract [49,50]. Although the initial dogma was that the response was driven by adhesion, it soon became clear that it required the activation of conventional second messenger cascades in growth cones [51]. Bioassays for CAM-stimulated axonal growth were established, and results with pharmacological and other tools led to the emergence of the 'CAM-FGFR' hypothesis proposing that axonal growth responses stimulated by the above three CAMs depended upon them acting as surrogate ligands for the fibroblast growth factor receptor (FGFR) [52,53]. The downstream signalling cascade was established to require activation of PLCγ to generate DAG, followed by a DAGL-dependent step to generate 2-AG, which is now understood to act upon CB_1 receptors in the same growth cone to promote motility [34]. There is now widespread support for the 'CAM-FGFR' hypothesis that has been extended to include additional CAMs [54] and has also been proposed to drive the metastasis of various cancer cells [55]. The pathway described earlier provides one detailed example of how cross-talk between a growth factor receptor and DAGL-dependent eCB signalling can modulate growth cone motility, and notably other studies on neuronal cell lines and primary neurons have alluded to additional ways in which eCB signalling can impact on axonal growth [56–59].

It is now clear that the DAGLs and the CB_1 receptors can modulate growth cone dynamics *in vitro*, and they are well placed to play a key role in axonal growth and guidance *in vivo* as they are expressed in advancing growth cones during development [34,58,60]. Still, mice that lack one of the DAGLs or CB_1 receptors are viable and relatively healthy—so, to what extent can the above pathway impact on brain development? In this context, there is considerable redundancy in terms of the molecules that lay down the general structure of the brain, with relatively subtle phenotypes found following the loss of some key guidance molecules [61]. Indeed, there are now a number of reports showing changes in the wiring of the brain in animals where DAGL and/or CB_1 receptor function is compromised by pharmacological tools and/or by genetic means [58,59,62,63]. Thus, the 'proof-of-principle' experiments for the DAGL-dependent eCB signalling playing an important role in the development of the brain are clearly emerging [64]. Nonetheless, this work is still in its infancy and much remains to be learned about how eCB signalling is regulated in the developing brain.

After synapses form, they need to function and generally do so by releasing an excitatory or inhibitory neurotransmitter from the presynaptic terminal that acts upon postsynaptic receptors to depolarize or hyperpolarize the next cell in the relay. Individual synapses in the central nervous system (CNS) do not function in a binary manner. The amount of transmitter that is released, and the postsynaptic sensitivity to the transmitter are both malleable, with changes in one or the other resulting in a change in synaptic strength, with this process often being described as 'synaptic plasticity'. Perhaps not surprisingly, there are numerous ways to produce a change in synaptic strength, and a wide range of molecular mechanisms, including eCB signalling, have been identified that contribute to this [47]. In this context, eCB signalling has been recognized for some time to regulate some forms of activity-dependent synaptic plasticity. In this scheme, high levels of presynaptic activity can stimulate the synthesis and/or release of an eCB from the postsynaptic site that acts back on presynaptic CB_1 receptors to limit the release of an excitatory or inhibitory neurotransmitter. This is a simple but elegant feedback loop that is widely used throughout the nervous system and can explain the effects that eCB signalling can exert on among other things, learning and memory, mood, appetite, pain and motility. The various types of eCB-dependent synaptic plasticity have been reviewed in detail by others [65,66]. Here, we concern ourselves only with the question of the enzyme(s) that makes the eCB(s) driving these responses. Although there was considerable circumstantial evidence that 2-AG was responsible for much of the CB_1-dependent synaptic plasticity, this work generally relied on the use of RHC 80267 and there are conflicting reports in the literature as to whether or not this drug inhibits some forms of CB_1-dependent plasticity [67]. In addition, there are no drugs to differentiate DAGLα from DAGLβ activity. The key here is that for the feedback loop to work, the enzyme responsible for the synthesis of the eCB needs to be specifically localized to the postsynaptic site. In this context, although the DAGLs are expressed in developing growth cones, they are lost from presynaptic terminals and axonal tracts sometime around or after synapse formation—with high levels of DAGLα expression becoming restricted to dendritic fields in the adult brain [42]. Numerous more precise studies have now shown an exquisite mirrored pattern of the CB_1 receptor and DAGLα expression at synapses throughout the nervous system, with the CB_1 receptor restricted to the presynaptic terminal and DAGLα to the complementary postsynaptic site [68–72]. Thus it perhaps came as no surprise when it was shown that CB_1-dependent synaptic plasticity is lost throughout the brain in DAGLα, but not in DAGLβ, knockout mice [45,46]. As predicted, a switch in the expression of the DAGLα from axon growth cones to dendritic spines allows for two fundamentally different eCB pathways to operate in the developing and adult brain [42]. Antibodies to DAGLβ have as yet not been validated as being specific by use of tissues from knockout mice; so the precise cellular location of this enzyme in the developing and adult brain is not clear.

