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Comunicato Stampa

Studio congiunto Fondazione Santa Lucia, Università Tor Vergata e Università di Teramo

DISFUNZIONI NERVOSE: RIVISTO IL RUOLO DEGLI ENDOCANNABINOIDI CAMBIA L'APPROCCIO FARMACOLOGICO A NUMEROSE MALATTIE

Interessate patologie come Alzheimer, Parkinson, sclerosi multipla, obesità e infertilità

La prestigiosa rivista "*Nature Neuroscience*" ha appena pubblicato on line una ricerca proveniente dall'Italia che potrebbe rimettere in discussione alcuni meccanismi fondamentali nella trasmissione nervosa e nel trattamento di sue importanti disfunzioni. Per i risultati di questo studio, cui il prossimo numero della rivista dedicherà anche un editoriale, s'ipotizza un notevole impatto sulle strategie di sviluppo di nuovi farmaci capaci di contrastare le malattie nervose e quelle periferiche. La ricerca si è focalizzata sulle alterazioni del sistema endocannabinoide e sull'azione svolta da due neurotrasmettitori implicati sia nelle tossicodipendenze sia nelle malattie infiammatorie e degenerative del cervello. Il lavoro scientifico si è svolto presso l'IRCCS **Fondazione Santa Lucia** di Roma in collaborazione con l'**Università di Roma Tor Vergata** e l'**Università di Teramo**; coinvolto, come supporto, anche lo statunitense **The Scripps Research Institute**.

Le aree del cervello si scambiano informazioni mediante una fitta rete di segnali generati dai neurotrasmettitori: tra questi vi sono i *cannabinoidi endogeni* o e*ndocannabinoidi*, la cui azione è simile a quella di alcuni estratti della canapa indiana (*cannabis)* come hashish e marijuana. Il sistema degli endocannabinoidi si attiva in diverse malattie infiammatorie e degenerative del cervello, presumibilmente per frenare il danno neuronale. Due sono gli endocannabinoidi più coinvolti in tali patologie: l'*AEA (anandamide*) e *2-AG* (*2-arachidonilglicerolo*). Come già osservato nella sclerosi multipla, sarebbe soprattutto AEA ad attivarsi in caso di malattie neurodegenerative ed infiammatorie, con un effetto neuroprotettivo. Per potenziare la sua azione attualmente sono usati a scopo terapeutico i cannabinoidi vegetali derivati dalla canapa indiana che, però hanno effetti collaterali tipici di queste sostanze psicoattive.

Finora AEA e 2-AG erano ritenuti cooperativi e capaci di svolgere, fondamentalmente, le stesse azioni biologiche. Per tale motivo erano bersagli quasi equivalenti per lo sviluppo di farmaci contro gravi patologie neurodegenerative quali la malattia di Alzheimer, il morbo di Parkinson, la còrea di Huntington, la sclerosi multipla e la sclerosi laterale amiotrofica, come pure per patologie periferiche quali obesità, cirrosi epatica e infertilità. Ora questa ricerca ha evidenziato, per la prima volta, che AEA e 2-AG possono inibirsi reciprocamente. In particolare si è dimostrata la capacità di AEA di ridurre i livelli endogeni di 2-AG. Quindi AEA svolgerebbe il ruolo di endocannabinoide "buono" e il suo effetto neuroprotettivo in certe patologie scaturirebbe dall'inibizione di quello "cattivo", 2-AG, che ha invece un ruolo prodegenerativo ed è in grado di bloccare alcune sinapsi che normalmente tendono a preservare l'integrità neuronale.

(segue)

La scoperta comporta una radicale rivisitazione delle azioni svolte da questi fondamentali neurotrasmettitori ed apre interessanti prospettive nell'approccio farmacologico a quelle patologie correlate a disfunzioni del nostro sistema endocannabinoide. I risultati della ricerca suggeriscono lo sviluppo di farmaci in grado di stimolare la produzione nel cervello di AEA ma non di 2-AG oppure, al contrario, di inibire la produzione di 2-AG: Infatti, le attuali terapie basate sui derivati della canapa indiana attivano indiscriminatamente i recettori dei cannabinoidi del cervello (sia quelli per l'anandamide che per il 2-arachidonilglicerolo), con un effetto contemporaneamente antidegenerativo e prodegenerativo, oltre ad avere degli effetti indesiderati.

