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DAGLα AND DAGLβ COOPERATIVELY REGULATE THE PRODUCTION OF 2-ARACHIDONOYL GLYCEROL IN AUTAPTIC HIPPOCAMPAL NEURONS

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Running title page

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DAGLbeta in neurons

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Δ^9-THC, Δ^9-tetrahydrocannabinol; DSE, depolarization induced suppression of excitation; 2-AG, 2-arachidonoyl glycerol; eCB, endocannabinoid; DAGLα, diacylglycerol lipase alpha; DAGLβ, diacylglycerol lipase beta; MSE, metabotropic suppression of excitation; DHPG, dihydroxyphenyl glycine; EPSC, excitatory post-synaptic current.

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ABSTRACT

Cannabinoids are part of an endogenous signaling system consisting of cannabinoid receptors and endogenous cannabinoids (eCBs) as well as the enzymatic machinery for their synthesis and degradation. Depolarization-induced suppression of excitation (DSE) is a form of cannabinoid CB_1 receptor-mediated inhibition of synaptic transmission that involves the production of the eCB 2-arachidonoyl glycerol (2-AG). Both DAGLα and DAGLβ can produce 2-AG *in vitro* but evidence from knockout animals argues strongly for a predominant, even exclusive, role for DAGLα in regulation of 2-AG-mediated synaptic plasticity. What role, if any, might be played by DAGLβ remains largely unknown. Cultured autaptic hippocampal neurons exhibit robust DSE. With the ability to rapidly modulate expression of DAGLα and DAGLβ in these neurons with shRNA they are well-suited for a comparative study of the roles of each isoform in mediating DSE. We find that RNAi knockdown of DAGLα substantially reduces autaptic DSE, shifting the 'depolarization response curve' from an ED50 value of 1.7 sec to 3.0 sec. Surprisingly, DAGLβknockdown diminishes DSE as much or more (ED50 6.4 sec), suggesting that DAGLβ is also responsible for a portion of 2-AG production in autaptic neurons. Similarly, the two DAGLs both contribute to the production of 2-AG via group I metabotropic glutamate receptors. Our results provide the first explicit evidence for a role of DAGLβ in modulating neurotransmission.

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INTRODUCTION

Cannabinoids first gained notoriety as the psychoactive ingredients of marijuana and hashish. These agents, chief among them Δ^9-tetradhydrocannabinol (Δ^9-THC, (Gaoni and Mechoulam, 1964)), act on endogenous targets, designated the CB_1 and CB_2 cannabinoid receptors (Matsuda et al., 1990; Munro et al., 1993). These receptors are found throughout much of the brain and body and are implicated in a host of physiological functions (Piomelli, 2003). The body also makes endogenous cannabinoids (eCBs), with 2-arachidonoyl glycerol (2-AG, (Stella et al., 1997)) deeply implicated in endogenous modulation of neurotransmission via CB_1 (Kano et al., 2009). Understanding the production and breakdown of 2-AG is necessary in order to appreciate its physiological role and develop therapeutics targeting 2-AG metabolism. As a lipid, 2-AG is unlikely to be packaged and released as a conventional neurotransmitter, but is instead produced enzymatically by cleavage from a precursor lipid (likely diacylglycerol) primarily by either of two diacylglycerol lipases (DAGLα and DAGLβ (Bisogno et al., 2003; Piomelli, 2003; Stella et al., 1997)). Several studies support a DAGL role in endocannabinoid-mediated neuronal plasticity (e.g.(Chevaleyre and Castillo, 2003; Jung et al., 2005; Straiker and Mackie, 2005)), but these all relied on pharmacological tools that do not distinguish between the two DAGLs. Separately, expression studies offered differing pictures of the relative prominence of these enzymes (Bisogno et al., 2003; Jung et al., 2005).

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Consequently, the question of which DAGL mediated cannabinoid modulation remained unresolved for several years.

Three independent reports of DAGL knockout mice appeared in 2010 and 2011 that seemed to resolve the question of the relative roles of DAGLα vs. DAGLβ in endocannabinoid-mediated synaptic plasticity (Gao et al., 2010; Tanimura et al., 2010; Yoshino et al., 2011). Tanimura et al. found that synaptic plasticity was absent in DAGLα knockout mice for each of eight forms of cannabinoid-mediated plasticity examined, while limited tests of DAGLβ knockout mice showed no changes. A separate study by Gao et al. (Gao et al., 2010) found that hippocampal depolarization-induced suppression of inhibition (DSI) was similarly dependent on DAGLα. Lastly a third study appeared the following year that showed a dominant role for DAGLα in synaptic plasticity in the prefrontal cortex (Yoshino et al., 2011). These three studies all strongly suggested that DAGLα, rather than DAGLβ, was the predominant 2-AG synthesizing enzyme in adults for endocannabinoid-mediated modulation of neurotransmission. A recent study (Hsu et al., 2012) implicates DAGLβ in inflammatory responses in macrophages.

However, questions remain regarding DAGLα and DAGLβ in synaptic transmission; particularly with respect to the functional role of DAGLβ, as it is more abundantly expressed in the developing CNS (Bisogno et al., 2003; Wu et al., 2010). Also, constitutive knockout mice are subject to adaptive responses that may obscure the true role of the targeted gene. As a consequence, there is

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a need to probe the relative roles of these two enzymes with other tools. This is particularly compelling for studies using DAGLα knockouts, because of the known role of this enzyme in neurodevelopment (Berghuis et al., 2007; Keimpema et al., 2011).

To explore this question in detail we developed RNA interference (RNAi) tools for use in autaptic hippocampal neurons. These cultured neurons express a robust, well-characterized CB_1-based cannabinoid signaling system, with multiple forms of endocannabinoid-mediated synaptic plasticity including depolarization induced suppression of excitation (DSE) and metabotropic suppression of excitation (MSE) (Straiker et al., 2009; Straiker and Mackie, 2005; Straiker and Mackie, 2007). This system has recently allowed a detailed dissection of the enzymes capable of participating in the breakdown of 2-AG (Straiker et al., 2009; Straiker et al., 2011). In the current experiments we examined the consequences of knocking down DAGLα or DAGLβ on autaptic DSE and MSE.

