CaMKII regulates diacylglycerol lipase-α and striatal endocannabinoid signaling

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The endocannabinoid 2-arachidonoylglycerol (2-AG) mediates activity-dependent depression of excitatory neurotransmission at central synapses, but the molecular regulation of 2-AG synthesis is not well understood. Here we identify a functional interaction between the 2-AG synthetic enzyme diacylglycerol lipase-α (DGLα) and calcium/calmodulin dependent protein kinase II (CaMKII). Activated CaMKII interacted with the C-terminal domain of DGLα, phosphorylated two serine residues and inhibited DGLα activity. Consistent with an inhibitory role for CaMKII in 2-AG synthesis, in vivo genetic inhibition of CaMKII increased striatal DGL activity and basal levels of 2-AG, and CaMKII inhibition augmented short-term retrograde endocannabinoid signaling at striatal glutamatergic synapses. Lastly, blockade of 2-AG breakdown using concentrations of JZL-184 that have no effect in wild-type mice produced a hypolocomotor response in mice with reduced CaMKII activity. These findings provide mechanistic insights into the molecular regulation of striatal endocannabinoid signaling with implications for physiological control of motor function.

Motor function and action selection are controlled by the basal ganglia1–2. Cortical inputs, forming glutamatergic synapses on ‘direct’ and ‘indirect’ pathway medium spiny neurons (MSNs) provide the major excitatory drive to the basal ganglia to facilitate and inhibit motor activity, respectively3. Endocannabinoid (eCB) signaling has a prominent role in the drive to the basal ganglia to facilitate and inhibit motor activity, respectively3. Endocannabinoid (eCB) signaling has a prominent role in the control of motor behavior, with abnormal eCB signaling linked to several movement disorders, including Parkinson’s disease4,5, Tourette’s syndrome6 and Huntington’s disease7.

The two best-studied eCBs are anandamide12 and 2-AG13. The eCB endocannabinoid signaling system involves neurotransmitter release and receptor activation through presynaptic and postsynaptic receptors. Presynaptic cannabinoid receptors are linked to the inhibition of neurotransmitter release, and postsynaptic cannabinoid receptors are linked to the modulation of synaptic activity. Two different mechanisms can initiate activity-dependent synaptic depression: depolarization-induced suppression of either excitation25 (DSE, depression of glutamatergic transmission) or inhibition23 (depression of GABAergic transmission). Second, pair activation of Gq/11 protein-coupled receptors, including group I metabotropic glutamate receptors (mGluRs), with less robust calcium signals triggers 2-AG mobilization in a process termed ‘calcium-assisted receptor-driven 2-AG release26–28. Finally, robust mGluR activation alone can initiate 2-AG signaling in a calcium-independent manner29. However, molecular mechanisms regulating DGLα activity in these different situations are not well understood.

Here we define a mechanism for DGLα regulation by CaMKII. CaMKII has critical roles in synaptic plasticity by regulating a broad range of proteins by phosphorylation and/or activity-dependent targeting. We found that CaMKII binds to and phosphorylates DGLα and inhibits 2-AG synthesis in vitro. Biochemical and electrophysiological data are consistent with a model in which CaMKIIα limits calcium-dependent eCB signaling at glutamatergic synapses onto both direct- and indirect-pathway MSNs. Behavioral studies indicate that CaMKIIα is a negative regulator of eCB signaling in vivo. Our data provide insights into fundamental mechanisms regulating eCB signaling at central synapses.

RESULTS
CaMKII interacts with DGLα
We initially screened for modulators of striatal DGLα by characterizing the DGLα interactome in mouse striatum using a shotgun

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proteomics approach. We immunoprecipitated DGLα from solubilized adult murine striatal extracts. We resolved immune complexes using SDS-PAGE and digested with trypsin the proteins in excised gel lanes. We analyzed extracted peptides by liquid chromatography–coupled tandem mass spectrometry (LC-MS/MS) and matched the amino acid sequences to the *mus musculus* subset of the UniProt database (Online Methods). We detected multiple CaMKIIα and CaMKIIβ peptides along with peptides derived from several other proteins (Supplementary Table 1). We confirmed that CaMKIIα interacts with DGLα by immunoblotting striatal DGLα immune complexes with an antibody to CaMKIIα (Fig. 1a). We detected no CaMKIIα in control IgG complexes (Fig. 1a), demonstrating the interaction specificity. To confirm the interaction and test the isoform specificity of CaMKIIα-DGL binding, we expressed CaMKIIα or CaMKIIβ either with DGLα or DGLβ containing a C-terminal V5-epitope tag in HEK293T cells. We found that DGLα-V5 immune complexes, but not DGLβ-V5 immune complexes, contained CaMKIIα (Fig. 1b). Moreover, both CaMKIIα and CaMKIIβ specifically associated with recombinant DGLα-V5 (Fig. 1c).

Whereas amino acid sequences of DGLα and DGLβ are very similar in transmembrane and catalytic domains, DGLα contains an extension of the C-terminal domain (Fig. 1d). CaMKIIα interacts with DGLα but not DGLβ, and the formation of physiological CaMKIIα complexes is often enhanced by autophosphorylation of Thr286 in CaMKIIα (30,31), which also generates constitutive CaMKIIα activity. Therefore, we tested the hypothesis that Thr286-autophosphorylated CaMKIIα directly interacts with the unique C-terminal domain of DGLα (residues 761–1042). Indeed, purified Thr286-autophosphorylated CaMKIIα specifically bound to a purified glutathione S-transferase (GST) fusion protein containing the DGLα C-terminal domain (Fig. 1d and Supplementary Fig. 1a). To explore the role of Thr286 autophosphorylation in modulating CaMKIIα interactions with full-length DGLα, we expressed full-length DGLα-V5 with either wild-type CaMKIIα or a CaMKIIα(T286A) mutant that cannot undergo autophosphorylation of Thr286. Wild-type CaMKIIα was phosphorylated at Thr286 when expressed in heterologous cells (Supplementary Fig. 2), and the T286A mutation significantly (P < 0.001) reduced CaMKII association with DGLα-V5 (Fig. 1e and Supplementary Fig. 1b). These data indicate that CaMKIIα is a DGLα-associated protein and that the interaction is enhanced by autophosphorylation of CaMKIIα at Thr286.

