

Endocannabinoids Generated by Ca^{2+} or by Metabotropic Glutamate Receptors Appear to Arise from Different Pools of Diacylglycerol Lipase

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Abstract

The identity and subcellular sources of endocannabinoids (eCBs) will shape their ability to affect synaptic transmission and, ultimately, behavior. Recent discoveries support the conclusion that 2-arachidonoyl glycerol, 2-AG, is the major signaling eCB, however, some important issues remain open. 2-AG can be synthesized by a mechanism that is strictly Ca^{2+} -dependent, and another that is initiated by G-protein coupled receptors (GPCRs) and facilitated by Ca^{2+} . An important question is whether or not the 2-AG in these cases is synthesized by the same pool of diacylglycerol lipase alpha (DAGL α). Using whole-cell voltage-clamp techniques in CA1 pyramidal cells in acute *in vitro* rat hippocampal slices, we investigated two mechanistically distinct eCB-mediated responses to address this issue. We now report that pharmacological inhibitors of DAGL α have quantitatively different effects on eCB-mediated responses triggered by different stimuli, suggesting that functional, and perhaps physical, distinctions among pools of DAGL α exist.

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Introduction

The cannabinoid system affects behavior and regulates many synaptic functions. There are two major endogenous ligands for CB1R (the main cannabinoid receptor in the brain): the eCBs N-arachidonoyl-ethanolamine (anandamide [1]) and 2-arachidonoyl-glycerol (2-AG) [2,3,4]. Anandamide and 2-AG have different synthetic and degradative pathways, and the eCB-dependent regulation of neuronal communication will be determined by the identity and subcellular sources of the eCB involved. Evidence is converging on the conclusion that 2-AG is the primary phasic signaling eCB at numerous synapses in the brain [5], whereas anandamide may regulate tonic eCB actions [6]. Strong support for the former inference comes from recent molecular genetic studies in which the primary synthetic enzyme for 2-AG, DAGL α , was knocked out in lines of mutant mice [7,8], causing a reduction of ~80% in basal 2-AG levels. Purely Ca^{2+} -dependent eCB signaling – depolarization-induced suppression of inhibition, DSI [9,10], and excitation, DSE [11] – and eCB signaling mediated by GPCRs, including group I metabotropic glutamate receptors (mGluRs), i.e., (eCB_{mGluR}) [12,13] were essentially abolished by DAGL α deletion. Yet, additional issues remain unresolved. For example, it is not known if the same DAGL α source (pool) provides 2-AG for both DSI and eCB_{mGluR}.

eCBs mediate different forms of synaptic plasticity [14], hence knowledge of the cellular source(s) of eCBs is an important issue, yet one that cannot be addressed with a global knock-out strategy. Accordingly, we have taken a pharmacological approach, using

two DAGL inhibitors to determine whether the pools of Ca^{2+} - and mGluR-dependent of 2-AG are distinguishable. If eCB responses to both stimuli were equally sensitive to the inhibitors, it would argue that the sources of 2-AG are the same, whereas marked differences in sensitivity would indicate that on a functional, and perhaps physical, level they differ. We report that the DAGL that mediates hippocampal DSI and eCB_{mGluR}, can be functionally separated into two pools. Understanding the differences in subcellular regulation of 2-AG may lead to new modes for controlling eCB actions.

Results

While recent molecular biological evidence supports the conclusion that 2-AG is the signaling eCB, pharmacological tools can be useful in teasing apart subtle features of the DAGL α /2-AG system that are not revealed by constitutive knock-out strategies. To test the hypothesis that both DSI and eCB_{mGluR} are mediated by the same source of 2-AG, we began by bath-applying DAGL inhibitors to voltage-clamped hippocampal CA1 cells in acute slices in which inhibitory post-synaptic currents (IPSCs) were pharmacologically isolated (see Methods). External application of the selective and potent inhibitor, OMDM-188 [15], 5 μM , or the less-selective inhibitor, tetrahydrolipstatin (THL), 10 μM , abolished DSI of evoked IPSCs (eIPSCs). As a percentage of baseline (100%) level, eIPSCs in the various conditions were: Vehicle: $60.2 \pm 4.0\%$, $n = 20$; OMDM-188: $95.7 \pm 1.5\%$, $n = 34$; THL: $92.8 \pm 1.4\%$, $n = 35$ (Fig. 1). We also tested two inhibitors of the