Lo studio ha visto la stretta collaborazione tra due laboratori della Fondazione Santa Lucia: quello di Neurochimica dei Lipidi, diretto dal prof. **Mauro Maccarrone***, e quello di Neurofisiologia, diretto dal dott. **Diego Centone****. Coinvolti due dipartimenti dell'Università di Roma Tor Vergata: Neuroscienze e Medicina Sperimentale e Scienze Biochimiche. Per l'Università di Teramo, ha partecipato il Dipartimento di Scienze Biomediche, anch'esso diretto dal prof. Maccarrone. Dagli USA The Scripps Research Institute ha messo a disposizione dei ricercatori italiani un modello di topo geneticamente modificato.

"Proprio l'interdisciplinarità e la condivisione di competenze scientifiche diverse – sottolinea Centonze - *ha permesso di dimostrare un aspetto inatteso dell'omeostasi degli endocannabinoidi che, se confermato anche in altre aree cerebrali, può davvero rivoluzionare il nostro modo di considerare la regolazione della trasmissione nervosa e il trattamento delle sue disfunzioni".*

In quanto alle ricadute pratiche della scoperta, il prof. Maccarrone evidenzia che *"ora la possibilità di ridurre in vivo i livelli di 2-AG tramite l'aumento di quelli di AEA è piuttosto concreta, visto che già esistono inibitori della degradazione dell'AEA molto efficaci. Più remota è invece la prospettiva di modulare direttamente il metabolismo del 2-AG, per il quale mancano ancora inibitori con potenziali applicazioni terapeutiche*".

Roma, 21 gennaio 2008

** **Mauro Maccarrone** è professore ordinario di biochimica e presidente del Corso di Laurea in Biotecnologie all'Università degli Studi di Teramo, dove dirige il Dipartimento di Scienze Biomediche. E' professore visitatore presso il Dipartimento di Medicina Sperimentale e Scienze Biochimiche dell'Università degli Studi di Roma Tor Vergata. E' stato insignito di due premi internazionali per la ricerca scientifica. E' autore di oltre 200 pubblicazioni su riviste scientifiche internazionali.*

*** **Diego Centone** è Ricercatore Confermato in Neurologia alla Clinica Neurologica dell'Università Tor Vergata; presso il policlinico di questa Università è responsabile del Centro di Riferimento Regionale sulla Sclerosi Multipla. Svolge la sua attività scientifica nella Fondazione Santa Lucia, presso il Centro Europeo di Ricerca sul Cervello. Ha pubblicato circa 130 lavori scientifici internazionali e la rivista* Science *lo ha incluso tra i "*Six stellar neuroscientists*" in Europa e Nord America.*

nature neuroscience

Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum

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Of the endocannabinoids (eCBs), anandamide (AEA) and 2-arachidonoylglycerol (2-AG) have received the most study. A functional interaction between these molecules has never been described. Using mouse brain slices, we found that stimulation of metabotropic glutamate 5 receptors by 3,4-diydroxyphenylglycol (DHPG) depressed inhibitory transmission in the striatum through selective involvement of 2-AG metabolism and stimulation of presynaptic CB1 receptors. Elevation of AEA concentrations by pharmacological or genetic inhibition of AEA degradation reduced the levels, metabolism and physiological effects of 2-AG. Exogenous AEA and the stable AEA analog methanandamide inhibited basal and DHPG-stimulated 2-AG production, confirming that AEA is responsible for the downregulation of the other eCB. AEA is an endovanilloid substance, and the stimulation of transient receptor potential vanilloid 1 (TRPV1) channels mimicked the effects of endogenous AEA on 2-AG metabolism through a previously unknown glutathione-dependent pathway. Consistently, the interaction between AEA and 2-AG was lost after pharmacological and genetic inactivation of TRPV1 channels.