MATERIALS AND METHODS

Culture preparation

All procedures used in this study were approved by the Animal Care Committee of the Indiana University and conform to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Mouse hippocampal neurons isolated

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from the CA1-CA3 region were cultured on microislands as described previously (Bekkers and Stevens, 1991; Furshpan et al., 1976). Neurons were obtained from animals (age postnatal day 0-2, of either sex) and plated onto a feeder layer of mouse hippocampal astrocytes that had been laid down previously (Levison and McCarthy, 1991). Cultures were grown in high-glucose (20 mM) medium containing 10% horse serum, without mitotic inhibitors and used for recordings after 8 days in culture and for no more than three hours after removal from culture medium.

Electrophysiology

When a single neuron is grown on a small island of permissive substrate, it forms synapses—or "autapses"—onto itself. All experiments were performed on isolated autaptic neurons. Whole cell voltage-clamp recordings from autaptic neurons were carried out at room temperature using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). The extracellular solution contained (in mM) 119 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.5 $MgCl_2$, 30 glucose, and 20 HEPES. Continuous flow of solution through the bath chamber (~2 ml/min) ensured rapid drug application and clearance. Drugs were typically prepared as stocks, then diluted into extracellular solution at their final concentration and used on the same day.

Recording pipettes of 1.8-3 MΩ were filled with (in mM) 121.5 KGluconate, 17.5 KCl, 9 NaCl, 1 MgCl2, 10 HEPES, 0.2 EGTA, 2 MgATP, and 0.5 LiGTP. Access resistance and holding current were monitored and only cells with both stable

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access resistance and holding current were included for data analysis. The membrane potential was held at –70 mV and excitatory postsynaptic currents (EPSCs) were evoked by triggering an unclamped action current with a 1.0 ms depolarizing step. EPSC size was calculated by integrating the evoked current to yield a charge value (in pC). Calculating the charge value in this manner yields an indirect measure of the amount of neurotransmitter released while minimizing the effects of cable distortion on currents generated far from the site of the recording electrode (i.e., the soma). Data were acquired at a sampling rate of 5 kHz and filtered at 2 kHz.

To evoke depolarization induced suppression of excitation (DSE), after establishing a 10-20 second 0.5 Hz baseline, DSE was evoked by depolarizing to 0 mV for 1-10 seconds, followed by resumption of a 0.5 Hz stimulus protocol for 10-80+ seconds, until EPSC's recovered to baseline values. In experiments investigating 2-AG, the likely endogenous mediator of DSE in this preparation, was applied at 5μM since this concentration was found to correspond to maximal DSE in autaptic cultures (Straiker and Mackie, 2005). Depolarization response curves derived from a depolarization series were used to derive an effective dose (depolarization) 50 (ED50). Curves were compared using 95% confidence intervals. We did not observe an effect of knockdown on average baseline EPSC size (EPSC size with DAGLα RNAi: 1.6 ± 0.3nA, n=10; with DAGLβ RNAi: 2.3 ± 0.24 nA, n=5; same-day untransfected controls: 2.3 ± 1.2 nA, n=6). Responses to exogenously applied 2-AG were intact in RNAi-transfected neurons (Relative

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EPSC charge for DAGLα RNAi: 0.60 ± 0.03, n=8; for DAGLβ RNAi: 0.52 ± 0.07, n=3).

Neuronal Transfection

We transfected neurons using a calcium phosphate-based method adapted from Jiang et al. (Jiang et al., 2004). Briefly, plasmids for the protein of interest and EYFP or mCherry (2μg/well) were combined with 2M $CaCl_2$ in water and gradually added to 2x HEPES buffered saline (HBS); the mixture was then added to serum-free neuronal media. Coverslips were incubated with this mixture for 2.5 hours while extra serum-free media was acidified in a 10% CO_2 incubator. At the end of 2.5 hours, the reaction mixture was replaced with acidified serum-free media for 20 mins. After this cells were returned to their home wells. Each data set was taken from at least two different neuronal platings.

Western Blot

HEK293 cells were grown to approximately 60% confluency in 6 well dishes. V5-tagged murine DAGLα or β (kindgiftsfromDanieliPiomelli) with or without DAGL RNAi expression plasmids were transfected into these cells using Lipofectamine 2000 as per manufacturer's instructions (Invitrogen, Carlsbad, CA). Following a 72 hour incubation, cells were removed from the incubator, chilled on ice, and washed with ice-cold 1X PBS. They were then covered with 200 μl lysis buffer containing (in mM) 100 Tris (pH 7.4), 150 NaCl, 8 CHAPS, 1 EDTA, 6 $MgCl_2$ and 0.1 PMSF) and incubated on ice 5 minutes. Cells were then scraped

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and suspensions were sonicated and centrifuged at 10,000 x g and 4°C. The supernatant was collected and protein concentration was determined using the Bradford assay. Samples (25μg protein) were run on a 10% discontinuous SDS-PAGE gel. The separated proteins were transferred to nitrocellulose and Westerns were performed using a mouse anti-V5 antibody (Cat# R960-25, Invitrogen, Carlsbad, CA). Primary antibody was diluted 1:1,000 in a 1:1 mixture of Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE.) and 1xPBS. A goat anti-mouse conjugated with an IR680 dye (Cat# 926-32220, Li-Cor, Lincoln, NE) was used as the secondary antibody (diluted 1:5,000 as above). Western blots were scanned on an Odyssey scanner, and the signal intensities determined using ImageJ (available at http://rsbweb.nih.gov/ij/). Background was subtracted from plots and the area under the curve was determined for each DAGL-expressing sample. Four separate experiments were performed and knockdown analyzed via two-tailed Student's t tests using Prism 4 (GraphPad, San Diego, CA).