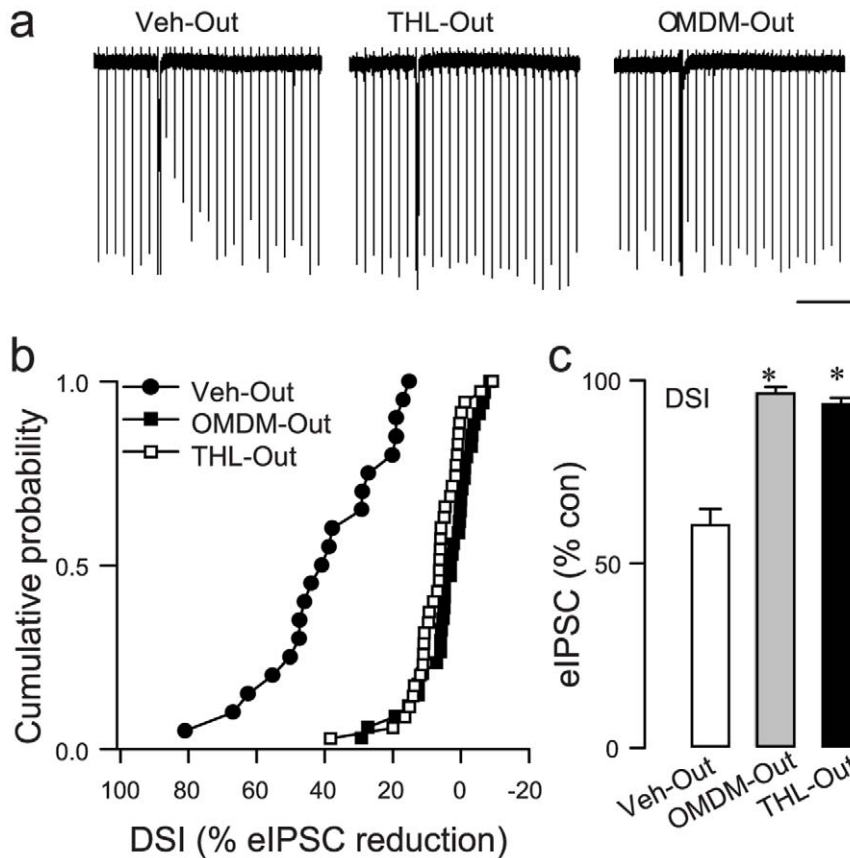


Figure 1. External application of DAGL inhibitors blocks DSI. (a) Representative DSI trial. Downward deflections are eIPSCs evoked at 4-s intervals; DSI was evoked by a 3-s voltage step to 0 mV from the holding potential of -70 mV; depression of eIPSCs after a step is the period of DSI (see text). Scale: 24 s/200 pA. (b) Bath application of OMDM-188 (5 μ M) or THL (10 μ M) essentially abolished DSI; K-S tests, $p < 0.01$. Note: values < 0 represent eIPSCs that were greater than baseline amplitudes, not enhanced DSI. (c) Group data. * $p < 0.001$, one way ANOVA on ranks. Vehicle, $n = 20$; OMDM-188, $n = 34$; THL, $n = 35$.

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2-AG degradative enzyme, monoglyceride lipase, as these inhibitors do not affect anandamide. Both JZL 184 [16], 1 μ M, and OMDM-169 [15], 2 μ M, significantly prolonged τ_{decay} of DSI (cf [17]), thus providing an independent cross-check on the hypothesis that DSI is mediated by 2-AG (Fig. 2).

Unlike DSI, eCB_{mGluR} -dependent eIPSC suppression (e.g., Fig. 3a) was highly resistant to external application of DAGL inhibitors. Responses to initial applications of DHPG were not different ($p > 0.05$) whether the slices were treated with vehicle, or DAGL inhibitors. Even when evoked by repeated 4-min bath-applications of a high concentration of the group I mGluR agonist, DHPG., 50 μ M, eCB_{mGluR} was only slightly, though statistically significantly, reduced by OMDM-188 (1st DHPG: eIPSC reduction to $54.4 \pm 4.3\%$ of baseline; 2nd DHPG: to $65.6 \pm 5.2\%$ of baseline; $n = 15$, $p < 0.005$, Figs. 3b, 3c) or THL (1st DHPG: to $58.9 \pm 3.1\%$ of baseline; 2nd DHPG: to $64.7 \pm 3.3\%$ of baseline; $n = 13$, $p < 0.05$; Figs. 3d, 3e). In vehicle-treated cells there were no significant reductions in the responses to repeated DHPG applications: 1st DHPG: to $47.5 \pm 4.5\%$ of baseline; 2nd DHPG: to $47.3 \pm 4.4\%$ of baseline; $n = 8$, $p > 0.5$, Figs. 3f, 3g). Though resistant to DAGL inhibitors, eCB_{mGluR} was strongly reduced by the CB1R antagonist, SR141716A (10 μ M) (1st DHPG: to $70.6 \pm 8.1\%$ of baseline; 2nd DHPG in SR141716A: to $89.9 \pm 5.2\%$ of baseline; $n = 5$, $p < 0.05$; data not shown), confirming previous reports [12,13], that eCB_{mGluR} is CB1R-dependent.

Because intracellular application could conceivably be more effective on eCB_{mGluR} [18], we tested the DAGL inhibitors by applying them intracellularly via infusion through the whole-cell pipette. We observed dose-dependent reduction in DSI with internal OMDM-188 (2–20 μ M) (Figs. 4a, 4b; $n = 94$), and similar significant reductions caused by internal application of THL (10 μ M, $n = 19$) (Fig. 4b). Internal DAGL inhibition did not alter eCB_{mGluR} in the same way (Figs. 4c, 4d). We examined the effects of OMDM-188 in detail and found that, even in the same cells in which DSI was reduced to negligible levels ($\leq 5\%$ eIPSC reduction, $n = 28/35$ cells; see Fig. 4d, dotted oval), eCB_{mGluR} still suppressed eIPSCs by $\sim 50\%$, i.e., OMDM-188 had almost no effect on eCB_{mGluR} . Internal infusion of 5 μ M OMDM-188 (filled triangles in Fig. 4d), reduced either eCB_{mGluR} or DSI only slightly. Interestingly, data from the cells in which 10 or 20 μ M OMDM-188 was least effective fell along a regression line around which the 5 μ M data also scattered. This could mean that in these cases diffusion of 10 or 20 μ M OMDM-188 out of the pipettes was incomplete, resulting in a lower-than-expected internal concentration of the drug.

To ensure that the DAGL antagonist had an opportunity to equilibrate throughout the cells, we extended the observations and reapplied DHPG at 15–20 min intervals with OMDM-188 (10 or 20 μ M) in the internal solution. Indeed, with repetitive DHPG application, eCB_{mGluR} suppression of eIPSCs diminished (1st DHPG: to $51.5 \pm 2.5\%$ of baseline; 2nd DHPG: to $80.4 \pm 4.4\%$ of baseline; 3rd DHPG: to $83.5 \pm 5.6\%$ of baseline; $n = 8$, $p < 0.001$,