Recent physiological studies have substantially contributed to clarifying the role of eCBs in the short- and long-term control of synaptic transmission, and have shown that these substances are released on demand from stimulated neurons and move backward to inhibit transmitter release[1–3]. Direct postsynaptic neuronal depolarization[4–6] and specific patterns of synaptic activation[7,8] induce eCB-mediated suppression of synaptic transmission in many brain areas. Notably, metabotropic glutamate (mGlu) receptors have been heavily implicated in the activity-dependent production and physiological effects of eCBs[8–11]. Accordingly, pharmacological stimulation of group I mGlu receptors induces *per se* synaptic effects that are mediated by eCBs in many brain areas[12–15].

Several eCBs have been identified to date[1], and AEA and 2-AG are the most studied of these[1–3]. The distinct synthesis, transport and degradation processes of AEA and 2-AG suggest that there are specific regulation mechanisms for these substances. Consistent with this, AEA and/or 2-AG have been implicated in the eCB-mediated actions that have been described throughout the brain[2]. To date, however, no study has addressed the possible metabolic and/or functional interaction between AEA and 2-AG, although this interaction might be a mechanism involved in the control of synaptic transmission.

The specific aim of our study was to address the possible interactions between AEA and 2-AG in the nucleus striatum, a brain region that is important for motor and cognitive activities and contains high levels of cannabinoid CB1 receptors[1,3]. In this context, eCB signaling has been shown to be triggered in the striatum by repetitive synaptic activation[9,15]. However, the identity of the eCB molecule involved in the resulting long-term depression of excitatory transmission is uncertain, and it is difficult to address with direct biochemical assays. Here, we have chosen to study the AEA-mediated modulation of 2-AG metabolism and physiological activity in the simplified context of striatal slices subjected to pharmacological stimulation of mGlu receptors.

RESULTS

DHPG inhibits GABA transmission via CB1 receptors

In the cerebellum[7], hippocampus[13] and brainstem[14], activation of group I mGlu receptors by DHPG results in presynaptic inhibition of GABA transmission via CB1 receptor stimulation. To see whether a functional coupling between mGlu receptors and eCB-mediated synaptic inhibition was also present in the striatum, we measured the effects of DHPG at both excitatory and inhibitory synapses in striatal slices. DHPG had no effect on evoked ($n = 12$, $P > 0.05$) and miniature ($n = 16$, $P > 0.05$) excitatory postsynaptic currents (eEPSCs, mEPSCs) (**Fig. 1a**,**b**), but significantly depressed evoked inhibitory postsynaptic currents (eIPSCs; $n = 18$, $P < 0.01$), increased the paired-pulse ratio (PPR) of eIPSCs ($n = 10$, $P < 0.05$) and reduced the frequency (but not the amplitude) of miniature IPSCs (mIPSCs) ($n = 20$, $P < 0.01$) (**Fig. 1c–f**). These effects, which are indicative of a presynaptic site of