**CaMKIIα phosphorylates DGLα at Ser808 and Ser782**

As CaMKII phosphorylates many binding partners (33,34), we compared the phosphorylation of DGL isoforms by CaMKIIα. We purified DGLα-V5 or DGLβ-V5 from lysates of HEK293T cells expressing DGLα-V5 or DGLβ-V5 using a V5 antibody and then incubated the immunoprecipitates with [γ-32P]ATP, either alone or with purified activated CaMKIIα. Autoradiography after SDS-PAGE of reactions containing both DGLα-V5 and CaMKIIα revealed a ~116-kDa 32P-labeled protein that aligns with the DGLα-V5 band on the anti-V5 western blot as well as a ~55-kDa band corresponding to autophosphorylated CaMKIIα (Fig. 2a). We did not detect the 116-kDa 32P-labeled protein in control reactions lacking either DGLα or CaMKIIα.

To identify CaMKII phosphorylation sites, we incubated immunoprecipitated full-length DGLα-V5 with or without activated CaMKIIα in the presence of ATP. LC-MS/MS analysis of proteolytic digests of the DGLα-V5 detected ten phosphorylated serine or threonine residues in one or both samples (Table 1). Only Ser782 and Ser808 exhibited a substantial increase in phosphorylation after incubation with CaMKIIα (Table 1 and Fig. 2b). All but one of these sites were located in the unique C-terminal domain of DGLα (Fig. 2c).
CaMKIα selectively phosphorylates DGLα at Ser782 and Ser808. (a) Purified DGLα-V5 or DGLβ-V5 was incubated with [γ-32P]ATP and recombinant CaMKIα and analyzed by SDS-PAGE, followed by autoradiography to detect 32P-labeled proteins (top) and immunoblotting using a V5 antibody (bottom). (b) Extracted ion chromatograms for phosphorylated and nonphosphorylated DGLα peptides containing Ser782 and Ser808. Monoisotopic mass-to-charge ratio (m/z) values for the observed [M+3H]3+ and [M+2H]2+ precursor ions for DGLα peptides 774–795 (top) and 805–815 (bottom), respectively, are provided adjacent to their corresponding chromatographic peak. Observed values are within 2 p.p.m. of theoretical values calculated for these precursor ions. AUC, area under curve. Intensity refers to total ion current detected for each peptide. (c) Phosphorylation sites identified by mass spectrometric analysis of full-length wild-type (WT) DGLα purified from HEK293 cells. Black circles indicate Ser782 and Ser808, the only two sites that were only detected after in vitro incubation with activated CaMKIα. GST-fusion proteins used in (d) are shown. (d) Phosphorylation of GST-fusion proteins containing C-terminal tail fragments of DGLα incubated with recombinant CaMKIα and [γ-32P]ATP. Error bars, s.e.m. (n = 3). (e) Time course for phosphorylation of GST-DGLα(761–870) (WT, S782A, S808A or S782A,S808A) or GST alone by CaMKIα. Error bars, s.e.m. (f) Representative reactions from (e) (10 min) analyzed by SDS-PAGE followed by autoradiography ([32P] and protein staining (Prot). (g) Alignment of residues 776–788 (top) and 802–814 (bottom) of human DGLα with conserved domains in DGLα from the indicated vertebrates.

Sites detected in the absence of added CaMKIα may be phosphorylated by endogenous HEK293 cell kinases either in intact cells or in vitro.

To confirm that CaMKII phosphorylates DGLα, we incubated GST fusion proteins containing C-terminal domain fragments of DGLα (Fig. 2c), or GST alone as a control, with [γ-32P]ATP in the presence of purified, activated CaMKIα. Two fragments, containing residues 761–1042 and 761–870, were phosphorylated with similar stoichiometries (Fig. 2d), yet a third fragment containing residues 871–1042 and two other fragments were not substantially phosphorylated. These data indicate that the CaMKII phosphorylation site(s) are between residues 761 and 870, consistent with LC-MS/MS data identifying Ser782 and Ser808 as CaMKII phosphorylation sites in the full-length protein. To determine whether these sites were selectively phosphorylated in vitro, we mutated Ser808 and Ser782 to alanine individually and in combination. The single mutation of Ser808 to alanine substantially reduced the extent of phosphorylation by CaMKII, whereas the Ser782 to alanine mutation had a more modest effect. Double mutation of Ser782 and Ser808 to alanines reduced CaMKII phosphorylation of this DGLα fragment to levels that were similar to those of the GST control. These data indicated that Ser808 was phosphorylated to a higher stoichiometry than was Ser782 (Fig. 2e,f and Supplementary Fig. 2b). To determine whether CaMKII phosphorylates these sites in intact cells, we purified DGLα-V5 from lysates of HEK293T cells that either did or did not coexpress constitutively active CaMKIIα and analyzed tryptic digests using targeted LC-MS/MS. We detected phosphorylation of Ser808 and Ser782 in digests of DGLα expressed with CaMKIIα, but not in digests of DGLα expressed alone (Supplementary Fig. 3). Ser808 and Ser782 are conserved across a broad range of vertebrate species (Fig. 2g). These data show that CaMKIα selectively phosphorylates Ser808 and Ser782 in the unique C-terminal domain of DGLα.