Immunocytochemistry

Cultured neurons were fixed in 4% paraformaldehyde for 30-60 mins, washed, treated with a detergent (Triton-X100, 0.3% or saponin, 0.1%) and milk (5%) in PBS. Neurons were incubated with antibodies against DAGLα or DAGLβ alone or in combination with monoclonal (mouse) antibodies MAP2 (to identify dendrites, Millipore, Temecula, CA, No. MAB3418, 1:500), or SV2 (to identify axon terminals, Developmental Studies Hybridoma Bank, Iowa City IA, 1:500) or PSD95 (to identify dendritic spines, Genetex, Irvine CA, GTX80682, 1:500)

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overnight at 4°C. For the staining of DAGLα and DAGLβ in combination, a DAGLα antibody raised in guinea pig was used. Antibodies against DAGLα or DAGLβ were developed in-house and have been previously characterized (Hu et al., 2010). After washing, secondary antibodies (Alexa 488, Alexa 594 or Alex 633, 1:500, Invitrogen, Inc., Carlsbad, CA) were applied the next day at room temperature for 1.5 hours. Images were acquired with a TCS SP5 confocal microscope (Leica, Wetzlar, Germany) using a 63x oil immersion objective (1.4NA). Images were processed using ImageJ and/or Photoshop (Adobe Inc., San Jose, CA). Images were modified only in terms of brightness and contrast. Knockdown of DAGLα or DAGLβ expression in neurons was determined as follows. For DAGLβ, neurons transfected with DAGLβ RNAi and then stained for DAGLβ and the dendritic marker MAP2, since DAGLβ showed relatively strong colocalization with MAP2. Transfected neurons expressed EYFP to allow identification relative to untransfected neurons. ROIs were identified in EYFP+/MAP2+ (knockdown condition) and EYFP-/MAP2+ (no knockdown) from the same coverslip. DAGLβ staining intensity in the ROI was then measured using ImageJ. At least three ROIs were identified in a given image and the DAGLβ intensity values averaged. Staining intensities from multiple images were used to obtain an overall average for each condition. For DAGLα we followed the same procedure except that we used PSD95 as the marker since this had somewhat better colocalization than MAP2.

RNAi Constructs

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DAGLα and DAGLβ small interfering RNA sequences were identified using Primer-Blast from NCBI. Short hairpin sequences were designed, commercially synthesized, and inserted downstream of the RNA polymerase III H1 promoter in a pSuper EGFP plasmid (Oligoengine, Seattle, WA) using BglII and HindIII restriction sites.

DAGLα sense:

GATCCCGggtgctggagaattacaacTTCAAGAGAgttgtaattctccagcaccTTTTTA

DAGLα antisense:

AGCTTAAAAAggtgctggagaattacaacTCTCTTGAAgttgtaattctccagcaccGGG

DAGLβ sense:

GATCCCGgctttcacgacaaggtgtaTTCAAGAGAtacaccttgtcgtgaaagcTTTTTA

DAGLβ antisense:

AGCTTaaaaagctttcacgacaaggtgtaTCTCTTGAAtacaccttgtcgtgaaagcCGG

RESULTS

Design and characterization of RNAi constructs

RNA interference (RNAi) is a cellular process used to control gene expression. Using the pSuper-EGFP expression plasmid, we specifically targeted mouse DAGLα and DAGLβ proteins for knockdown. RNAi-containing plasmids were transfected into Human Embryonic Kidney (HEK293) cells along with a plasmid containing either mouse V5-DAGLα or V5-DAGLβ (kindgiftsfromDanieliPiomelli).

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αRNAi reduced DAGLα but not DAGLβ expression (Fig.1A, lane 1 vs. 2 and 6). Conversely, RNAi specific for DAGLβ decreased DAGLβ expression, but not DAGLα expression (Fig. 1A, lane 4 vs. 5 and 3). RNAi for DAGLα and DAGLβ resulted in decline of their respective detectable DAGL proteins of 97% and 88%, respectively (Fig. 1A,C, n=4; significance determined via two-tailed Student's t test). To assess knockdown in neurons we examined expression of DAGLα or DAGLβ protein in cells transfected with the appropriate RNAi or a pSuper transfection control. We found that in each case the DAGL signal declined in RNAi-transfected cells relative to untransfected cells but not in pSuper-transfected cells relative to untransfected cells (Fig. 1E-F, n=6; significance determined via two-tailed Student's t test).

DAGLα and DAGLβ both regulate 2-AG production in autaptic hippocampal neurons.

In order to test whether DAGLα plays a role in DSE, we transfected autaptic hippocampal neurons with RNAi constructs for DAGLα. These neurons express presynaptic CB_1 receptors as well as the machinery to produce and to degrade endocannabinoids, likely 2-AG (Straiker and Mackie, 2005; Straiker and Mackie, 2009).

A simple way to quantify DSE and thereby assess CB_1 signaling is to assemble a "depolarization-response curve." Cells are depolarized for increasing durations (50ms, 100ms, 300ms, 500ms, 1s, 3s, 10s), resulting in increasing synthesis of endocannabinoids (likely 2-AG) and progressive EPSC inhibition (Straiker and Mackie, 2009). The resulting inhibition can be measured and analyzed in a

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manner very similar to a classical dose-response curve. The duration of depolarization required to reach a 50% of maximal inhibition is referred to here as the effective depolarization 50 (ED50).

We have previously shown that the non-selective DAGL blocker RHC80267 diminishes DSE (Straiker and Mackie, 2005). But because RHC80267 does not distinguish between DAGLα and DAGLβ the possibility remains that either (or both) plays a role in DSÊ If DAGLα is required for 2-AG production, then a knockdown of DAGLα should result in diminished DSE. We found that DAGLα RNAi did indeed substantially diminish DSE, shifting the depolarization-response curve to the right (Fig. 2A,B; pSuper ED50 1.5 sec (95% CI: 1.2-1.8); DAGLα ED50: 3.0 sec (95% CI: 2.4-3.8, n=5; non-overlapping 95% CI). This suggests that DAGLα is responsible for a substantial proportion of the DSE-related 2-AG release.