Adult neurogenesis constitutes a form of cellular plasticity in the developed brain that impacts on memory, depression and neurodegenerative diseases [73]. This involves the maintenance of neural stem cell populations in the hippocampus and the subventricular zone (SVZ) of the lateral ventricle. These cells seed the continuous production of neuroblasts that differentiate into new neurons and can integrate into the existing synaptic circuits. In the case of the hippocampus, this is localized to the dentate gyrus, whereas in the SVZ, the stem cells generate migratory neuroblasts that have to traverse the rostral migratory stream (RMS) to populate the olfactory bulb (OB) with new neurons. A role for the CB_1 and CB_2

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receptors in adult neurogenesis is supported by studies that show that antagonists of these receptors, or genetic deletion of them, reduce the number of proliferating stem cells in the hippocampus, whereas agonists can increase the number [74–76]. Interestingly, when eCB hydrolysis is blocked by genetic deletion or pharmacological inhibition of the fatty acid amide hydrolase (FAAH), neurogenesis is enhanced in both the hippocampus and SVZ [35,77]. Furthermore, DAGLα is highly expressed in proliferating neural stem cells in the SVZ and this prompted a detailed pharmacological study into the role of DAGL-dependent eCB signalling in this stem cell niche [35]. In young adult animals, blocking DAGL activity (with RHC 80267 or THL) reduced stem cell proliferation in the SVZ by 70 to 80 per cent, and surprisingly this effect could be mimicked by blocking CB_2 receptors and not CB_1 receptors. Adult neurogenesis in the SVZ declines dramatically with age, and treatment with CB_2 agonists could circumvent this, suggesting that a run-down in eCB tone might account for this age-related decline. Importantly, changes in the number of proliferating stem cells in the SVZ correlated highly with the actual number of new neurons appearing in the OB two weeks later. Confirmation of the importance of DAGLα and DAGLβ for adult neurogenesis has come from an examination of the knockout mice. In the SVZ, there is a 50 per cent reduction in the number of proliferating stem cells in the young adult DAGLα knockout mice, with no difference in the DAGLβ knockout mice. However, there is a 50 per cent reduction in the hippocampus in DAGLα and DAGLβ knockout mice [45]. Thus both DAGLs regulate the number of proliferating neuronal stem cells in the adult brain, and this directly impacts on the number of new neurons appearing in the OB or hippocampus. Interestingly, in addition to promoting stem cell proliferation, DAGL-dependent eCB signalling via both the CB_1 and CB_2 receptors also promotes the migration of neuroblasts along the RMS towards the OB [36].

5. MONOACYLGLYCEROL LIPASE GENERALLY LIMITS DIACYLGLYCEROL LIPASE-DEPENDENT ENDOCANNABINOID SIGNALLING

2-AG can be hydrolysed by several serine lipases, including FAAH, MAGL, and the more recently identified ABHD6 and ABHD12, and all of these could in principle modulate DAGL-dependent eCB signalling; however, lipase profiling experiments suggest that MAGL is responsible for the hydrolysis of at least 85 per cent of the 2-AG in the brain. Studies with selective MAGL inhibitors have clearly shown that this enzyme can limit eCB signalling at synapses throughout the brain [31,33,78–80]. The important role for MAGL in regulating synaptic plasticity has been confirmed in studies on knockout mice; however, the picture here is complicated as the very large increases in 2-AG that are found in these animals can lead to the loss of some CB_1-dependent responses in some brain regions, presumably due to 2-AG dependent internalization or desensitization of the receptors [81]. Nonetheless, all of these results point to MAGL as being the enzyme that generally limits eCB signalling by terminating the action of 2-AG.