CaMKII inhibits DGL activity in vitro

To explore the functional consequences of DGLα phosphorylation at Ser782 and Ser808, we first compared the activities of full-length wild-type DGLα and DGLα(S782E,S808E) proteins in membrane fractions from transfected HEK293T cells using various concentrations of the physiological DGL substrate, 1-steroyl-2-arachidonylglycerol (SAG; Online Methods). Mutations of phosphorylation sites to glutamate often mimic the phosphorylated form. This phosphomimetic mutation significantly (P < 0.05) decreased the apparent maximum velocity (Vmax) of 2-AG production without affecting the apparent Michaelis
Table 1 Detection of DGLα phosphorylation sites

| Residue | Without CaMKIIα | With CaMKIIα |
|---------|----------------|--------------|
| Thr411  | 0.27           | 0.22         |
| Ser725  | CD             | CD           |
| Ser727  | CD             | CD           |
| Ser743  | 42.4           | 27.5         |
| Ser782  | ND             | 12.0         |
| Ser808  | ND             | 21.2         |
| Ser845  | 0.16           | 0.72         |
| Thr866  | 2.4            | 2.5          |
| Thr1023 | 5.7            | 4.4          |
| Ser1030 | 1.9            | 2.0          |

DGLα-V5 was immunoprecipitated from HEK293T cell lysates and incubated with Mg-ATP, calcium and calmodulin in the absence or presence of purified CaMKIIα. LC-MS/MS analysis detected phosphorylation at the sites listed; phosphorylation at the total area calculated for both nonphosphorylated and phosphorylated peptides. ND, not detected. CD, peptide and phosphorylation site were identified but the peptide was not present in sufficient amount to calculate relative abundance.

constant (K_{eq}, Fig. 3a). After subtracting background activity in membranes from mock-transfected cells, the S782E, S808E double mutation reduced the V_{max} by ~40%. These data suggest that CaMKIIα could inhibit DGLα activity through phosphorylation at Ser782 and Ser808.

We next investigated the regulation of striatal DGL. Production of 2-AG from a striatal membrane fraction was approximately linear with DGL activity. We tested the hypothesis that CaMKIIα negatively modulates DGL activity in situ. As Thr286 autophosphorylation activated CaMKIIα in the presence of ATP (10 min, 30 °C) in a concentration-dependent manner, which was significant (P < 0.01) with ≥500 nM CaMKIIα (Fig. 3b). Heat-inactivation of CaMKIIα (70 °C, 30 min) prevented inhibition of DGL activity (Fig. 3c).

We explored the role of CaMKII in regulating striatal DGL activity in situ. As Thr286 autophosphorylation activated CaMKIIα and facilitated CaMKII-DGLα binding, we tested the effect of a homozygous Thr286 to Ala knock-in mutation (which reduces CaMKIIα activity by preventing Thr286 autophosphorylation) on striatal DGL activity. DGL activity in striatal membranes from these Camk2αtm^{Sva} (here called Camk2αT286A) knock-in mice was significantly (P < 0.01) higher than the activity in membrane fractions from their wild-type littermates (Fig. 3d), consistent with the hypothesis that CaMKIIα inhibits DGLα in wild-type mice. We next investigated whether the reduced CaMKIIα activity and enhanced DGLα activity in Camk2αT286A mice affected total endogenous levels of striatal 2-AG. Amounts of 2-AG in dorsolateral striatal tissue from Camk2αT286A mice were significantly (P < 0.05) increased relative to those in their wild-type littermates (Fig. 3e). Monoacylglycerol lipase (MGL) degrades 2-AG into arachidonic acid and glycerol. The lack of difference in arachidonic acid levels between wild-type and Camk2αT286A tissue (Fig. 3f) suggests that CaMKIIα does not regulate 2-AG degradation. However, additional studies are needed to conclusively exclude CaMKIIα effects on MGL. Moreover, there was no difference in total striatal levels of anandamide between genotypes (Fig. 3g). Taken together, these data show that CaMKIIα inhibits DGLα in vitro and that mice with impaired CaMKIIα activity have increased DGL activity and a selective increase in striatal 2-AG levels in vivo. Thus, CaMKIIα is a negative regulator of 2-AG signaling in the striatum.

CaMKII negatively modulates striatal DSE

As our data indicate that CaMKIIα constrained DGL activity in vivo, we tested the hypothesis that CaMKII modulates 2-AG–mediated synaptic signaling in the striatum. We focused on DSE, a purely Ca^2+–dependent regulator of 2-AG degradation. Depolarization of 8 out of 8 randomly selected MSNs in the dorsolateral striatum resulted in a short-term depression of evoked excitatory postsynaptic currents (eEPSCs), and this depression was blocked by the CB1 antagonist rimonabant (Supplementary Fig. 5a–c). Because emerging evidence suggests that striatal CB1-dependent long-term depression may be specific to indirect pathway (D2 receptor expressing) MSNs^21,39,40, we examined DSE in slices from drd2-eGFP BAC transgenic mice.