We next turned to DAGLβ. Using the previously characterized RNAi construct specific for DAGLβ, we transfected neurons and tested their DSE response profiles. Surprisingly, we found that knockdown of DAGLβ also diminished DSE (Figure 2A,C; DAGLβ ED50: 6.4 sec (95% CI 5.7-7.1) n=5; non-overlapping CI relative to pSuper). The ED50 of 6.4 seconds (relative to 3.0 after DAGLα knockdown) suggests that in these neurons DAGLβ plays a major role in 2-AG production.

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This unexpected finding suggests that both enzymes synthesize the 2-AG that mediates DSE in autaptic hippocampal neurons. If both DAGLα and DAGLβ independently produce 2-AG in response to depolarization, one would expect knockdown of both enzymes to further diminish DSE. We found this to be the case (Figure 3A; DAGLα/DAGLβ ED50: 8.7 sec (95% CI (6.1-12.4), n=6)), non-overlapping confidence interval relative to control and DAGLα knockdown).

DAGLα and DAGLβ share in the production of 2-AG by group I metabotropic glutamate receptors.

We have previously shown that autaptic hippocampal neurons not only express depolarization-dependent DSE but also metabotropic suppression of excitation (MSE), particularly in response to activation of Group I metabotropic glutamate receptors (Straiker and Mackie, 2007). In autaptic hippocampal cultures, MSE is mediated by 2-AG acting at presynaptic CB_1 receptors (Straiker and Mackie, 2007). We investigated the impact of RNAi knockdown of DAGLα or DAGLβ on 50 μM DHPG-induced MSE. Similarly to DSE, DHPG inhibition was substantially diminished after knockdown of either DAGL (Fig 3. DHPG results: DAGLα: 0.85 ± 0.06; DAGLβ: 0.89 ± 0.01; Control: 0.63 ± 0.08, n=5; $p<0.05$ for DAGLα and DAGLβ vs. control, 1 way ANOVA with Dunnett's post hoc test).

DAGLα and DAGLβ are expressed post-synaptically in mature autaptic hippocampal neurons.

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As noted above, the localization of DAGLα and DAGLβ has been previously described, with a generally axonal expression for both in embryonic neurons and dendritic expression in adult neurons (Bisogno et al., 2003). However, their expression has not previously been detailed in autaptic hippocampal neurons. Using antibodies developed against DAGLα and DAGLβ, we examined the localization of these proteins relative to markers for dendrites (MAP2), postsynaptic terminals (PSD95), and axon terminals (SV2). We found that both DAGLα and DAGLβ frequently colocalize with the dendritic marker MAP2, consistent with a post-synaptic localization (Figure 4 C,D). DAGLβ was not generally seen colocalized with PSD95, in contrast to DAGLα (Figure 4A,B), suggesting DAGLβ is not enriched in dendritic spines. DAGLβ does not reliably colocalize with the presynaptic marker SV2 (Figure 4E) but is often associated with membranes of neuronal somata (Figure 4F). Therefore DAGLβ and DAGLα exhibit a similar but not identical post-synaptic expression profile consistent with a role in retrograde neuronal signaling (Figure 4G-H).

DISCUSSION

Our chief finding using an RNAi approach is that both DAGLα and DAGLβ cooperate to produce 2-AG in a cultured neuron model system for endocannabinoid signaling. Furthermore each of these isozymes participate in both depolarization-dependent and Group I mGluR-mediated production of 2-AG.

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Our results offer the first evidence that DAGLβ is capable of playing an active role in synaptic plasticity, coupling electrical and metabotropic stimulation to the production of 2-AG, the consequent activation of presynaptic CB_1 receptors, and inhibition of neurotransmitter release. Our results also indicate that DAGLα and DAGLβ are comparably coupled to both electrical and metabotropic stimulation in cultured neurons and that they act cooperatively.

Though DAGLα and DAGLβ were identified in 2003, it remains challenging to distinguish them pharmacologically. Preliminary investigations of DAGL expression did not strongly favor one over the other as a candidate to play a role in mediating cannabinoid plasticity. For instance, in immunohistochemical studies, expression of DAGLβ appeared to be lower than that for DAGLα in cerebellum, but mRNA expression was 150-fold higher for DAGLβ than for DAGLα in striatum (Bisogno et al., 2003; Jung et al., 2005). However, the recent publication of results from three independent lines of DAGL knockout mice seemed to largely settle the matter in the favor of DAGLα being the predominant synthetic enzyme for 2-AG involved in synaptic plasticity (Gao et al., 2010; Tanimura et al., 2010; Yoshino et al., 2011), thus making our results surprising.

One possible explanation for the discrepancy in our results vs. those of Tanimura et al., Gao et al., and Yoshino et al. has to do with the use of constitutive knockout animals. As noted earlier, knockout mice are valuable tools but come with the risk that over the course of development the animal has compensated in

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some manner for the absence of the gene or that secondary effects of gene deletion have an indirect impact on the final phenotype. For instance, Tanimura et al. measured the levels of 2-AG in cerebellum, striatum and hippocampus, finding that the levels of 2-AG were markedly decreased in DAGLα but not DAGLβ mice. This is consistent with expectations regarding the role of DAGLs in 2-AG production. However, they also observed changes in anandamide and arachidonic acid levels in DAGLα-/- mice. Gao et al. (2010) reported that both anandamide and arachidonic acid decreased in DAGLα-/- mice, though Yoshino et al. (2011) did not report such a change. Anandamide is not synthesized by DAGL, suggesting that knockout of DAGLα has profound effects on the profiles of arachidonic acid-containing lipids. Conversely, it is possible that in the absence of DAGLβ, DAGLα is upregulated or has its trafficking altered, accounting for the lack of apparent effect on cannabinoid plasticity. Arguing against the former possibility is that Gao et al. reported whole-brain DAGLα mRNA levels were unchanged in DAGLβ knockout animals. Because only limited experiments were conducted in DAGLβ knockouts, it is more difficult to assess whether DAGLβ might play at least a supporting role in synaptic modulation. The risk of compensatory effects is inherent to the use of knockout mice; such risks are lessened by the use of RNAi with its transient diminishment of gene function at specific developmental time points.