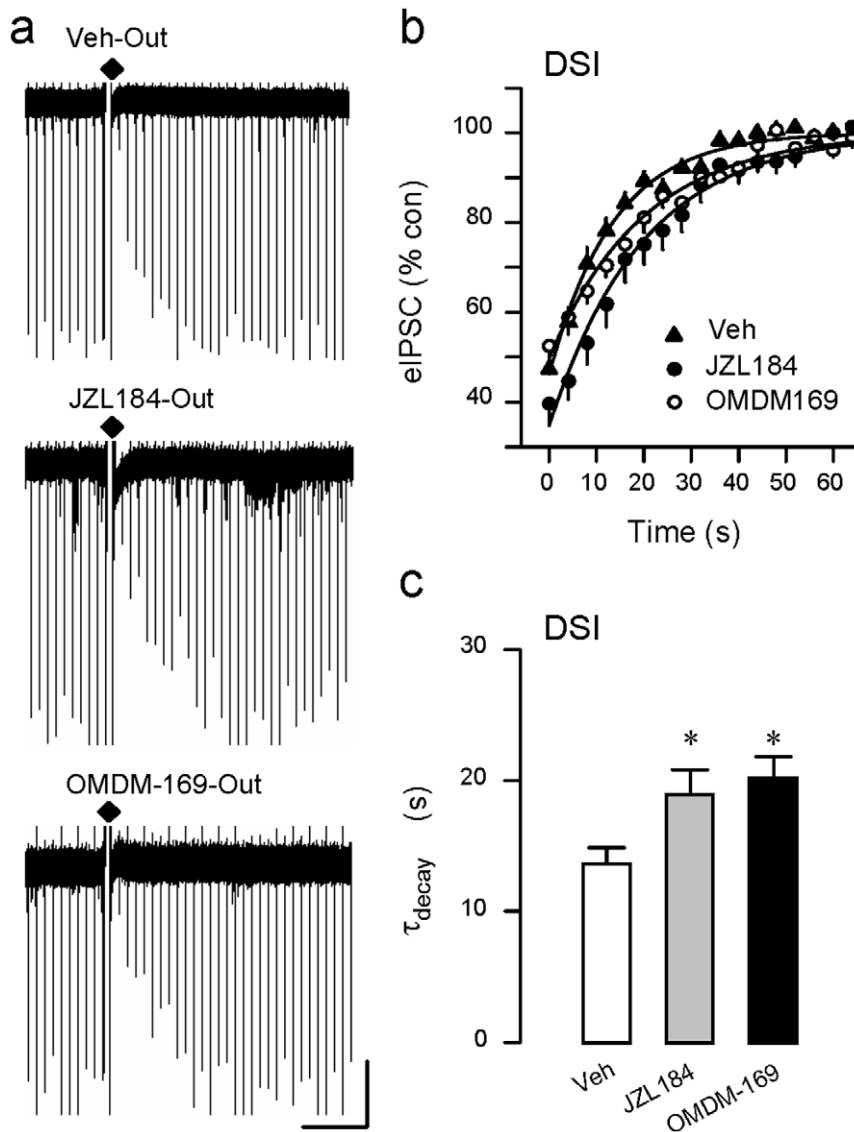


Figure 2. Antagonists of the primary catabolic enzyme for 2-AG, monoacylglycerol lipase (MAGL), prolong DSI. Diamonds indicate delivery of DSI-inducing voltage steps. Scale: 30 s/150 pA. (a) Bath application of MAGL inhibitors, JZL184 (1 μ M) or OMDM-169 (2 μ M), prolong DSI. (b) Group data showing recovery DSI in the presence of DMSO (Veh), JZL184, or OMDM-169. The DSI-inducing voltage step ended 1 s prior to time 0. The solid lines are best fitting single-exponential functions; the time constants of these functions were taken as the decay time constants (τ_{decay}) of DSI. (c) Group data showing increases in τ_{decay} of DSI in the indicated conditions. When applied for 40–120 min, JZL184 or OMDM-169, prolonged DSI; τ_{decay} was increased by \sim 40% (DMSO: 13.9 ± 1.1 s, $n = 21$; JZL184: 19.2 ± 1.7 s, $n = 15$; OMDM-169: 20.4 ± 1.6 s, $n = 15$; $p < 0.01$, one way ANOVA). doi:10.1371/journal.pone.0016305.g002

Figs. 5a, 5b). The decrease in eCB_{mGluR} did not reflect spontaneous response ‘rundown’, because responses were stable with infusion of vehicle (DMSO or ethanol) (1st DHPG: to $44.0 \pm 4.7\%$ of baseline; 2nd DHPG: to $44.4 \pm 4.0\%$ of baseline; 3rd DHPG: to $48.1 \pm 5.4\%$ of baseline; $n = 7$, $p > 0.5$; Figs. 5e, 5f).

It was not clear whether the passage of time alone accounted for the increased inhibitor efficacy, or whether suppression of eCB_{mGluR} by DAGL inhibition was use-dependent, i.e., whether it was enhanced by repetitive stimulation. To distinguish the effects of longer OMDM-188 infusions from those of repeated DHPG application, in another group of cells we delayed the 1st DHPG application until 30–40 min after break-in, i.e., it was given at the same relative time after break-in as the 2nd DHPG application in the original group (cf, Figs. 5b, 5d). The eCB_{mGluR} eIPSC suppression was the same in the two 1st DHPG groups: (30–

40 min post-break-in: to $50.5 \pm 4.3\%$ of baseline, $n = 11$; 10–20 min post-break-in: to $49.5 \pm 2.5\%$ of baseline, $n = 16$; n.s. $p > 0.1$). A 2nd DHPG application (i.e., 50–60 min post-break-in) given to cells receiving a delayed 1st DHPG application, induced less eIPSC depression (2nd DHPG: to $73.4 \pm 4.3\%$ of baseline; 1st DHPG: to $46.8 \pm 1.7\%$ of baseline; $n = 5$, $p < 0.01$; Figs. 5c, 5d). As a final check, we compared the magnitudes of the 2nd DHPG responses, obtained either 30–40 or 50–60 min post-break-in, and found that they were indistinguishable ($p > 0.1$, Figs. 5b, 5d). Hence, gradual diminution in the eCB_{mGluR} response depended on the presence of both the DAGL inhibitor and repeated DHPG stimulation, and could not be explained simply by the duration of the inhibitor application.