Detailed studies on the localization of MAGL give insights into the 'gate-keeper' role that this enzyme plays in some fundamentally important eCB pathways. For example, in the developed brain, MAGL is present in presynaptic terminals [44,82,83] and excluded from the postsynaptic density (PSD), presumably allowing for depolarization-induced accumulation of 2-AG that drives synaptic plasticity. Thus a picture emerges of an elegant 'molecular architecture' where the synthesis of 2-AG is protected within, as well as restricted to, the postsynaptic site, on the basis of segregation of the DAGL from the MAGL in a manner that then allows the privileged access of 2-AG to the presynaptic CB_1 receptor. Interestingly, a similar segregation of the DAGLs from MAGL is important for axonal growth and guidance; here the DAGLs and CB_1 receptors are found enriched within the growth cones with MAGL excluded from the highly specialized compartment but found further back along the axon [56]. Again, this will protect 2-AG generated within the growth cone from hydrolysis and make it more readily available to activate CB_1 receptors in an autocrine manner.

6. NON-CANNABINOID FUNCTIONS FOR THE DIACYLGLYCEROL LIPASES

As discussed above, DAGL activity was initially studied in the context of a DAGL/MAGL pathway releasing AA in cells. The pivotal role ascribed to the PLA2 family of enzymes in the generation and maintenance of AA levels in tissues, together with the emergence of 2-AG as an eCB whose actions could be terminated by MAGL activity, gave succour to the notion that there might be little cross-talk between these pathways. However, a number of observations now challenge this view. The relatively large increases (10–30 fold) in 2-AG levels that are seen when MAGL activity is inhibited or when the enzyme is knocked out [81,84] do not sit well with the idea that MAGL is simply terminating the action of a small signalling pool of 2-AG. Still, conclusive proof for the important role that the DAGL/MAGL pathway plays in generating and/or maintaining AA levels came from the studies on the DAGL knockout mice [45]. These studies reported parallel reductions in 2-AG and AA levels in some, but not all, tissues. For example, in the brain and spinal cord, there was an approximately 80 per cent reduction in 2-AG and AA in the DAGLα knockout mice. Similar parallel reductions were seen in the liver, with the most dramatic effect being the approximately 90 per cent reduction in 2-AG levels in the DAGLβ knockout animals being accompanied by an approximately 80 per cent reduction in AA. However, in adipose tissue, AA levels remained unchanged despite a 50 per cent reduction in 2-AG, in the DAGLα knockout animals.

Given that it is now established that the DAGL/MAGL pathway is largely responsible for generating and/or maintaining AA levels in the brain and other tissues, perhaps the most pertinent question is to what extent this pathway drives AA-dependent responses in cells and tissues. In this context, there are indications

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that DAGL activity is required for a wide range of responses, including the vasopressin-induced release of noradrenaline and adrenaline from the adrenal medulla [85] and other AA-dependent responses such as vasodilation [86,87]. However, perhaps the most exciting result is the observation that 2-AG hydrolysis by MAGL generates the prostaglandins that drive inflammatory responses in the brain, including those that lead to neurodegeneration in a mouse model of Parkinson's disease [88]. Interestingly, a MAGL inhibitor has shown excellent results in this pre-clinical model of Parkinson's, owing to the dampening of an AA-dependent inflammatory response as opposed to stimulating a 2-AG-mediated eCB response [88]. This is perhaps a clinically relevant finding that again challenges the dogma that the PLA2 pathway generates the AA for the COX-mediated biosynthesis of prostaglandins.

In addition to being the precursor for AA biosynthesis in the COX pathway, 2-AG itself is a substrate for COX-2 and this might not only limit eCB signalling in some circumstances, but also generate a novel family of bioactive prostaglandins that act on as yet uncharacterized receptors [89]. Likewise, 2-AG can be oxygenated by a number of lipooxygenases to generate a ligand that can activate peroxisome-activated receptors [90]. There is also emerging evidence that DAGL-dependent pathways can inhibit L- and N-type calcium currents [91], and regulate a form of synaptic plasticity mediated by a TRPV-like receptor [92]; however, it remains unclear whether these effects are mediated by 2-AG or by a 2-AG metabolite.

A final proof that the DAGLs have major roles that are unrelated to cannabinoid signalling comes from the observation that the DAGLs are expressed in species that do not express cannabinoid receptors. A good example of this is represented by protostomian invertebrates (e.g. *Drosophila*) that harbour a DAGL but show no evidence for orthologues of the vertebrate cannabinoid receptors [93]. Interestingly, in *Drosophila*, DAGL activity is required for the pathway that couples photo-excitation of rhodopsin to the opening of TRP/TRPL channels in photoreceptors—but again it is not known whether this response to light is mediated directly by 2-AG or by a 2-AG metabolite [94].