The distribution of DAGLα and DAGLβ has previously been shown to shift over the course of development from a predominantly axonal distribution, consistent

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with a role in axonal pathfinding, to dendritic enrichment (Berghuis et al., 2007; Bisogno et al., 2003). The latter profile is consistent with the generally accepted model of the majority of endocannabinoid signaling whereby 2-AG is synthesized post-synaptically but then travels across the synapse to act on presynaptically expressed CB_1 receptors. Our evidence for functional postsynaptic DAGLβ is consistent with this model.

Our results demonstrate that DAGLβ is *capable* of playing a role in neurotransmission via 2-AG production, but whether/when/where this capability is realized remains an open question. The prominent role of DAGLβ may be restricted to autaptic cultures or the immature nervous system. Also, DSE in autaptic neurons is quite robust and it is possible that the DAGLβ system is selectively engaged when DSE is especially strong. The enrichment of DAGLα in spines points to a more localized production, while the diffuse dendritic/somatic expression pattern of DAGLβ suggests that it may be more involved in 'volume' production of 2-AG, or during periods of more pronounced activation. However, the question of whether DAGLβ acts in DSE and/or MSE 'in the wild' must await more sophisticated tools (such as inducible knockouts or more selective pharmacological blockers) capable of dissecting the roles of DAGLβ and DAGLα in these preparations.

In summary, we have found that in contrast to expectations, DAGLα and DAGLβ share roles in production of 2-AG for two forms of cannabinoid-mediated synaptic

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plasticity in cultured autaptic hippocampal neurons. This represents the first explicit evidence supporting a role for DAGLβ in the regulation of synaptic transmission. Our results invite further investigation of DAGLβ in the context of synaptic modulation and offer a novel therapeutic target for cannabinoid related studies.

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AUTHORSHIP CONTRIBUTION
Participated in research design: Straiker, Mackie

Conducted experiments: Jain, Straiker, Wager-Miller

Contributed new reagents or analytic tools: Jain, Wager-Miller

Performed data analysis: Straiker

Wrote or contributed to the writing of the manuscript: Straiker, Mackie

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Footnotes.

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FIGURE LEGENDS:

Figure 1. Characterization of DAGL RNAi constructs. A) V5-DAGLα or βwas expressed in HEK293 cells with or without RNAi constructs. Expression of the enzyme was significantly reduced in cells co-expressing the targeted DAGL RNAi but not by RNAi against the other DAGL. B) Ponceau S stain showing protein levels from blot in A. C) Summary of results from experiments like A showing substantial knockdown of DAGLα. D) Summary of results showing knockdown of DAGLβ. *, $p<0.05$, **, $p<0.01$ two-tailed Student's t test vs. DAGLα (C) or DAGLβ (D) control. Thus, RNAi for DAGLα specifically suppressed expression of DAGLα, but not DAGLβ and vice versa (Figure 1C and D). E) Summary of results showing knockdown of DAGLα in neurons. Left bars show DAGLα staining intensity in neurons untransfected (-) vs. transfected with RNAi for DAGLα (+). Right bars show DAGLα staining intensity in neurons untransfected (-) vs. transfected with PSuper (+). F) Same as in E but for DAGLβ. *, $p<0.05$, **, $p<0.01$ two-tailed Student's t test

Figure 2. Both DAGLs regulate 2AG release in depolarization-induced suppression of excitation (DSE).

A) DSE depolarization-response curves, representing progressive inhibition in response to increasing durations of depolarization (50ms, 100ms, 300ms, 500ms,

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1s, 3s, 10s). Depolarization response curves for DSE in control PSuper-transfected (filled triangles), transfected DAGLα RNAi (squares), transfected DAGLβ RNAi (circles), and transfected DAGLα/DAGLβ (inverted open triangles). B) Sample DSE time course of a DAGLα/RNAi-transfected neuron (black circles) and a non-transfected wild-type neuron (red circles) in response to a 3-second depolarization. Inset: corresponding traces for baseline and DSE-inhibited conditions in RNAi (upper) and control conditions (lower). Scale bars 1nA, 10ms. C) Sample DSE time course of a DAGLβ/RNAi-transfected neuron (black circles) and a non-transfected wild-type neuron (red circles) in response to a 3-second depolarization. Inset as in B. Scale bars 500pA, 10ms.

Figure 3. Both DAGLs regulate metabotropic suppression of excitation (MSE)

A) Bar graph shows relative EPSC charge (1.0 = no inhibition) after treatment with the group I metabotropic glutamate receptor agonist DHPG (50μM). Conditions: untransfected neurons (Control) and DAGLα/RNAi or DAGLβ/RNAi transfected neurons. B) Sample time courses for DHPG response in control (black circles) and DAGLα/RNAi transfected neuron (red circles). *, $p<0.05$ one way ANOVA with Dunnett's post hoc test. Inset: corresponding traces for baseline and DHPG-treated conditions in RNAi (upper) and control conditions (lower). Scale bars 300pA, 10ms.