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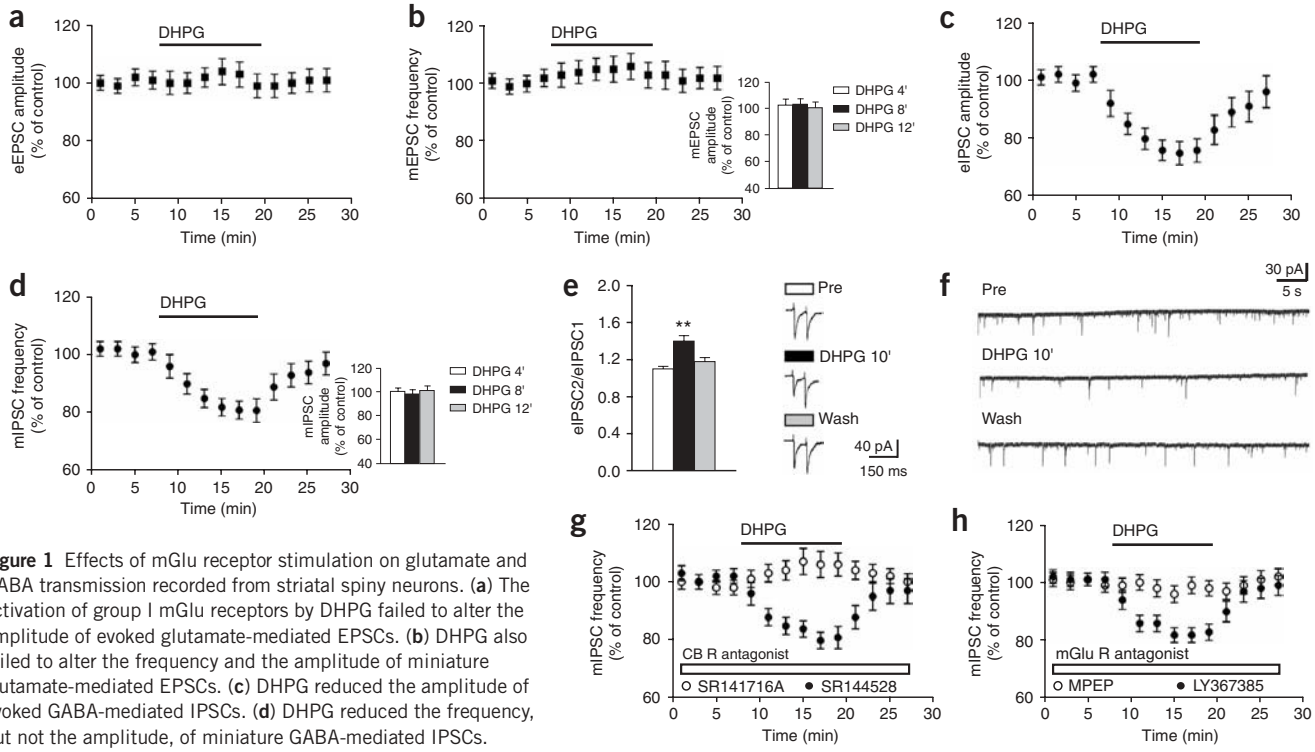

Figure 1 Effects of mGlu receptor stimulation on glutamate and GABA transmission recorded from striatal spiny neurons. (**a**) The activation of group I mGlu receptors by DHPG failed to alter the amplitude of evoked glutamate-mediated EPSCs. (**b**) DHPG also failed to alter the frequency and the amplitude of miniature glutamate-mediated EPSCs. (**c**) DHPG reduced the amplitude of evoked GABA-mediated IPSCs. (**d**) DHPG reduced the frequency, but not the amplitude, of miniature GABA-mediated IPSCs. (**e**) DHPG enhanced GABAergic PPR. Examples of evoked paired-pulse experiments before and during DHPG application are shown on the right. (**f**) The electrophysiological traces shown are examples of voltage-clamp recordings in the presence of tetrodotoxin. The mIPSC frequency was reduced by DHPG in striatal neurons. (**g**) SR141716A, but not SR144528, prevented the depressant action of DHPG on mIPSCs. (**h**) Blockade of mGlu 5 receptors with MPEP prevented the effects of DHPG. Blockade of mGlu 1 receptors with LY367385 failed to affect the action of DHPG on striatal mIPSCs. ** indicates $P < 0.01$.

action, were reversible at the wash of the drug, and were prevented by SR141716A ($n = 8$, $P > 0.05$), but not by SR144528 ($n = 8$, $P < 0.01$), which are selective inhibitors of CB1 and CB2 receptors, respectively (**Fig. 1g**). Pharmacological inhibition of mGlu 5 receptors by MPEP ($n = 14$, $P > 0.05$), but not of mGlu 1 receptors by LY367385 ($n = 10$, $P < 0.01$), also blocked the DHPG-mediated suppression of mIPSCs (**Fig. 1h**).