Figure 3 CaMKII inhibits DGL activity. (a) Effects of phosphomimetic mutation of Ser782 and Ser808 to glutamate on the activity of DGLα at the indicated concentrations of SAG (n = 4 independent experiments; V_{max} for wild-type (WT), 9.8 ± 1.1 pmol min^{-1} μg^{-1} and for S782E, S808E, 6.1 ± 0.4 pmol min^{-1} μg^{-1}; unpaired two-tailed t-test: t_{6} = 3.059, P = 0.022; K_{eq} for WT, 158 μM ± 24 μM and for DGLα (S782E, S808E), 114 μM ± 8.7 μM, P > 0.05). (b) Effects of purified recombinant CaMKIIα on striatal DGL activity in the presence of Ca^{2+}, calmodulin, ATP and Mg^{2+} (0 nM CaMKIIα, 100.0 ± 0.8 pmol μg^{-1} protein; 100 nM, 97.7 ± 2.0 pmol μg^{-1} protein; 500 nM, 85.1 ± 1.1 pmol μg^{-1} protein; 1 μM, 56.2 ± 2.7 pmol μg^{-1} protein; 2 μM, 55.4 ± 2.3 pmol μg^{-1} protein; n = 4 independent experiments; one-way ANOVA F_{6,10} = 161.8, P < 0.0001; Bonferroni post-hoc analysis: 0 versus 0.1: t_{10} = 0.9609, P > 0.05; 0 versus 0.5: t_{10} = 6.16, **P < 0.01; 0 versus 1: t_{10} = 18.07, ***P < 0.001; 0 versus 2: t_{10} = 18.38, ****P < 0.0001). (c) Effects of prior heat treatment of CaMKIIα (1 μM) on DGL activity in striatal membranes (heat-inactivated CaMKIIα, 244.9 ± 6.5 pmol μg^{-1} protein; active CaMKIIα, 146.0 ± 14.5 pmol μg^{-1} protein; unpaired two-tailed t-test: t_{6} = 6.245, ****P < 0.0008, n = 4 independent experiments). (d) DGLα activity in striatal membranes from Camk2αT286A knock-in mice (KL; 594 ± 64 pmol μg^{-1} protein, n = 5 mice) and WT littermates (349 ± 38 pmol μg^{-1} protein, n = 6 mice); unpaired two-tailed t-test: t_{6} = 3.440, **P = 0.0074. (e) Total amounts of 2-AG in dorsolateral striatal punches from WT and Camk2αT286A mice (KL; 73.8 ± 12.4 pmol μg^{-1} tissue, n = 6 mice) and WT littermates (44.6 ± 6.8 pmol μg^{-1} tissue, n = 7 mice); unpaired two-tailed t-test: t_{10} = 2.396, *P = 0.0355. (f, g) Amounts of arachidonic acid (AA; f) and anandamide (AEA; g) in dorsolateral striatal punches from WT and Camk2αT286A mice (unpaired two-tailed t-test, P > 0.05). All error bars, s.e.m.
Figure 4  Inhibiting CaMKII or preventing CaMKII autophosphorylation at Thr286 enhances endocannabinoid-mediated retrograde transmission in striatal MSNs. (a,b) DSE in D2(−) MSNs from Camk2aT286A knock-in mice (KI; 65% ± 2%, n = 10 cells from 4 mice) relative to wild-type mice (WT; 74% ± 2%, n = 15 cells from 4 mice) and when the CaMKII inhibitory peptide AIP (10 μM) was included in the patch pipette solution (61% ± 2%, n = 9 cells from three mice); one-way ANOVA: F2,31 = 8.30, P = 0.0013; Bonferroni’s post-hoc analysis: WT versus KI: t31 = 2.764, *P < 0.05; WT versus AIP: t31 = 3.830, **P < 0.01. (c,d) DSE in D2(+) MSNs from WT (67% ± 2%, n = 15 cells from 4 mice) and Camk2aT286A MSNs (69% ± 2%, n = 11 cells from 5 mice), and with loading of AIP in WT cells (53% ± 5%, n = 7 cells from 3 mice). One-way ANOVA: F2,30 = 8.769, P = 0.001. Bonferroni’s post-hoc analysis: WT versus KI: t30 = 0.5626, P > 0.05; WT versus AIP: t30 = 3.654, **P < 0.01. (e,f) Effects of DHPG (10 μM) on 10-s DSE in D2(−) MSNs from WT and Camk2aT286A mice (WT, 57% ± 3%, n = 7 cells from 3 mice; KI, 54 ± 1, n = 9 cells from 3 mice; unpaired two-tailed t-test, P > 0.05). (g,h) DSE in D2(+) MSNs from WT and Camk2aT286A mice (WT, 57% ± 3%, n = 7 cells from 4 mice; KI, 53% ± 2%, n = 8 cells from 4 mice; unpaired two-tailed t-test, P > 0.05). Representative traces are shown for each condition. Calibration bars, 20 ms and 150 pA. All error bars, s.e.m.

(Tg(Drd2-EGFP)S118Gsat)41. Significant (P < 0.05) DSE was induced in both GFP-expressing (D2(−)) and nonfluorescent D2(−)) MSNs by as little as a 3-s depolarization, although longer depolarizations induced more robust DSE in both cell types (Supplementary Fig. 5d–g). Comparison of DSE between D2(−) and D2(+) MSNs across all depolarization times by two-way ANOVA did not reveal a significant (P > 0.05) cell type by depolarization time interaction, indicating that the magnitude of DSE was similar in both cell types.

We investigated the role of CaMKII in striatal DSE by comparing DSE in wild-type and Camk2aT286A mice, which have reduced CaMKII activity, that both also carried the drd2-eGFP BAC transgene. DSE was enhanced in D2(−) MSNs in Camk2aT286A mice relative to their wild-type littermates (Fig. 4a,b), consistent with the increased DGL activity and striatal 2-AG levels in these mice. We also investigated the role of CaMKII in DSE by perfusing an inhibitory peptide that inhibits both Ca2+-dependent and Ca2+-independent CaMKII activity (AIP, 10 μM) into the postsynaptic cell via the recording pipette. Similar to the effect of Camk2aT286A mutation, AIP caused a significant (P < 0.01) enhancement of DSE in wild-type D2(−) MSNs (Fig. 4a,b). Although the Camk2aT286A mutation had no effect on DSE in D2(+) MSNs, intracellular perfusion of AIP also significantly (P < 0.01) enhanced DSE in D2(+) MSNs (Fig. 4c,d). Taken together, these data suggest differences between direct and indirect pathway MSNs in either how CaMKII is regulated or how it interacts with the 2-AG signaling pathway. Furthermore, the fact that AIP was applied only to the postsynaptic cell indicates that DSE is modulated by postsynaptic CaMKII. Consistent with this interpretation, there is no change in presynaptic CB1 receptor signaling in Camk2aT286A mice, as evidenced by a similar decrease of eEPSCs in MSNs from wild-type and Camk2aT286A mice after bath application of WIN55,212-2 (3 μM), a CB1R agonist (Supplementary Fig. 6g).

Synthesis and release of 2-AG can also be induced by activation of group I mGluRs in combination with a depolarization-induced Ca2+ signal26–28. Although this mode of 2-AG release still requires DAGλα, it also involves signaling via phospholipase C-β (PLCβ)27,28 and in some cases may be Ca2+-independent42. To determine whether...
CaMKII has a role in these pathways, we pretreated slices from wild-type and Camk2aT286A mice with the mGluR1/5 agonist DHPG (10 μM) before induction of DSE. There was no significant difference in DSE induced by either a 10 s (Fig. 4e–h) or 3 s (Supplementary Fig. 6a–d) depolarization between Camk2aT286A mice and their wild-type littermates in either D2(−) or D2(+) MSNs in the presence of DHPG. Thus, the lack of DSE enhancement in Camk2aT286A mice in the presence of DHGP was not because DSE was maximally induced by a 10 s depolarization. In addition, we saw no effect of postsynaptic AIP loading in D2(−) cells using a 3 s depolarization in DHPG-treated slices (Supplementary Fig. 6e,f). Together these results suggest that the mGluR1/5 activation overcame the negative modulatory role of postsynaptic CaMKII in striatal 2-AG release.