The decline in eCB_{mGluR} just described might reflect use-dependent depletion of a pool of 2-AG. Two predictions would

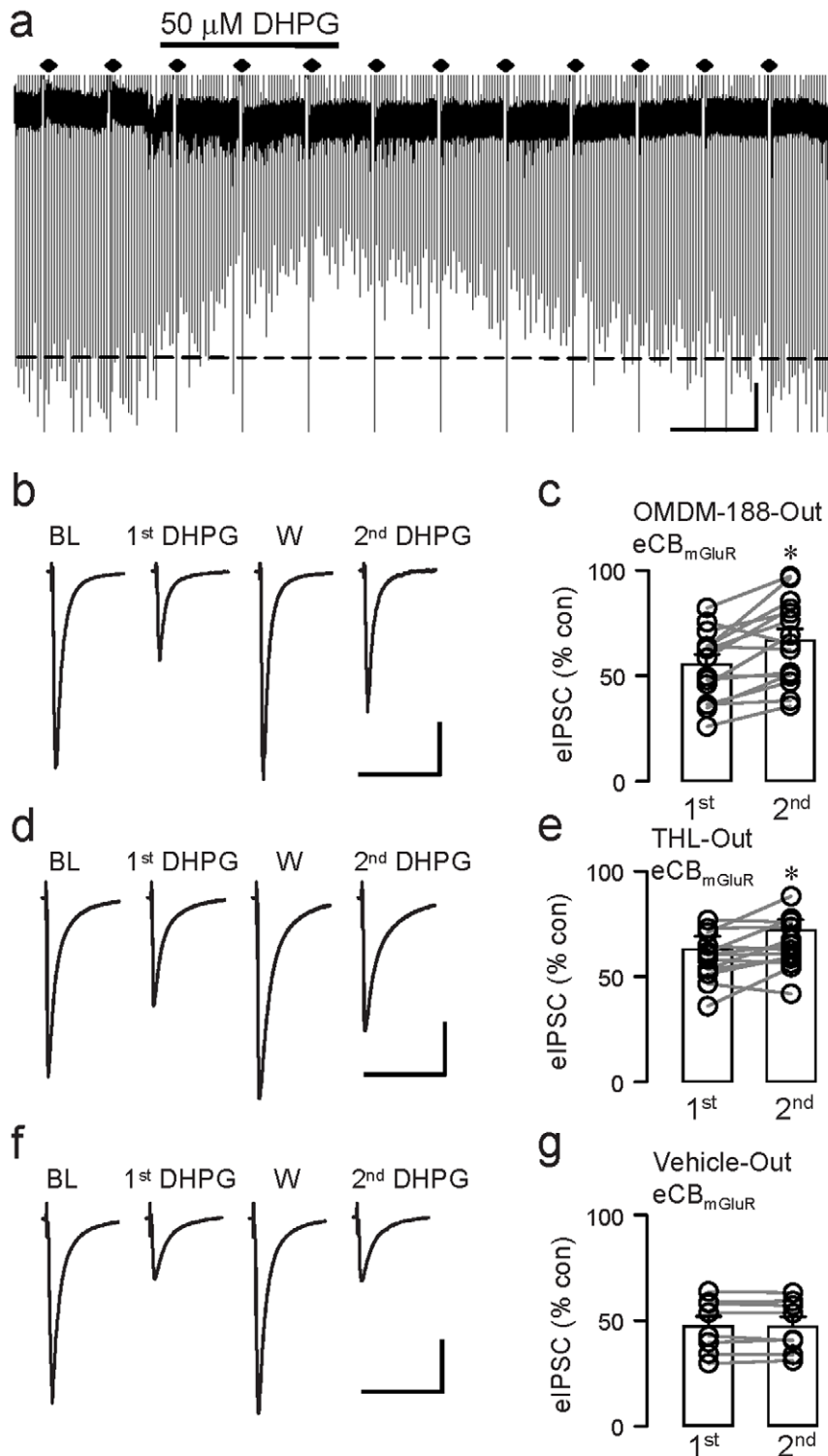


Figure 3. External application of DAGL inhibitors has minimal effects on eCB_{mGluR}. (a) Sample trace showing eIPSCs (downward deflections) and DSI trials (diamonds) in external OMDM-188, 5 μM. Note DSI is abolished despite continued suppression of eIPSCs by DHPG. (b) Sample eIPSCs (each trace is the mean of 3) from the same cell in external OMDM-188. BL denotes the baseline response, and W, the response obtained after washing out DHPG. DHPG was applied twice at 20-min intervals starting ~15–20 min after break-in. (d) As in (b), with THL in the saline. (f) As in (b), with vehicle only in the saline. (c)(e)(g) Group data for experiments in (b), (d), and (f), respectively (paired-t-tests). OMDM-188-Out, n = 15, p < 0.01; THL-Out, n = 13, p < 0.05; Vehicle-Out, n = 8, p > 0.5. Scale: 20 ms/200 pA. doi:10.1371/journal.pone.0016305.g003