Despite the lack of effect of DHPG on eEPSCs and mEPSCs, functional CB1 receptors were also expressed at glutamate synapses, as application of the CB1 receptor agonist HU210 reduced eEPSC and eIPSC amplitudes, as well as mEPSC and mIPSC frequencies ($n =$ at least 7, $P < 0.05$ for each experimental group, data not shown)[16].

DHPG upregulates 2-AG synthesis and physiological effects

Consistent with the idea that DHPG engages the eCB system, we found that this agent increased CB1 receptor binding in striatal slices (pre-DHPG, 120 ± 14 fmol mg^{-1} protein; in DHPG, 170 ± 18 fmol mg^{-1} protein; $n = 4$, $P < 0.05$). To see whether the DHPG-stimulated eCB effects were mediated by AEA or by 2-AG, we compared the levels of both eCBs in basal conditions and following the application of DHPG. DHPG significantly increased 2-AG levels ($P < 0.01$) without altering AEA concentrations ($n = 4$, $P > 0.05$) (**Fig. 2a**). Notably, the time frame of DHPG treatment (a few minutes) does not seem to be compatible with protein synthesis, suggesting that elevated 2-AG increases the number of receptors on the plasma membrane by acting on its recycling[17].

Consistent with the electrophysiological data, MPEP ($n = 4$, $P > 0.05$), but not LY367385 ($n = 4$, $P < 0.01$), prevented the DHPG-induced upregulation of 2-AG (**Fig. 2b**). Increased synthesis and reduced degradation were probably responsible for the observed effects of DHPG on 2-AG levels, as DHPG stimulated the activity of the 2-AG–synthesizing enzyme diacylglycerol lipase[18] (DAGL; $n = 4$, $P < 0.05$) and reduced the activity of monoacylglycerol lipase (MAGL), the main responsible for 2-AG degradation[19] ($n = 4$, $P < 0.05$). Instead, 2-AG uptake through a purported 2-AG membrane transporter (2-AGMT)[20], AEA synthesis via *N*-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (NAPE-PLD)[21], transport via a purported AEA membrane transporter (AMT)[22] that might be the same entity as 2-AGMT[23], and degradation via fatty acid amide hydrolase (FAAH)[24] were unaffected by DHPG ($n = 4$, $P > 0.05$ for each experimental group; **Fig. 2c,d**).

We also measured the effects of DHPG on both eIPSCs ($n = 6$) and mIPSCs ($n = 7$) in the presence of O-3841, a selective inhibitor of DAGL[25]. Consistent with our biochemical results, O-3841 blocked the inhibitory effects of DHPG on striatal GABA transmission ($P > 0.05$ for both experimental groups; **Fig. 2e,f**). Notably, treatment of striatal slices with O-3841 selectively inhibited DAGL activity (~20% of the control value) without affecting the activity of MAGL (~95%), NAPE-PLD (~110%) or FAAH (~95%) in the same samples (100% values were as shown in **Fig. 2c,d** for unstimulated samples).

Inhibition of FAAH interferes with DHPG-mediated effects

We measured the physiological effects of DHPG in the presence of URB597, a selective inhibitor of FAAH[26], the enzyme that mediates AEA degradation[24]. Preincubation with URB597 failed to affect the amplitude of eIPSCs and the frequency of mIPSCs, but fully prevented