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**Camk2aT286A mice have enhanced sensitivity to MGL inhibition**

Excitatory inputs to striatal MSNs have a critical role in controlling motor activity, and cannabinoids have potent inhibitory effects on movement. Released 2-AG is degraded by presynaptic MGL, and pharmacological inhibition of MGL with JZL-184 enhances 2-AG levels in the brain to reduce motor activity in a dose-dependent manner. Therefore, to investigate the behavioral relevance of CaMKII modulation of 2-AG signaling in vivo, we compared the effects of JZL-184 in wild-type and Camk2aT286A mice. We reasoned that elevated striatal DGL activity in Camk2aT286A mice should amplify the build-up of 2-AG at synapses in Camk2aT286A mice in the presence of JZL-184. Thus, we predicted that JZL-184 should cause a more robust suppression of locomotor activity in Camk2aT286A mice compared to wild-type littermates. To test this hypothesis, mice were habituated to a home-cage monitoring environment for 2 d, treated with vehicle and then monitored for 24 h (Online Methods). Mice then received a submaximal dose of JZL-184 (12 mg kg⁻¹) and were monitored for an additional 24 h. The distances these mice traveled in each 1-h period across the entire 48-h monitoring period are shown in Figure 5a. Although elevated baseline motor activity of vehicle-treated Camk2aT286A mice relative to that of wild-type littermates (because of an unidentified mechanism) somewhat confounds direct comparisons, this submaximal dose of JZL-184 significantly (P < 0.01) decreased motor activity in Camk2aT286A mice but not wild-type littermates (Fig. 5b). On average, JZL-184 decreased locomotor activity in Camk2aT286A mice by 32% ± 7% but had no net effect on locomotor activity in wild-type mice (0.04% ± 14.4%; Fig. 5c). This effect of JZL-184 in Camk2aT286A mice was not due to differences in habituation to the testing environment between genotypes because circadian locomotor patterns of Camk2aT286A and wild-type mice were reliable and stable when monitored for three consecutive days without any manipulations (Supplementary Fig. 7). There was no significant (P > 0.05) difference in eating, drinking, grooming or sleeping in the home-cage environment between genotypes, and JZL-184 treatment had no effect on these behaviors in either genotype (Fig. 5d–g).

**DISCUSSION**

We found that CaMKIIα interacted with DGLα in mouse striatum, phosphorylated DGLα at Ser782 and Ser808, and inhibited DGLα activity. Consistent with the expression pattern of DGLα, DSE can be induced in both direct-pathway and indirect-pathway MSNs, unlike striatal anandamide-mediated long-term depression. CaMKII α-containing immune complexes. This effect of JZL-184 in Camk2aT286A mutation were more sensitive to the locomotor suppressant effect of MGL inhibition, which may reflect an enhanced 2-AG synthetic pathway in these mice. These data suggest that CaMKIIα is important in controlling striatal functions by modulating Ca²⁺-dependent 2-AG signaling at corticostratial glutamatergic synapses.

DGLα interacts with Homer1 (ref. 19), but interactions of DGLα with other proteins that may modulate the subcellular targeting or enzyme activity are poorly understood. Our initial proteomics screen of striatal DGLα complexes detected Homer1 and several other postsynaptic density (PSD)-associated proteins including Shank3, a known binding partner of Homer1, PSD-95, PSD-93, SAPAP3 and SAPAP2. Additional studies will be required to determine the biochemical basis and roles for these interactions with DGLα, but we speculate that they may contribute to the known targeting of DGLα to dendritic spines. CaMKIIα was the only kinase detected in striatal DGLα immune complexes.

Residues 1–673 of DGLα are well-conserved in DGLβ, but DGLα contains a unique C-terminal tail. We found that CaMKIIα directly interacts with the C-terminal tail of DGLα, and also interacted with full-length DGLα but not full-length DGLβ. Using a proteomics-based approach, we found that in HEK293 cells DGLα was phosphorylated at several serine and threonine residues, mostly in the unique C-terminal tail, suggesting that DGL isoforms may be differentially regulated. Although these sites may be targeted by many kinases, we found that CaMKIIα specifically phosphorylated Ser782 and Ser808 of DGLα. Moreover, mutation of Ser782 and Ser808 to glutamate, which mimics phosphorylation, decreased DGL activity in vitro. In agreement with this, we found that incubation of striatal membranes with activated CaMKIIα reduced DGL activity by ~50% in vitro. Although additional studies are required to understand the relative roles of CaMKII binding.
to DGLα and phosphorylation at Ser782 and Ser808, these data are the first to our knowledge to implicate CaMKIIα as a negative regulator of eCB synthesis via modulation of DGLα activity.

Previous studies indicate that eCBs have distinct roles in long-term modulation of excitatory inputs onto direct and indirect pathway MSNs. In contrast, we found that striatal DSE can be expressed in both subpopulations of MSNs. Consistent with our biochemical data showing that CaMKIIα inhibits DGLα activity, DSE in both MSN subtypes was enhanced by acute postsynaptic blockade of CaMKII activity by postsynaptic perfusion of AIP. These data show that postsynaptic CaMKIIα can inhibit eCB retrograde signaling at glutamatergic striatal synapses. Given the calcium dependence of both DGLα and CaMKIIα, our data suggest a scenario whereby strong calcium signals initiate eCB mobilization via activation of DGLα but also recruit CaMKIIα to inhibit DGLα to limit and/or terminate eCB-mediated synaptic signaling.