7. STRUCTURAL FEATURES OF THE DIACYLGLYCEROL LIPASES AND POTENTIAL REGULATORY MECHANISMS

A schematic of the DAGLα and DAGLβ exons is shown in figure 1. These have been colour-coded to highlight the various domains of the DAGLs that are also shown in the figure. In brief, both enzymes have a 4TM domain that contains a short cytoplasmic N-terminal sequence leading to the 4TM helices, followed by a canonical α/β hydrolase domain that harbours the catalytic activity (referred to hereafter as the catalytic domain), followed by a considerable carboxyl-terminal 'tail' domain in the case of DAGLα, but not DAGLβ.

(a) *The four transmembrane domain*

Within the 4TM domain, the leading 19 amino acid N-terminus cytoplasmic sequence differs only in one residue between the single invertebrate (*Drosophila*) DAGL and the vertebrate DAGLα, suggesting an important as yet to be elucidated function. However, the sequence is less well conserved between DAGLα and DAGLβ, with only 63 per cent residue identity between the human enzymes. The 4TM helices are separated by short, relatively un-conserved loops, with the two extracellular ones being potential sites for glycosylation. The TM helices might facilitate packing of the enzymes at the membrane in analogy with tetraspanins into micro-domains that contain other signalling components [95]. Furthermore, they may serve as docking sites for other proteins in a functional complex, or form part of a channel that regulates DAG access to the catalytic domain, or indeed 2-AG release from cells. However, catalytic activity and the availability of 2-AG to activate CB_1 receptors in the same cell is not apparently compromised by the deletion of this region [96].

(b) *The catalytic domain*

The α/β hydrolase fold family is characterized by a core set of eight mutually hydrogen-bonded β-sheet strands linked in a multiplicity of ways, mostly via α-helices, but also through β-sheets and loops. A schematic showing the structure of this domain in DAGLα/β relative to two well-characterized lipases, namely the pancreatic lipase and hormone sensitive lipase (HSL), is shown in figure 2. Key features include conservation of the presence and critical spacing of the serine, aspartic acid and histidine catalytic triad within a hydrophobic active site. In this context, the catalytic serine and aspartic acids were straightforward to identify within the catalytic triad in the DAGLs and they have been verified experimentally [42]. The catalytic histidine was less obvious but it has been identified and confirmed in a later study [97]. The next feature is the presence of a variable size insert in close proximity to the catalytic triad that functions as a 'cap' or 'lid' to shield the hydrophobic catalytic cavity from water, but opens upon membrane adsorption to allow lipid access [98,99]. In the case of the pancreatic lipase, this is governed by the association of the enzyme with the membrane [100], whereas in the case of the HSL, it is more complex and is regulated by phosphorylation within both the core α/β hydrolase domain and the regulatory module [101].

The DAGLα/β hydrolase domain has a substantial 50–60 amino acid insert of unknown structure between the 7th and 8th canonical β-sheets that, on the basis of consideration of the structure and function of a wide range of α/β hydrolases [99] including the HSL (figure 2), can be considered as the regulatory loop likely to control substrate access to the catalytic site. The crystal structure of the minimal fold fungal DAGL has been solved [102], and homology modelling of DAGLα and DAGLβ against this structure confirms that this regulatory loop is well placed to shield the active site (not shown). The regulatory loop in DAGLα and DAGLβ is considerably larger than the lid/cap regions of most other lipases (e.g. pancreatic lipase; figure 2) and this suggests the potential for substantial conformational changes determining whether the lid is open or closed. A detailed analysis of the loop suggests phosphorylation as the most