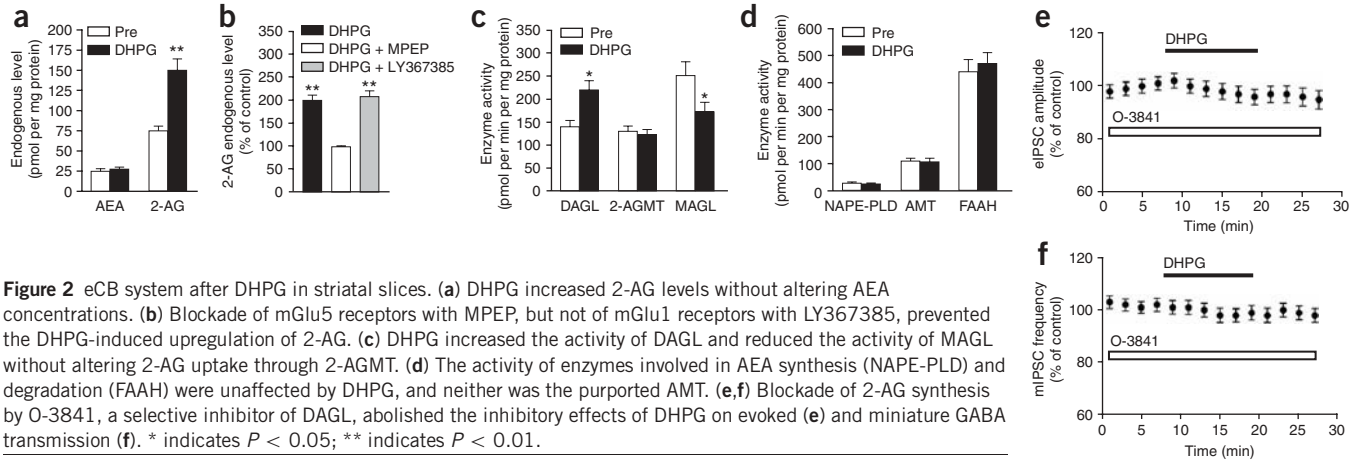

Figure 2 eCB system after DHPG in striatal slices. (**a**) DHPG increased 2-AG levels without altering AEA concentrations. (**b**) Blockade of mGlu5 receptors with MPEP, but not of mGlu1 receptors with LY367385, prevented the DHPG-induced upregulation of 2-AG. (**c**) DHPG increased the activity of DAGL and reduced the activity of MAGL without altering 2-AG uptake through 2-AGMT. (**d**) The activity of enzymes involved in AEA synthesis (NAPE-PLD) and degradation (FAAH) were unaffected by DHPG, and neither was the purported AMT. (**e**,**f**) Blockade of 2-AG synthesis by O-3841, a selective inhibitor of DAGL, abolished the inhibitory effects of DHPG on evoked (**e**) and miniature GABA transmission (**f**). * indicates $P < 0.05$; ** indicates $P < 0.01$.

the effects of DHPG on both of these neurophysiological parameters ($n = 9$, $P > 0.05$ for both experimental groups; **Fig. 3a**,**b**). URB597 prevented DHPG-driven upregulation of CB1 receptor expression ($n = 4$, $P > 0.05$; **Fig. 3c**), and contrasted DHPG-mediated action on 2-AG levels and metabolism. In the presence of this FAAH inhibitor, in fact, 2-AG levels were markedly downregulated, either in the absence ($n = 4$) or in the presence ($n = 4$) of this mGlu receptor agonist ($P < 0.01$ for each experimental group). As expected, URB597 significantly increased AEA levels in striatal slices, either in the absence ($n = 4$, $P < 0.01$) or in the presence of DHPG ($n = 4$, $P < 0.01$) (**Fig. 3d**). However, the URB597-dependent inhibition of DHPG's physiological effects on striatal GABA transmission was probably not caused by occlusion effects of the two eCBs on CB1 receptors, as stimulation of these receptors with HU210 reduced eIPSCs to a similar extent ($P > 0.05$) in the absence ($n = 7$, 79 ± 5%) and in the presence of the FAAH inhibitor ($n = 6$, 82 ± 8%).

We also confirmed that URB597, alone or in combination with DHPG, inhibited AEA degradation ($P < 0.01$) in our slices without altering AEA synthesis ($P > 0.05$) or transport ($P > 0.05$) ($n = 4$ for each experimental group). Moreover, the observed URB597-driven AEA increase was associated with reduced 2-AG synthesis ($n = 4$, $P < 0.01$), and normal 2-AG transport ($n = 4$, $P > 0.05$) and degradation ($n = 4$, $P > 0.05$) (**Fig. 3e**,**f**). However, URB597 did not inhibit DAGL activity in striatal slice homogenates when added directly to the assay mixture (145 ± 16 versus 140 ± 14 pmol min^{-1} mg^{-1} protein in the presence or absence of the drug, respectively; $n = 4$, $P > 0.05$), suggesting that its effect on DAGL in intact slices was indirect. As in control conditions, DHPG significantly reduced the activity of MAGL ($P < 0.01$), even in the presence of URB597 (**Fig. 3f**). This result was not further analyzed, as the levels of 2-AG did not appear to be substantially modified by this effect.