The relative prominence of CaMKII feedback control of DGLα may vary subtly between striatal MSN subtypes. Whereas acute inhibition of both calcium-dependent and calcium-independent activity of both CaMKIIα and CaMKIIβ using AIP enhanced DSE in both MSN subtypes, the inhibition of only calcium-independent CaMKIIα activity in Camk2α+/- mice selectively enhanced DSE in direct pathway MSNs. The T286A mutation of CaMKIIα does not affect calcium/calmodulin-stimulated CaMKIIα activity and does not directly affect CaMKIIβ. There may be several explanations for these apparently discrepant findings. First, this may reflect a difference between acute kinase inhibition compared to long-term genetic disruption in Camk2α−/− mice. Compensatory mechanisms may overcompensate the loss of calcium-independent CaMKIIα activity in indirect pathway Camk2α+/-MSNs but not in direct pathway Camk2α+/-MSNs. Second, the ‘set point’ of 2-AG signaling may differ in the two cell types, perhaps owing to differences in the levels of Thr286 autophosphorylation of CaMKIIα. Moreover, more robust inhibition of CaMKIIα activity by AIP might be required to suppress DSE in MSNs with lower levels of Thr286 autophosphorylation.

Neither acute CaMKII blockade nor the Camk2α+/- mutation affected calcium-assisted mGluR-mediated 2-AG release. The different regulation of these distinct forms of eCB mobilization by CaMKIIα may be related to mechanistic differences in the two signaling pathways. DSE is mediated by calcium-dependent activation of DGLα, whereas calcium-assisted mGluR-mediated 2-AG signaling is mediated via calcium enhancement of PLCβ activity, which provides diacylglycerol substrate for DGLα. These two forms of 2-AG mobilization may depend on distinct pools of DGLα46. For example, a complex between DGLα and mGLRs mediated by Homer proteins may recruit a specific pool of DGLα for mGluR-mediated 2-AG signaling19. Purely calcium-driven DSE may involve distinct pools of DGLα46 that are not associated with mGLRs or Homer but that can be regulated by CaMKIIβ77, perhaps associated with calcium channels47. Thus, in this putative model, CaMKIIα may not be able to inhibit DGLα molecules in complex with mGLRs and Homer. Additional studies will be required to determine the molecular basis for the different regulation of calcium-dependent and calcium-assisted, mGLR-driven eCB mobilization by CaMKIIα. Although our biochemical and electrophysiological findings strongly support the conclusion that CaMKIIα regulates DGLα-mediated synthesis of 2-AG, at this time we cannot rule out an additional role for CaMKII in release of 2-AG.

Lastly, we have begun to evaluate the importance of CaMKIIα-eCB signaling interactions in vivo using Camk2α+/- mice. Inhibition of 2-AG hydrolysis using JZL-184 reduced locomotor activity in Camk2α+/- mice, as measured using a home-cage monitoring system that reduces potential confounding effects of novelty or anxiety. As Camk2α+/- mice have elevated DGLα activity, one explanation for these data is that blockade of 2-AG hydrolysis results in enhanced 2-AG-mediated and CB1-mediated inhibition of glutamatergic drive to direct pathway neurons in Camk2α+/- mice. However, several caveats to this interpretation remain. Camk2α+/- mice exhibit a baseline hyperactive phenotype, which is unlikely to be explained by alterations in basal 2-AG signaling because the enhanced 2-AG levels and enhanced direct pathway DSE would predict a hypoactive rather than hyperactive phenotype. Furthermore, we cannot conclusively exclude contributions from deficits in spatial and working memory displayed by Camk2α+/- mice34,48 to the changes in locomotor activity. Nevertheless, these data clearly support the overall notion that CaMKIIα-eCB interactions occur in vivo and could regulate striatal function under physiological and possibly pathophysiological conditions in which striatal CaMKIIα function is enhanced, such as experimental parkinsonism49,50.

In summary, these studies provide evidence for a functional link between CaMKIIα and 2-AG signaling in the striatum. The data indicate that CaMKIIα is a negative modulator of short-term calcium-dependent eCB signaling at corticostriatal glutamatergic synapses, adding to the diverse synaptic functions of CaMKIIα. Future studies need to be directed at understanding how distinct modes of 2-AG signaling are regulated by CaMKIIα signaling and the precise molecular determinants of CaMKIIα modulation of DGLα activity. Exploration of these questions could provide strategies to fine-tune synaptic transmission in the central nervous system in multiple neurological and psychiatric disorders.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.C.S. and J.C. prepared the manuscript with input from all other authors. R.J.C. prepared the manuscript with input from all other authors. V.S.C. carried out the GST cosedimentation assay. B.C.S. and X.W. generated the GST-fusion proteins and DGLα assay. V.S.C. carried out the GST coexpressionification assay. B.C.S. and X.W. generated the GST fusion proteins and DGLα mutants, and performed the phosphorization site identification studies. B.C.S. and K.L.R. performed the LC/MS analysis of phosphorylation sites. B.C.S. and K.L.R. performed the LC/MS analysis of phosphorylation sites. B.C.S. and T.S.R. conducted the electrophysiology experiments. B.C.S. and T.S. conducted the behavioral experiments. B.C.S. analyzed data from all experiments. N.J.-S. performed the T286 phosphorylation measurements in HEK293 cells. K.M. contributed the DGLα activity assays. V.S.C. and J.C. designed the experiments. B.C.S., S.P. and R.J.C. performed the manuscript with input from all other authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. All mice were on a C57Bl6 background and were housed on a 12 h light-dark cycle with food and water ad libitum. Male wild-type (WT) and homozygous Camk2αα (Camk2αα) mice35 were generated by breeding heterozygous mice. Camk2αα transgenic mice (homozygous) to generate drd2-eGFP (Fig. 2c) on a heterozygous Camk2αα background. The mutant mice were bred with drd2-eGFP / Camk2αα mice to produce the drd2-eGFP / Camk2αα mice and drd2-eGFP / Camk2αα used for electrophysiology experiments. Homozygous Camk2αα mice are referred to as Camk2αα throughout. Behavioral experiments were performed at postnatal days 43–50; biochemical and electrophysiological experiments were performed at postnatal days 21–27. All experiments with mice were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were carried out in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cloning and protein expression. Plasmids encoding DGLα-V5 and DGLβ-V5 were gifts from B. Cravatt (The Scripps Research Institute). Sequences encoding DGLα mutants were generated by site-directed mutagenesis and verified by sequencing. Sequences encoding GST-fusion proteins were generated by PCR amplification followed by restriction digest cloning into PGE6P-1 using BamHI and XhoI sites. Vectors were transformed into BL21 DE3 pLysS and XhoI sites. Vectors were transformed into BL21-DE3 pLysS E. coli and XhoI sites. Vectors were transformed into BL21-DE3 pLysS E. coli, and proteins were purified as previously described30. Recombinant mouse CaMKIIα purified from baculovirus-infected Sf9 insect cells was autophosphorylated at Thr286 essentially as described previously30.