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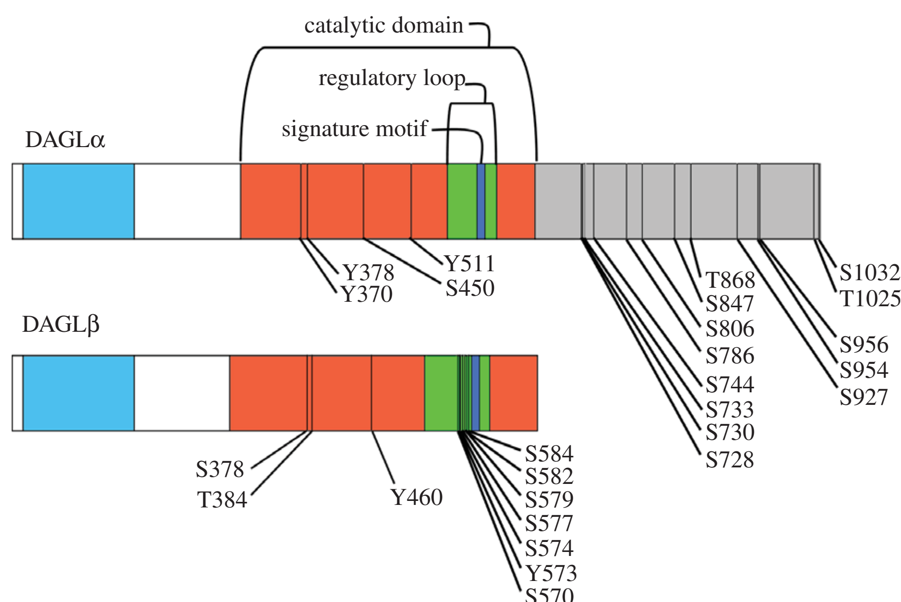

Figure 3. The current DAGL phospho-map (DAGLα top and DAGLβ bottom and numbering according to human enzymes). Phosphorylation sites identified by mass-spectrometry analysis of various cells and tissues are highlighted in the catalytic domain (red) with a large cluster identified within the regulatory loop (green) close to the signature motif (blue) found in DAGLβ. Several of the phosphorylated residues in DAGLβ are conserved in DAGLα (and vice versa), and we would predict that these can also be phosphorylated. The carboxyl-terminal tail region of DAGLα has been found to be especially heavily phosphorylated.

probable regulatory mechanism. In this context, numerous mass spectrometry studies provide direct evidence for the regulated phosphorylation of the DAGLs within the catalytic domain and regulatory loop [103–109]. We have collated this data to build a DAGL phospho-map (figure 3). Interestingly, the DAGL purified from brain microsomes was shown to be positively regulated by direct phosphorylation by PKA [39] and phosphorylation of bovine rod membranes by PKA resulted in an approximately 70 per cent increase in DAGL activity [110]. Likewise, activation of PKA and PKC stimulates 2-AG synthesis in cells presumably via a DAGL-dependent mechanism [111], and glucocorticoid-stimulated increases in 2-AG levels in hypothalamic slices are dependent on PKA activity [112]. The activity of the HSL is also regulated by phosphorylation through the regulatory module highlighted in figure 2 [101]. Notably, numerous kinases that include PKA, ERK, GSK4, CaMKII and AMPK can regulate HSL activity by phosphorylating sites both within the canonical α/β hydrolase fold and the regulatory module [113], and the DAGLs contain consensus phosphorylation motifs for many of these kinases, including PKA, PKC, CaMKII and Src (not shown). Thus it is reasonable to postulate that, like that of the HSL, DAGL activity will be regulated by phosphorylation of the catalytic domain, including the regulatory loop.

In principle, phosphorylation of the catalytic domain and/or regulatory loop might stabilize an ‘open’ and ‘closed’ conformation and, based on analogy with general mechanisms this suggests that the regulatory loop is likely to be a flexible linker that contains a binding motif for either an inter- or intra-molecular interaction [114]. In this context, the regulatory loop harbours a 10 amino acid poly-proline ‘signature motif’ (PLYPPGRIIH) that is invariant between DAGLα, DAGLβ, and the invertebrate DAGLs and is even found conserved in plant DAGLs (G. Williams 2012, unpublished observation). On this basis, we postulate that it has an essential binding function, and that an intra-molecular interaction is the more parsimonious hypothesis, as this does not require the conservation of an interacting protein across this wide phylogenetic range. Nonetheless, future studies are required to provide direct experimental evidence for this model.