Genetic inactivation of FAAH prevents DHPG effects

The impact of AEA degradation on endogenous levels and physiological actions of 2-AG was also tested in FAAH knockout mice[27]. In these mutants ($n = 26$), the frequency (wild type, 0.80 ± 0.1 Hz; knockout, 0.85 ± 0.07 Hz; $P > 0.05$) and amplitude (wild type, 27.2 ± 2.7 pA; knockout, 28.9 ± 3 pA; $P > 0.05$) of mIPSCs were unaltered (**Fig. 4a**), whereas the DHPG-mediated modulation of eIPSCs and of mIPSCs was absent ($n = 12$, $P > 0.05$; **Fig. 4b**,**c**).

As with URB597, expression of CB1 receptors was normal in striatal slices from FAAH knockout mice (wild type, 135 ± 14 fmol mg^{-1} protein; knockout, 145 ± 14 fmol mg^{-1} protein; $n = 4$, $P > 0.05$), endogenous levels of AEA were increased ($n = 4$, $P < 0.01$) and endogenous levels of 2-AG were decreased ($n = 4$, $P < 0.01$) (**Fig. 4d**). Furthermore, application of DHPG increased 2-AG levels in slices from wild-type mice ($n = 4$, 204 ± 12%, $P < 0.01$), but not from FAAH knockout animals ($n = 4$, 100 ± 3%, $P > 0.05$). Conversely, in slices