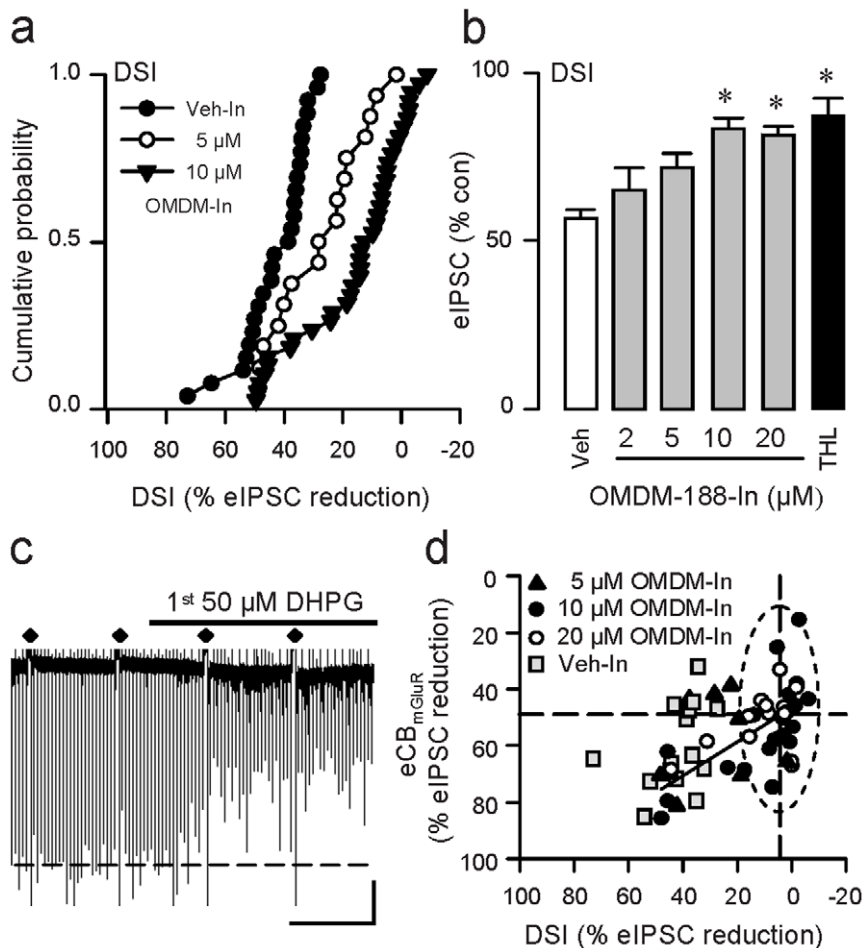


Figure 4. Intracellular application of DAGL inhibitors reduces DSI to a greater extent than it reduces eCB_{mGluR} . (a) Intracellular infusion of OMDM-188 reduced DSI; each symbol represents averaged DSI values (3 trials) from one cell. Only data for 5 μ M (n = 16) and 10 μ M (n = 38) groups shown for clarity; both differ from Veh-In group, K-S test, $p < 0.01$. (b) Group data. Includes results in (a), plus 2 μ M (n = 6), 20 μ M (n = 40) groups. * $p < 0.001$, one-way ANOVA on ranks. (c) Sample continuous trace showing DSI trials and eIPSC suppression by eCB_{mGluR} . Scale: 2 min/200 pA. (d) Group data for experiments as in (c) with internal 5, 10 or 20 μ M OMDM-188, or Veh only. DSI and eCB_{mGluR} were measured, and eCB_{mGluR} was plotted against DSI for each cell. For the cells (n = 28/35) within dotted oval, mean eIPSC reduction from baseline by DSI is $4.3 \pm 1.06\%$, mean eCB_{mGluR} eIPSC reduction is $49.0 \pm 2.32\%$. The straight line is a linear regression fit to the data for 10 and 20 μ M DHPG (see text for discussion). doi:10.1371/journal.pone.0016305.g004

follow from this hypothesis: 1) evoking eCB_{mGluR} with a low concentration of DHPG should cause less of, or a slower onset of, a reduction in the DHPG effect in the presence of a DAGL inhibitor, and 2) inhibiting 2-AG synthesis by blocking another major synthetic enzyme in this pathway, phospholipase C_{β} [19], should also give rise to a use-dependent decline in eCB_{mGluR} .

In experiments thus far, we used 50 μ M DHPG, which is a high concentration. To test the prediction that, in the presence of a DAGL inhibitor, weaker stimulation of mGluRs would induce less decline in eCB_{mGluR} , we used 10 μ M DHPG, with 20 μ M OMDM-188 in the internal solution. In this case, we did not observe significant reduction in eCB_{mGluR} , even with four applications of DHPG given to the same cell (1st DHPG: to $69.9 \pm 8.3\%$ of baseline; 2nd DHPG: to $72.9 \pm 7.5\%$ of baseline; 3rd DHPG: to $70.1 \pm 9.9\%$ of baseline; 4th DHPG: to $74.6 \pm 9.2\%$ of baseline; n = 5, $p > 0.1$, data not shown). Hence, the declines in eCB_{mGluR} seen with the higher DHPG concentration were not caused simply by repetitive activation of mGluRs.

In testing the second prediction, we found that both DSI and eCB_{mGluR} were induced in the presence of the PLC inhibitor,

U73122 (6 μ M), however, when elicited repeatedly, eCB_{mGluR} suppression of eIPSCs diminished (1st DHPG: eIPSCs were reduced to $40.1 \pm 3.8\%$ of baseline; 2nd DHPG: to $67.3 \pm 6.9\%$ of baseline; 3rd DHPG: to $62.1 \pm 5.8\%$ of baseline; n = 5, $p < 0.01$, Figs. 6a, 6b). As expected, since DSI is normal (though eCB_{mGluR} is abolished) in the $PLC_{\beta 1}^{-/-}$ mouse [19], DSI was not altered by the PLC inhibitor (eIPSC reduction to $66.8 \pm 3.5\%$ of baseline, n = 8).

If DSI and eCB_{mGluR} arise from the same source of 2-AG, then strong activation of one mechanism could alter the response of the other. Because strong stimulation of mGluRs with bath-applied DHPG has persistent effects on eIPSCs and DSI [14,20] that could confound interpretation, we tested this prediction by determining if repetitive elicitation of DSI would affect eCB_{mGluR} . Pipettes contained normal intracellular solution. We used two 4-min applications of 50 μ M DHPG separated by a 4-min period during which DSI was elicited with 1-s depolarizing steps given at 12-s intervals. The 12-s interval is too short to permit full recovery from each DSI episode, with the result that eIPSCs are continually suppressed by the DSI mechanism for the period of stimulation (cf, [20]). As we have reported, an even longer period of repetitive