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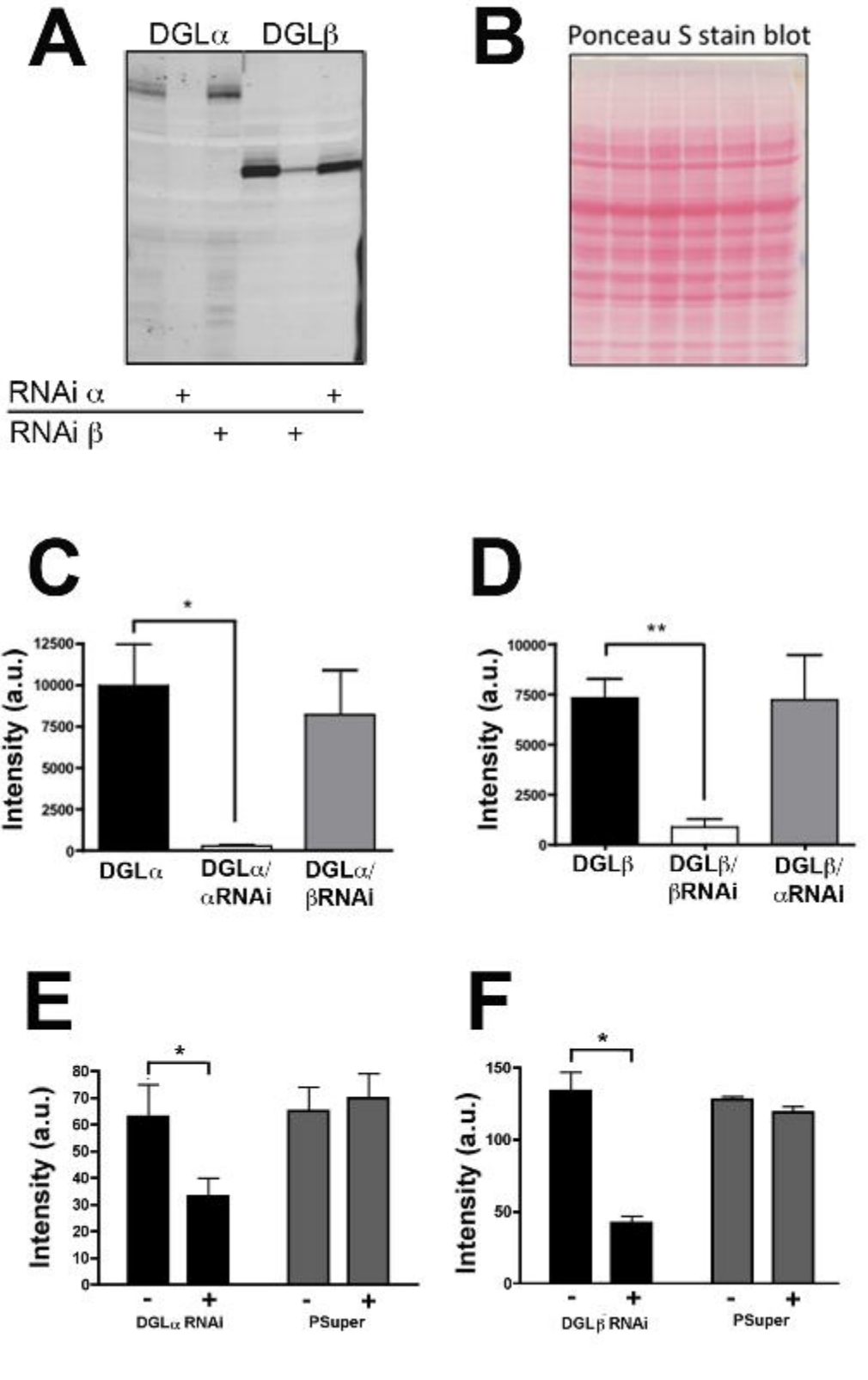
Figure 4. DAGLα and DAGLβ are post-synaptically expressed in autaptic hippocampal neurons.

Micrographs examining expression of DAGLα and DAGLβ in autaptic cultured hippocampal neurons. A) DAGLα (red) strongly co-localizes with the dendritic spine marker PSD95 (green, overlap in yellow), though some PSD95 labeled spines lack detectable DAGLα (green). Inset (within frame) shows 2x magnification with points of overlap and non-overlap. B) DAGLβ (red) does not colocalize with PSD95 (green). Inset (below) shows detail from B. C) DAGLβ (red) colocalizes in part with MAP2 (green, overlap in yellow). D) DAGLα (red) colocalizes with MAP2 (green). Middle and right panels show the corresponding DAGLα and DAGLβ staining, respectively. E) DAGLβ (red) does not colocalize with the synaptic terminal marker, SV2 (green). F) DAGLβ is frequently found in the membrane at or near the neuronal soma (arrows). G) Double staining for DAGLα and DAGLβ shows a similar though not identical expression profile in the cell soma. H) DAGLβ in same neuron as G, with DIC image for context; scale same as G. I) Another double staining for DAGLα and DAGLβ in neurites shows substantial overlap between DAGLα and DAGLβ. J) Same staining as I, but with MAP2 (blue, arrows) showing the predominantly dendritic staining. Scale bars: A: 10μm, B: 15μm, B(inset): 3μm, C: 5μm, D: 5μm, E: 5μm, F: 10μm, G-H: 5μm. I-J: 8μm

Figure 1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DGLα
DGLβ
RNAi α + +
RNAi β + +
B
Ponceau S stain blot
C
Intensity (a.u.)
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10000
7500
5000
2500
0
*
DGLα
DGLα/ αRNAi
DGLα/ βRNAi
D
Intensity (a.u.)
10000
7500
5000
2500
0
**
DGLβ
DGLβ/ βRNAi
DGLβ/ αRNAi
E
Intensity (a.u.)
80
70
60
50
40
30
20
10
0
*
- + - +
DGLα RNAi
PSuper
F
Intensity (a.u.)
150
100
50
0
*
- + - +
DGLβ RNAi
PSuper