Three independent proteomic studies have also shown that both DAGLα [115] and DAGLβ [116, 117] can be palmitoylated. One of these studies mapped this to cysteines 610/611 in the regulatory loop region within the DAGLβ catalytic domain [117]; however, the authors noted the possibility of false positives in terms of the accurate localization of the palmitoylated site. Interestingly, following the first two helices in DAGLα, DAGLβ and the invertebrate DAGL genes, there is a poorly conserved cysteine rich insert lying on the membrane proximal face of the enzyme that in principle could also be a site of palmitoylation (figures 1 and 2). Palmitoylation is a reversible and dynamic post-translational modification regulated by a large family of palmitoylating enzymes involving the covalent linkage of a long-chain fatty acid to a cysteine residue of membrane proteins. This increases the hydrophobicity of the protein, enhances the membrane association and can serve to modulate a wide range of processes such as protein–protein interaction, subcellular trafficking and substrate access [118–120]. One prominent member of the lipase family known to be palmitoylated is Phospholipase D (PLD), and this palmitoylation has been shown to be essential for its correct membrane localization and endocytosis [121]. Interestingly, during development, a number of molecules that collaborate with the DAGLs to promote axonal growth and guidance (e.g. L1 and NCAM, as well as GAP-43) are also palmitoylated, and this might

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allow for coordinated function between these proteins, most-likely within membrane microdomains, favouring the assembly of 4TM and/or palmitoylated proteins into ordered signalling complexes and promoting efficient signal transduction [95,119,122].

(c) *The diacylglycerol lipase α tail*

The most obvious structural difference between DAGLα and DAGLβ is the presence of a substantial carboxyl-terminal tail that does not directly contribute to catalytic activity [42], with the most obvious functional difference being an exclusive role for DAGLα in retrograde synaptic signalling [45,46]. In this context, the DAGLα tail contains a consensus motif (PPxxF) for binding the coiled-coil domain of Homer proteins, a family of adaptors that interact with many proteins, including the metabotropic glutamate receptors (mGluR) that localize to the PSD [123]. An interaction between Homer-1b and Homer-2 with DAGLα, mediated by the PPxxF motif, is important for DAGLα association with the plasma membrane in Neuro-2A cells, but not for 2-AG generation [124]. Cocaine inhibition of eCB-dependent synaptic plasticity has also been attributed to changes in the expression of Homer proteins [125], and Homer-1a can differentially modulate two types of eCB-mediated synaptic plasticity [126]. Finally, the 'molecular reconstruction of mGluR5a-mediated eCB signalling cascade' in sympathetic neurons required the expression of Homer-2b, together with DAGLα, the CB_1 receptor and the glutamate receptor [96]; however, the tail of DAGLα appeared to be dispensable for this. Nonetheless, the interaction of the DAGLα tail with Homer proteins is likely to play an important role in localizing and/or retaining this enzyme within the PSD. Importantly, there are other poly-proline clusters in the tail region, and detailed inspection of phospho-proteomic databases also shows that the tail is highly phosphorylated [107,127–129] (see figure 3 for the current phosphomap), suggesting that the tail will mediate other protein–protein interactions involved in perhaps the trafficking of the enzyme to, or within, the PSD.

In summary, the DAGLs regulate a wide range of biological responses via the generation of a cannabinoid receptor signalling pool of 2-AG, but also serve as 'hub' enzymes in pathways that generate and/or maintain signalling pools of AA and various prostanoids. The development of selective inhibitors for DAGLα and DAGLβ, perhaps based upon triazole urea compounds that have been shown to be a privileged chemotype for serine hydrolase inhibition [130], is a realistic goal and would greatly aid determining the function of each enzyme. Nonetheless, on the basis of the use of knockout animals, the role that each enzyme plays in brain development, in the generation of new neurons in the adult brain and in synaptic plasticity throughout the nervous system is becoming clear. They are also emerging as key enzymes with regulatory roles in pathological processes, including driving inflammatory responses that lead to neurodegeneration. In addition to transcriptional control of their expression, there needs to be a sophisticated mechanism in place to govern the activity of these enzymes. On the basis of analogy with tetraspanins, other α/β hydrolase lipases, and the collation of results from various proteomic studies, we have now proposed several possible regulatory mechanisms for DAGLs. These include assembly into specific membrane microdomains mediated by the 4TM domain and/or palmitoylation, regulation of substrate access via phosphorylation-dependent opening and closing of a regulatory loop, and the trafficking and/or retention within specific cellular compartments mediated by the tail domain. These mechanisms are likely to 'collaborate' with other fundamental processes, such as dynamic endocytosis, to regulate the activity and function of the DAGLs.

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