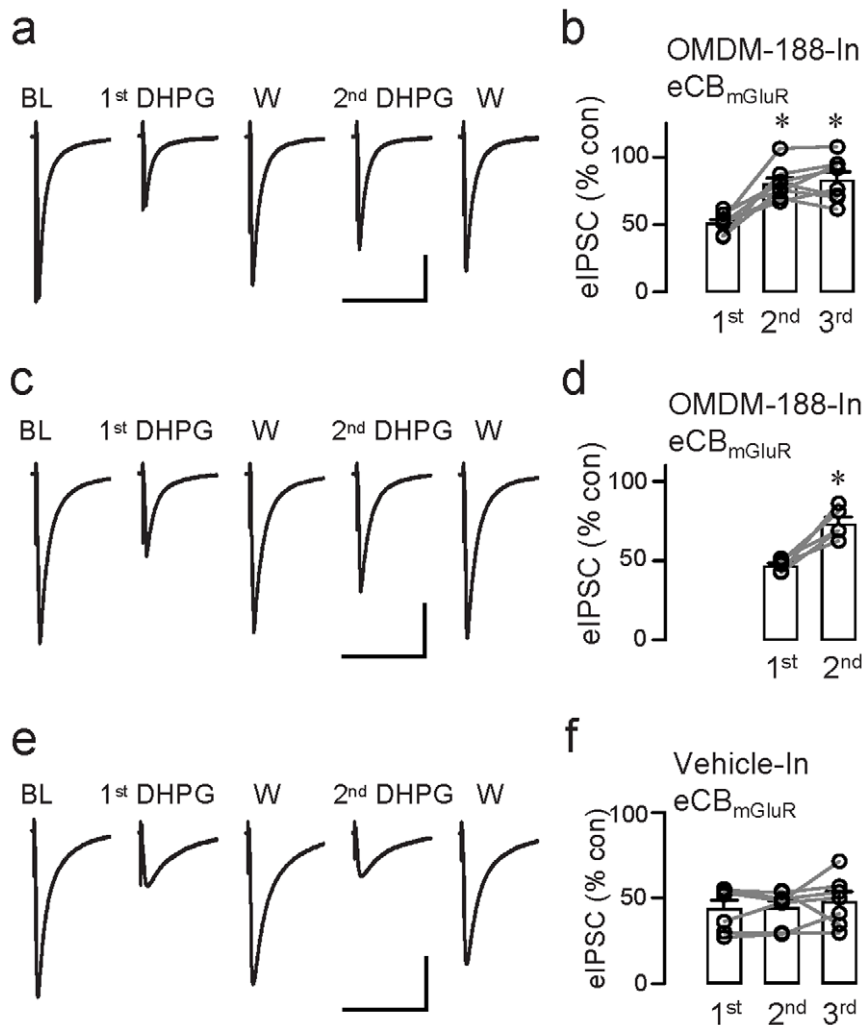


Figure 5. Use-dependent reduction of eCB_{mGluR} with internal DAGL inhibitor. (a) Sample eIPSCs (each trace is the mean of 3) from the same OMDM-188-loaded (20 μ M in the pipette) cell in the indicated conditions. BL denotes the baseline response, and W, the response obtained after washing out DHPG. DHPG was applied 3 times at 20-min intervals starting \sim 15–20 min after break-in. (c) As in (a) except that the 1st DHPG application was given \sim 40 min after break-in – i.e., at the same time as the 2nd DHPG application in (a) – and the 2nd one at 50–60 min after break-in. (e) As in (a), with vehicle only present in the internal solution. (b)(d)(f) Group data for experiments in (a), (c), and (e), respectively. * p <0.001, one way repeated ANOVA; Scale: 20 ms/200 pA. doi:10.1371/journal.pone.0016305.g005

elicitation does not persistently diminish DSI [20], nevertheless, it was possible that repetitive DSI stimulation could reduce the magnitude of the subsequent eCB_{mGluR} interval if a common 2-AG pool was being tapped. Nevertheless, we found no evidence that this occurred. The eIPSCs were suppressed to $49.8 \pm 5.8\%$ of baseline by 50 μ M DHPG before the repetitive DSI stimulation and to $48.4 \pm 4.8\%$ of baseline afterwards; $n = 6$, $p > 0.1$; Figs. 6c, 6d). We also tested the possibility that repetitive DSI stimulation might somehow alter eCB_{mGluR} if DSI expression was first blocked by OMDM-188. Again, after a 4-min period of repetitive DSI, 50 μ M DHPG induced an eIPSC suppression to $48.6 \pm 5.2\%$ of baseline, not significantly different from the 1st DHPG responses obtained either 10–20 or 30–40 min post break-in, $p > 0.5$ (data not shown).

Discussion

During a previous investigation [20], we noticed differences in the efficacy of THL on DSI or eCB_{mGluR}, however, in view of the non-specific effects of THL, no definite conclusions could be

drawn. Moreover, it was unclear if both DSI and eCB_{mGluR} were mediated by the same eCB. In showing that DAGL α , and by implication 2-AG, are involved in both processes, the recent studies on DAGL α ^{-/-} mice [7,8] prompted an examination of whether or not the same sources of 2-AG mediate eCB responses evoked by different stimuli. The hypothesis that a unitary pool of DAGL α supplies 2-AG for DSI and eCB_{mGluR} predicts they should be similarly affected by pharmacological inhibitors of DAGL α . Using different inhibitors and modes of drug application, we observed marked quantitative distinctions between the responses produced by DSI and eCB_{mGluR}. In particular: 1) eCB_{mGluR} is much less sensitive to block by DAGL-inhibitors than is DSI, and 2) repetitive activation of the eCB_{mGluR} system enhanced the effect of DAGL inhibition, whereas such use-dependence was not a feature of the block of DSI.