Figure 2

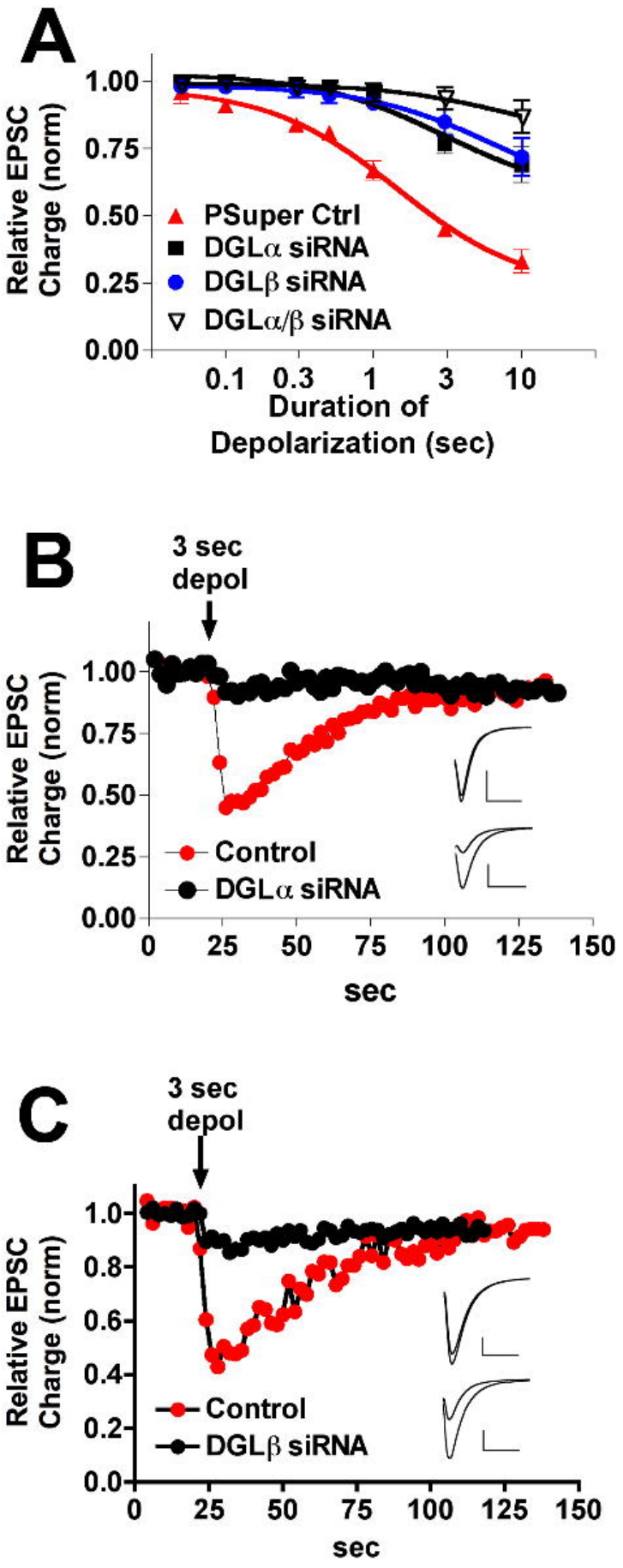

A
Relative EPSC Charge (norm)
1.00
0.75
0.50
0.25
0.00
PSuper Ctrl
DGLα siRNA
DGLβ siRNA
DGLα/β siRNA
0.1
0.3
1
3
10
Duration of Depolarization (sec)
B
3 sec depol
Relative EPSC Charge (norm)
1.00
0.75
0.50
0.25
0.00
Control
DGLα siRNA
0
25
50
75
100
125
150
sec
C
3 sec depol
Relative EPSC Charge (norm)
1.0
0.8
0.6
0.4
0.2
0.0
Control
DGLβ siRNA
0
25
50
75
100
125
150
sec