Immunoprecipitation. HEK293T cells. Lysates were prepared in cold lysis buffer (2 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1 µM microcin). Goat anti-V5 IgG (Bethyl Laboratories, A900-119A, 1:5) was cross-linked to protein-G magnetic beads using dimethyl pimelimidate (DMP; 10 µg/ml) following the manufacturer’s instructions (Dynabeads, Invitrogen). Beads were incubated with lysates overnight and were then washed with IP buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5 and 1% Triton X-100). Inputs and precipitates were resolved by SDS-PAGE and analyzed by western blotting using anti-V5 (Bethyl Laboratories, A190-120A, 1:10,000), mouse anti-CaMKIIα (761–870) (Pierce, MA1-048, 1:3,000) or mouse anti-CaMKIIβ (Invitrogen, 13–9800, 1:3,000).

Mouse striatum. Mice were decapitated without anesthetics, and the striatum was homogenized in lysis buffer (150 mM KCl, 50 mM Tris-HCl, 1% Triton X-100 (v/v), 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 10 µg/ml leupeptin, 10 µM pepstatin, 1 mM Na2VO4, 1 mM NaF and 1 µM microcin). After centrifugation (9,000g, 10 min), supernatants were incubated overnight with anti-DGLα (validated in previous studies51,52), cross-linked to magnetic protein-G beads (see above). After washing with IP wash buffer, immune complexes were resolved by SDS-PAGE and either analyzed by western blotting or used for proteomic studies.

GST cosedimentation assay. GST cosedimentation assays were performed as previously described30.

Proteomics methods. For phosphorylation-site analysis, DGLα-V5 was isolated from transfected HEK293 cells, phosphorylated by CaMKII (see below) and resolved by SDS-PAGE. Excised gel bands were incubated with 100 mM ammonium bicarbonate, pH 8, reduced with 4 mM DTT or TCEP, alkylated with 8 mM iodoacetamide and finally digested overnight with trypsin, elastase or endoproteinase AspN (10 ng/µl; 37 °C). Extracted peptides were reconstituted in 0.1% formic acid and fractionated on a C18 reverse-phase column (360 µm outer diameter and 100 µm inner diameter columns, 3-µm beads, 300 Å, Phenomenex) equipped with a laser-pulled emitter tip (flow rate: 500 nl/min). Mobile phase solvents consisted of 0.1% formic acid, 99.9% water (solvent A) and 0.1% formic acid, 99.9% acetonitrile (solvent B). A 90-minute gradient was used: 0–10 min, 2% B; 10–50 min, 2–35% B; 50–60 min, 35–90% B; 60–65 min, 90% B, 65–70 min, 90–2% B, 70–90 min, 2% B. The eluted peptides were analyzed on either LTQ Orbitrap XL or LTQ Orbitrap Velos mass spectrometers (Thermo Scientific), using a data-dependent method with dynamic exclusion enabled. Full scan (m/z 300–2,000) spectra were acquired with the Orbitrap as the mass analyzer, and the 5 most abundant (LTQ Orbitrap XL) or 12 most abundant ions (LTQ Orbitrap Velos) in each MS scan were selected for fragmentation via collision-induced dissociation (CID) in the LTQ. For analyses performed on the LTQ Orbitrap XL, the data-dependent method included neutral loss–triggered (MS/MS/MS) scan events, where MS/MS/MS scans were performed when neutral losses of 97.98 m/z, 48.99 m/z or 32.66 m/z (neutral losses specific for phosphorylation) were detected in the preceding MS2 spectra. All tandem mass spectra were converted into DTA files using Sconst and were searched against a human subset of the UniProtKB protein database, also containing reversed (decoy) protein sequences. Database searches were performed using a custom version of SEQUEST on the Vanderbilt ACCRE Linux cluster, and results were assembled in Scaffold 3 (Proteome Software) with minimum filtering criteria of 95% peptide probability. All searches were configured to use variable modifications of carbamidomethylation on cysteine, oxidation of methionine, and phosphorylation of serine, threonine and tyrosine. Sites of modification were validated by manual interpretation of the raw tandem mass spectra using QualBrowser software (Xcalibur 2.1.0, Thermo Scientific).

To determine the relative abundance of phosphorylated peptides in digests of in vitro phosphorylated DGLα-V5, accurate mass measurements acquired in the Orbitrap were used to generate extracted ion chromatograms (XICs). A window of 10 p.p.m. around the theoretical monoisotopic m/z values of the observed precursor ions was used for making XICs of the unmodified and phosphorylated peptide pairs. Using QualBrowser, the integrated area under each XIC peak was determined, and the percentage relative abundance of each phosphorylated peptide was calculated as a percentage of the total area under the curve (AUC) obtained for both the phosphorylated and unmodified forms for each DGLα peptide. AUC values were calculated for the following phosphorylated peptides: DGLα residues 405–416, 741–751, 774–785, 805–815, 838–848, 859–874, 1,021–1,033 and 1,021–1,042.