The differences in sensitivity to the DAGL inhibitors were obvious even when both responses were recorded in the same cell, ruling out systematic differences between experiments. Differences in inhibitor-enzyme interactions are also ruled out, as DAGL α

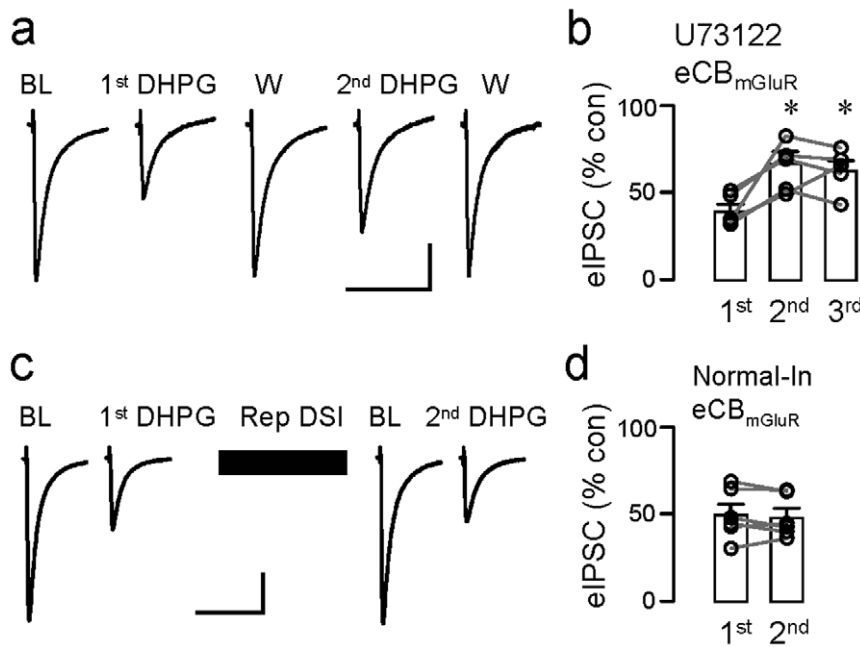


Figure 6. Regulation of eCB_{mGluR}. (a) Sample eIPSCs (each trace is the mean of 3) from the same U73122-loaded cell in the indicated conditions (U73122, 6 μ M, also present in the bath). BL denotes the baseline response, and W, the response obtained after washing out DHPG. DHPG was applied 3 times at 20-min intervals starting \sim 15–20 min after break-in. (b) Group data for experiments in (a); $p < 0.01$, one way repeated ANOVA; Scale: 20 ms/200 pA. (c) Sample eIPSCs (each trace is the mean of 3) response to 50 μ M DHPG before and after repetitive DSI stimulation (1-s depolarizing steps were given at 12-s intervals continuously for 4 min). (d) Group data showing that eCB_{mGluR} was not affected by repetitive DSI stimulation. Scale: 20 ms/200 pA.

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mediates both responses. A reasonable interpretation is that different pools of DAGL α provide 2-AG in the two cases. The hypothetical pools would not simply represent differences in spatial localization along the pyramidal cell: with external application the inhibitors have equal access to the surface of the cells, but had only slight effects on eCB_{mGluR}, despite abolishing DSI. A plausible explanation for the differing efficacy of internal and external application on eCB_{mGluR} is that the DAGL α responsible for eCB_{mGluR} is much less accessible to externally applied inhibitor.

The suggestion of different pools of DAGL α is in good agreement with previous observations. For example, the DAGL α involved in eCB_{mGluR} is found in dendrites apposed to glutamate releasing nerve terminals [21,22,23]. In contrast, DAGL α has not been reported near perisomatic GABAergic synapses like those we have studied [21,22]. Since eCBs can spread longitudinally along cell structures for only \sim 10 μ m [24], 2-AG produced by DAGL α near excitatory synapses, which are located on CA1 pyramidal cell dendrites >50 μ m from the somata, is most unlikely to account for DSI. While the failure to have detected DAGL α in pyramidal cell somata may reflect technical limitations in available morphological tools, it does highlight the possibility that different parts of a cell employ different pools of DAGL α for generating eCBs. We also note, however, that while this seems to be a parsimonious proposal, the lack of identification of the DAGL α responsible for DSI means that other possibilities are not ruled out. For example, our data would be compatible with differences in DAG (rather than DAGL α) pools. DAG can be produced by several mechanisms besides PLC [5]. We confirm the 2-AG produced by mGluRs is dependent on PLC β , but knocking out [19], or inhibiting [20] PLC β does not affect hippocampal DSI. Since DSI is dependent on DAGL α , and therefore probably on 2-AG, it could be mediated by a source of DAG that is distinct

from that underlying eCB_{mGluR}. Interestingly, use-dependence of eCB_{mGluR} reduction was seen when PLC, rather than DAGL, was inhibited, supporting the concept that the DAGL α -PLC pathway is upstream of the depletable source of 2-AG for eCB_{mGluR}.

Attempts to use DAGL α inhibitors to probe the eCB system have produced controversial results [18,20,25,26]. We had observed that external, though not internal, THL application affected DSI [26], and Min et al. [25] arrived at conclusions that are diametrically opposed to our present observations. Probably the use of higher concentrations of the inhibitors, longer application times, and repetitive activation of the 2-AG-dependent responses are the primary explanations for the reported variability. In particular, difficulties in bath- or internally-applying these lipophilic agents to cells in brain slices could account for the requirement for higher concentrations, especially with intracellular techniques, because restricted efflux from whole-pipettes can result from partial electrode occlusion or adherence of the drugs to pipette glass. Nevertheless, the possibility of non-specific effects must also be kept in mind.

The use-dependence of the reduction in eCB_{mGluR} is puzzling, and while direct evidence is not available, one speculative scenario is intriguing: decoupling of 2-AG synthesis and release could partly account for the data. For example, if the pool of DAGL α -dependent 2-AG that is present in unstimulated cells [4,7,8] could be mobilized by mGluR activation, its release would not be closely tied to DAGL α stimulation. Release from such a pool could persist after DAGL α was inhibited, decreasing in a use-dependent way as the preformed 2-AG pool diminished. If such decoupling were greater for eCB_{mGluR} than DSI, it could also account for their different sensitivities to DAGL inhibitors. Finally, 2-AG release from an existing pool could explain why knocking out PLC β abolishes eCB_{mGluR} [19], while a potent PLC inhibitor was

ineffective [20,26] (unless eCB_{mGluR} is repetitively elicited, Fig. 6). Thus, our present data is consistent with, and extends, previous proposals [26,27,28].