Figure 3

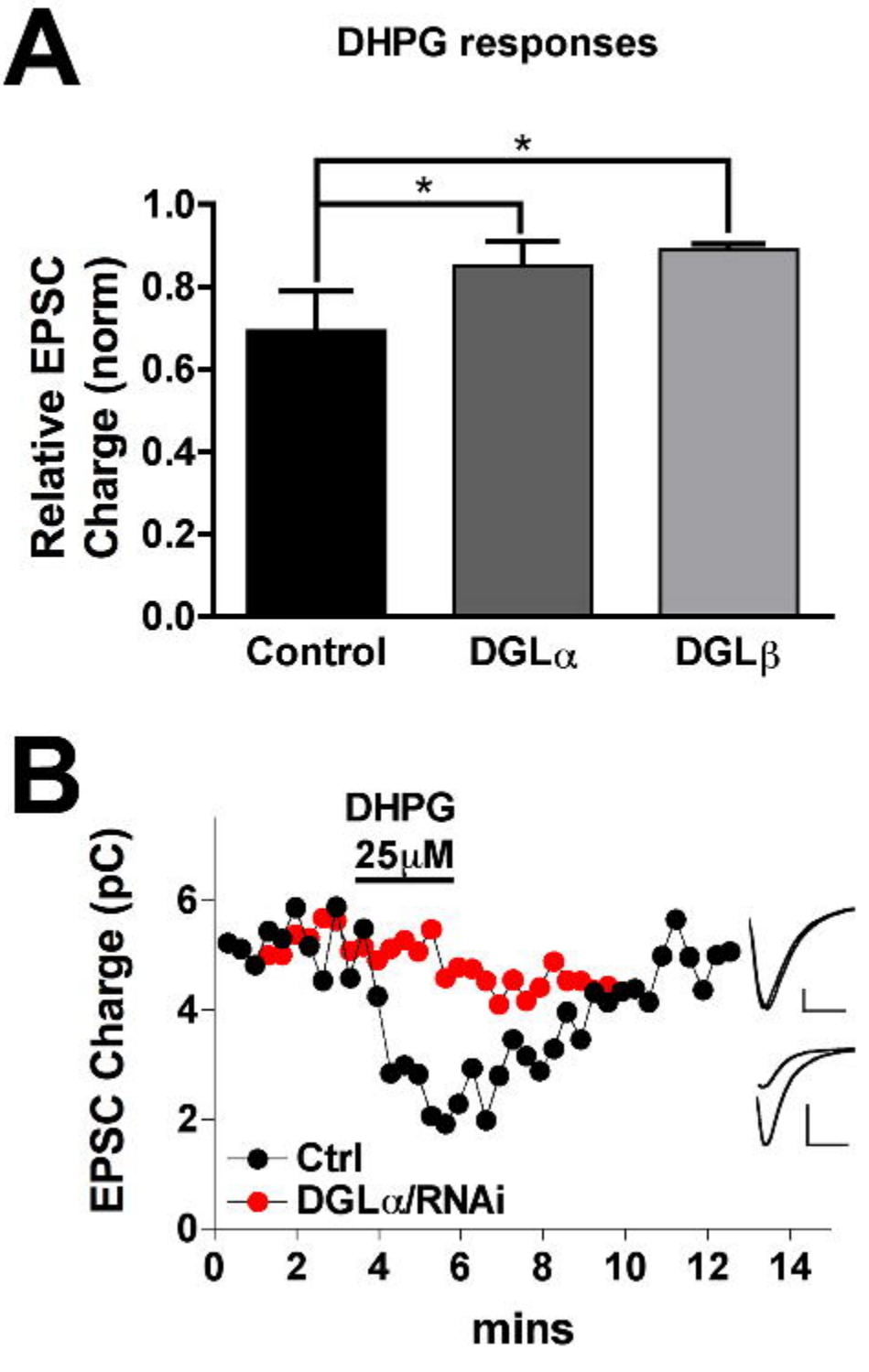
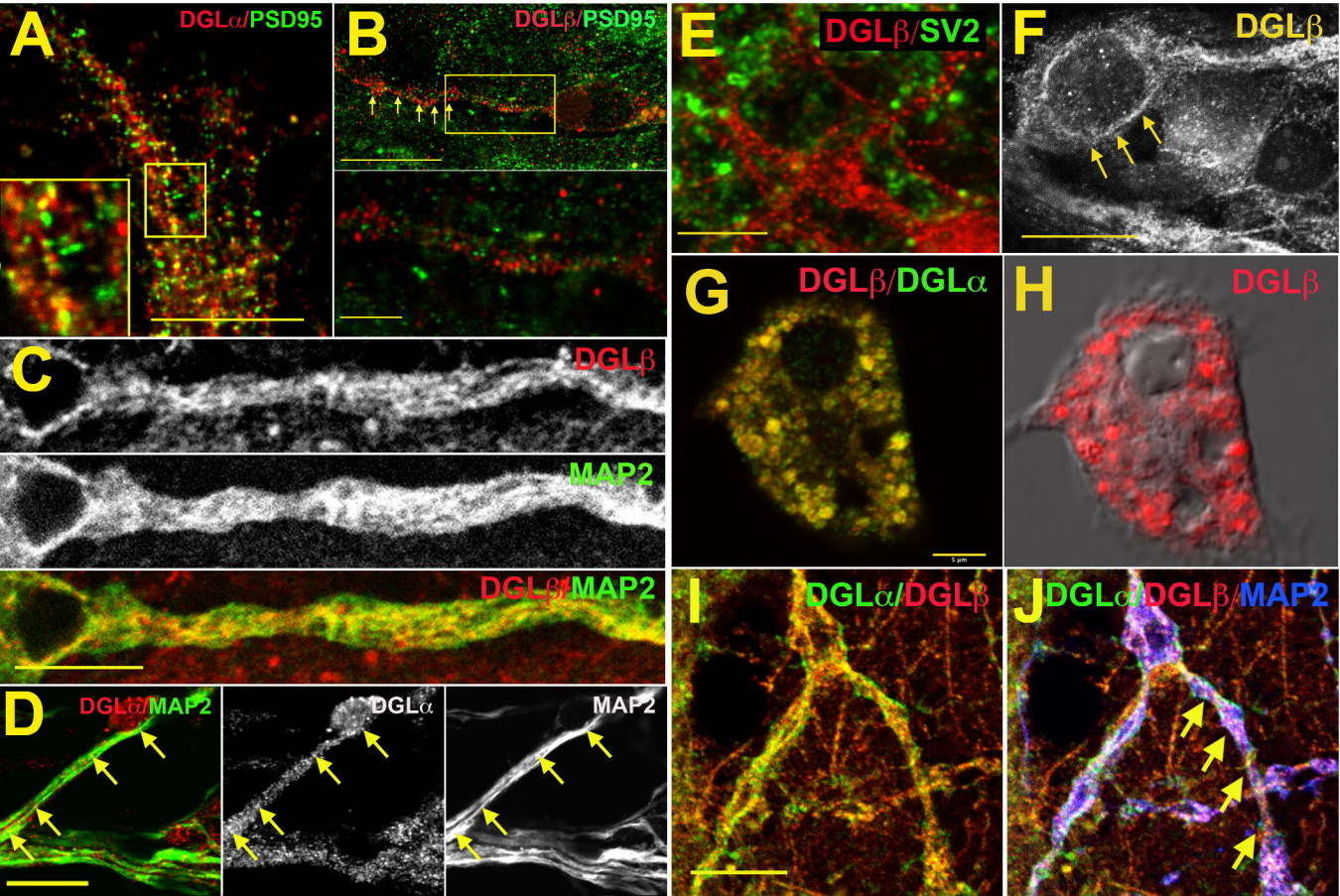

A
DHPG responses
*
*
1.0
0.8
0.6
0.4
0.2
0.0
Relative EPSC Charge (norm)
Control
DGLα
DGLβ
B
DHPG
25μM
EPSC Charge (pC)
6
4
2
0
Ctrl
DGLα/RNAi
0
2
4
6
8
10
12
14
mins

Figure 4


A
DGLα/PSD95
B
DGLβ/PSD95
C
DGLβ
MAP2
DGLβ/MAP2
D
DGLα/MAP2
DGLα
MAP2
E
DGLβ/SV2
F
DGLβ
G
DGLβ/DGLα
H
DGLβ
I
DGLα/DGLβ
J
DGLα/DGLβ/MAP2