For identification of protein in mouse striatal DGLα immune complexes, samples were resolved by SDS-PAGE and entire gel lanes were excised for in-gel tryptic digestion. All immune complex data were acquired on the LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Data-dependent methods were used where the five most abundant ions were selected for fragmentation, and dynamic exclusion was applied. SEQUEST was similarly used for database searching against a Mus musculus subset of the UniProtKB protein database, concatenated with reversed (decoy) sequences. Resulting peptide identifications were assembled using IDPicker 2.6 (ref. 54) with a target false discovery rate of 5% and a minimum requirement of two distinct peptides per protein. For analysis of phosphorylation in intact cells, DGLα-V5 was purified from HEK293T cell lysates in the presence of protein phosphatase inhibitors after expression with or without constitutively active CaMKIIα. Two DGLα-V5 peptides, GSPSGLHAVLER (residues 805–815) and RAPLATMESLSDTSELYSFDSR (residues 774–795), were analyzed using a targeted LC-MS/MS multiple-reaction monitoring (MRM) method, which was optimized using peptides derived from in vitro phosphorylated GST-DGLα (761–870) (Fig. 2c,d). DGLα-V5 was resolved via SDS-PAGE and in-gel digested with trypsin (see above); extracted peptides were resolved on a C18 column using a Waters NanoAcuity LC (flow rate, 450 nl/min). Peptides were eluted using a 60-min gradient (0–1 min, 1% B; 1–40 min, 1–40% B; 40–45 min, 40–90% B; 45–46 min, 90% B; 46–48 min, 90–1% B; 48–60 min, 1% B) and then analyzed on a TSG Vantage triple quadrupole mass spectrometer, equipped with a nanoelectrospray ionization source (Thermo Scientific). Instrument method parameters included Q2 collision cell gas of 1.5 mTorr, 0.02 m/z window, scan time of 50 ms, and Q1 and Q3 resolution of 0.7 Th at full-width half-maximum (FWHM). Targeted DGLα peptides and their corresponding precursor and product m/z values empirically developed for MRM analysis using in vitro–phosphorylated DGLα are listed in Supplementary Table 2. This optimized method with a refined subset of selected MRM transitions for each peptide was then used to analyze DGLα that had been phosphorylated in intact cells (Supplementary Fig. 3). The MRM data were imported into Skyline software55 for analysis, where co-eluting transition peaks were grouped by peptide precursor.

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In vitro phosphorylation assay. DGLα-V5 or DGLβ-V5 bound to protein-G beads (as above) were incubated (20 min; 30 °C) in the presence or absence of purified CaMKIIα (10 nM) in assay buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 2 mM CaCl₂, 2 μM calmodulin, 1 μM dithiothreitol, 400 μM [γ-32P]ATP (700–1,000 c.p.m./pmol)). Reactions were stopped and proteins were eluted by heating (10 min; 70 °C) in LDS sample buffer (Invitrogen). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes, which were then exposed overnight to X-ray film. To verify expression and the specificity of immunoprecipitation, membranes were then blotted with mouse anti-V5. Essentially the same procedure was carried out in reactions involving GST-fusion proteins, except that 15 μl aliquots of the reactions were stopped on P82 Whatman paper. The papers were then washed and phosphorylation stoichiometries were determined by quantifying 32P incorporation using a scintillation counter. To visualize phosphorylated products by autoradiography, some GST-fusion protein reactions were quenched with sample buffer and resolved by SDS-PAGE (see above).

DGL activity assay. Mouse striata or transfected HEK293T cell pellets were Dounce-homogenized in lysis buffer containing 20 mM HEPES (pH 7.5), 2 mM DTT, 250 mM sucrose, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF and 1 μM microcystin. After homogenization, membranes were pelleted by centrifugation at 10,000g for 25 min at 4 °C. The membrane pellet was resuspended in a Dounce homogenizer in membrane-resuspension buffer containing 20 mM HEPES (pH 7.5), 2 mM DTT, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF and 1 μM microcystin.

Aliquots of membrane protein (5 μg), containing equal amounts of DGLα protein as analyzed by immunoblot, were incubated (final volume, 50 μl; 37 °C or room temperature, as indicated in the main text,) with 1-steroyl-2-arachidonoylglycerol (SAG; Cayman Chemical), which was added directly from a 100% methanol stock (final methanol concentration, 5%). Reactions were stopped by adding 200 μl of 100% methanol containing 125 pmol 2-AG-d₄ and 50 pmol arachidonic acid-d₄. Insoluble material was removed by centrifugation (2,000g, 10 min, 4 °C), and 20 μl of supernatant was analyzed by LC/MS.

Analysis of CBs and arachidonic acid in dorsolateral striatum. After rapid decapitation without anesthesia, 1.0 mm blocks of tissue containing precommisural striatum were rapidly frozen on dry ice. Punches (1.0 mm) of dorsolateral striatum were rapidly frozen on dry ice. Punches (1.0 mm) of dorsolateral striatum were rapidly frozen on dry ice. Pumps into a C-18 column (50 × 2 mm, 3 μm) using the following reactions (the two-way ANOVA, which was followed by Bonferroni's post hoc test to compare each cell. Responses were normalized to an average of the baseline responses. Average normalized response amplitudes during the baseline were compared to the first time point following depolarization and statistical significance between groups was determined by an unpaired Student's t-test unless otherwise indicated. Series and input resistance was monitored throughout the experiment and recordings were discarded if series resistance changed by >20%.

Home-cage behavioral monitoring. All mice were housed separately for 48 h before and throughout home-cage monitoring. Mice were given an intraperitoneal vehicle (1:1:18 ethanol:emulphor:saline) injection 30 min before the beginning of the dark cycle and were monitored continuously for the next 24 h. The following day, the same mice were given a 12 mg/kg dose of JZL-184 (Cayman Chemical) 30 min before the dark cycle. Inhibition of MAGL by JZL-184 was reported to elevate brain 2-AG levels for more than 8 h. The automated Homecage Analysis monitoring system (CleverSys Inc.) was set to monitor distance traveled as well as several other relevant behaviors. The time spent eating and drinking were defined as time spent at the food bin or water bottle. Significance was determined by two-way ANOVA, which was followed by Bonferroni’s post hoc test to compare groups if the two-way ANOVA revealed a significant interaction.

Statistics. The statistical tests and parameters used are indicated in figure legends. Results were considered significant if they reached P < 0.05. Sample sizes for each experiment are based on previously published studies from our laboratories30,56,57. No blinding was done in any experiments. All data are presented as mean ± s.e.m.