Factors that could be responsible for maintaining the hypothetically distinct DAGL α (or DAG) pools are unknown. Physical separation of protein components of the biosynthetic cascades, perhaps sequestration on lipid rafts [29] or other factors related to the heterogeneity of the lipid bilayer could be involved [28]. In this context, it may also be worth noting that DAGL $\alpha^{-/-}$ mice also showed a reduction of \sim 50% in the levels of anandamide [7,8]. Therefore, although the greater reduction in 2-AG supports the concept that 2-AG is the eCB that mediates many phasic CB1R-dependent responses, the possibility cannot be eliminated that anandamide might play a contributory role in some cases.

The model of 2-AG release from a pre-existing pool differs from the conventional “on-demand” model of eCB production in which synthesis and release are necessarily tightly coupled to each other. The hypothesis may account in part for some of the multiple mechanisms of eCB responses [20,26], and may help reconcile the negative pharmacological reports with DAGL inhibitors, e.g. [25], with data from the DAGL $\alpha^{-/-}$ studies [7,8]. The hypothesis would also be consistent with evidence that 2-AG release is a regulated step, perhaps involving the eCB transporter [30,31]. Testing this idea and investigating the subcellular distribution and regulation of DAGL in general will be important future tasks.

Methods

Preparation of slices

All experimental protocols were reviewed and approved by the University of Maryland School of Medicine IACUC (IACUC approval #0609001), and all animal handling was conducted in accordance with national and international guidelines. The number of animals used was minimized, and all necessary precautions were taken to mitigate pain or suffering.

Hippocampal slices were obtained from 4- to 6-week-old male Sprague-Dawley rats. After rats were sedated with isoflurane and decapitated, the hippocampi were removed and 400- μ m-thick slices were cut on a Vibratome (model VT1200s, Leica Microsystems, Inc., Bannockburn, IL) in an ice-cold extracellular recording solution. Slices were stored in a holding chamber on filter paper at the interface of this solution and a moist, oxygenated atmosphere at room temperature for \geq 1h before transfer to the recording chamber (RC-27L, Warner Instruments, CT) and warmed to 30–31°C. The extracellular solution contained (mM): 120 NaCl, 3 KCl, 2.5 CaCl₂, 2 MgSO₄, 1 NaH₂PO₄, 25 NaHCO₃, and 20 glucose, and was bubbled with 95% O₂, 5% CO₂ (pH 7.4).

Electrophysiology

Whole-cell voltage-clamp recordings of CA1 pyramidal cells were made using the blind patch method. Pipettes were pulled from thin walled glass capillaries (1.5 O.D., World Precision Instruments, Sarasota, FL). Electrode resistances in the bath were 3–6 M Ω with internal solution containing (mM): 90 CsCH₃SO₄, 1 MgCl₂, 50 CsCl, 2 MgATP, 0.2 Cs₄-BAPTA, 10 HEPES, 0.3 Tris

GTP and 5 QX314. If the series resistances, which was checked by -2 mV voltage steps throughout experiments, changed $>$ 20%, the data were discarded. The holding potential was -70 mV in all experiments. Monosynaptic eIPSCs were elicited by 100- μ s-long extracellular stimuli delivered at 0.25 Hz with concentric bipolar stimulating electrodes placed in *s. radiatum*. NBQX (10 μ M) and D-AP5 (20 μ M) were present in all experiments to block glutamatergic EPSCs. Slices were pretreated in the holding chamber with the irreversible P/Q-type voltage-gated Ca²⁺-channel toxin, ω -agatoxin GVIA (agatoxin, 300 nM) for \geq 1 h to reduce the contribution of eCB-insensitive eIPSCs in all experiments [32]. Data were collected with an Axopatch 1C amplifier (Molecular Devices, Sunnyvale, CA), filtered at 1 kHz and digitized at 5 kHz using a Digidata 1200 (Molecular Devices) and Clampex 8 software (Molecular Devices).

The bath solution was oxygenated with 95% O₂/5% CO₂ gas, and perfused continuously through the recording chamber at \sim 1 ml/min. For external applications, slices were preincubated for $>$ 40 min with OMDM-188, THL, OMDM-169, DMSO, or ethanol, and the drug was also present in the bath solution throughout the recording. The final concentration of the solvent, DMSO or ethanol, was 0.05% (v/v) or less for both OMDM-188 and THL. JZL184 was obtained from Cayman Chemical, OMDM-188 and OMDM-169 was synthesized by Giorgio Ortora and Enrico Morera, and all other chemicals were purchased from Sigma (St. Louis, MO).

Data analysis

To measure DSI, we evoked IPSCs at 4-s intervals and depolarized the postsynaptic cell to 0 mV for 1–5 s at 90-s intervals. The magnitude of DSI was calculated as follows: DSI (%) = 100 \times [1 - (mean of 4 IPSCs after depolarization/mean of 5 IPSCs before depolarization)]. Values of 2–3 DSI trials were averaged for a given condition. The decay time constant of DSI (τ_{decay}) was determined by fitting the data with a single-exponential decay function in SigmaPlot 10.0. Two-tailed paired t-tests were used whenever appropriate; otherwise unpaired t-tests were used for single comparisons. Statistical tests among groups were done with one-way ANOVA. For comparison of results from repeated DHPG applications, we used one-way repeated ANOVA. The significance level for all tests was $p < 0.05$ (*). Group means \pm SEMs are shown for display purposes. For comparison of cumulative distributions, we used the Kolmogorov-Smirnov (K-S) test, available at http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html.

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Author Contributions

Conceived and designed the experiments: BEA LZ VDM. Performed the experiments: LZ MW. Analyzed the data: LZ MW BEA. Contributed reagents/materials/analysis tools: TB VDM. Wrote the paper: LZ MW VDM BEA.

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