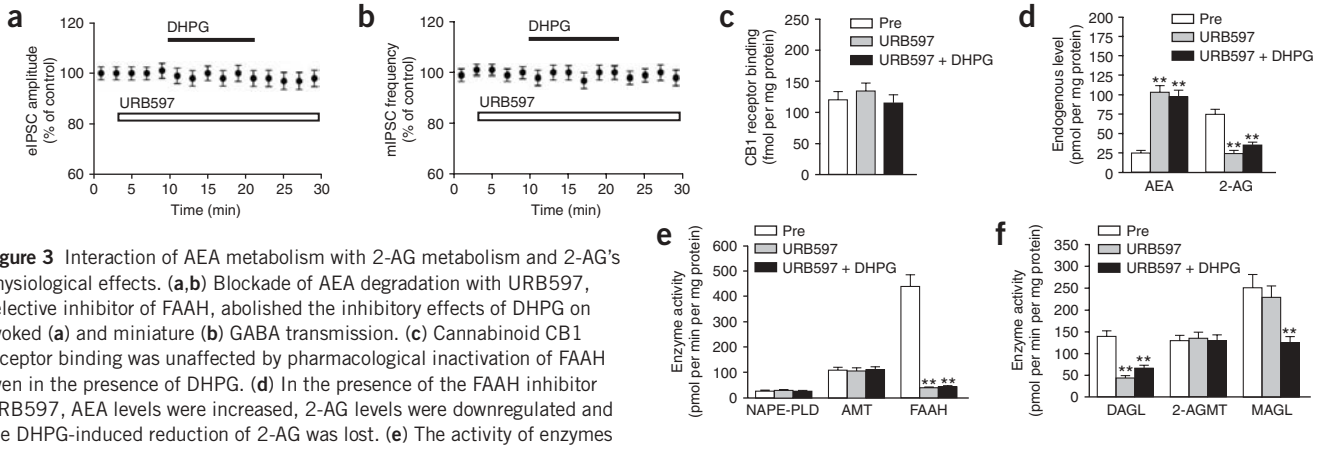

Figure 3 Interaction of AEA metabolism with 2-AG metabolism and 2-AG's physiological effects. (**a**,**b**) Blockade of AEA degradation with URB597, selective inhibitor of FAAH, abolished the inhibitory effects of DHPG on evoked (**a**) and miniature (**b**) GABA transmission. (**c**) Cannabinoid CB1 receptor binding was unaffected by pharmacological inactivation of FAAH even in the presence of DHPG. (**d**) In the presence of the FAAH inhibitor URB597, AEA levels were increased, 2-AG levels were downregulated and the DHPG-induced reduction of 2-AG was lost. (**e**) The activity of enzymes involved in AEA metabolism in the presence of URB597, alone or with DHPG. FAAH activity was reduced, whereas AEA synthesis and transport were unaffected. (**f**) The activity of enzymes involved in 2-AG metabolism in the presence of URB597, alone or with DHPG. ** indicates $P < 0.01$.

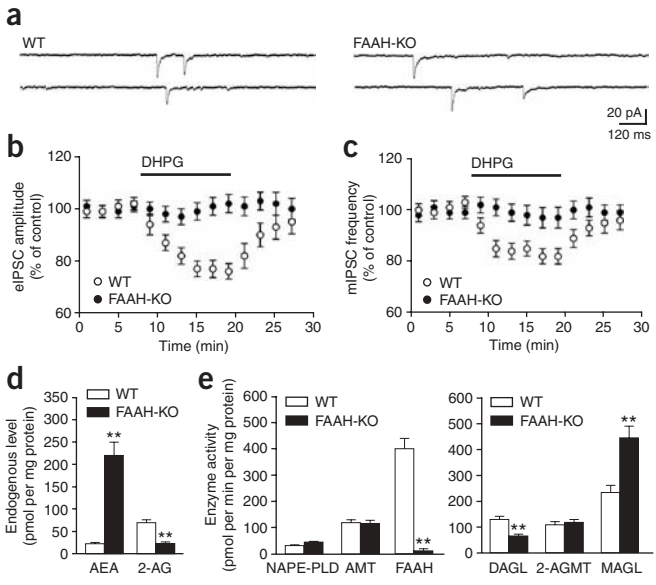

Figure 4 GABA transmission, mGlu receptor modulation and endocannabinoid metabolism in striatal slices of FAAH knockout and wild-type mice. (**a**) The electrophysiological traces are examples of mIPSC voltage-clamp recordings in wild-type and FAAH knockout mice. (**b**) The inhibitory effects of DHPG on eIPSC amplitude were absent in FAAH-KO mice. (**c**) The inhibitory effects of DHPG on mIPSC frequency were absent in FAAH knockout mice. (**d**) The genetic inactivation of FAAH markedly increased AEA levels and reduced 2-AG levels. (**e**) The activity of enzymes involved in AEA (left) and 2-AG (right) metabolism in FAAH knockout mice. DAGL activity was reduced and MAGL activity was increased. ** indicates $P < 0.01$.

from both wild-type ($n = 8$) and FAAH knockout mice ($n = 8$), HU210 produced similar ($P > 0.05$) inhibition of GABAergic eIPSCs (wild type, 77 ± 4%; knockout, 82 ± 7%), arguing against an occlusion mechanism as the basis for the loss of DHPG effects in these mutants.

As expected, FAAH activity was extremely low ($n = 4$, $P < 0.01$) in FAAH knockout mice, and the activity of DAGL was downregulated ($n = 4$, $P < 0.01$), providing a plausible explanation for the low levels of 2-AG that we found in these mutants. In FAAH knockout mice, we observed increased activity of MAGL ($n = 4$, $P < 0.01$), which might also contribute to the reduction of 2-AG levels (**Fig. 4e**).

AEA reduces 2-AG levels through TRPV1 channels

To directly address the role of AEA in the control of basal and DHPG-stimulated 2-AG levels, we pre-incubated the slices with the stable AEA derivative methanandamide (Met-AEA). Met-AEA produced *per se* inhibition of GABAergic eIPSCs ($n = 4$) and mIPSCs ($n = 4$) through the stimulation of CB1 receptors (data not shown), thereby preventing the analysis of the electrophysiological effects of DHPG on these parameters. Met-AEA, however, significantly reduced 2-AG levels in basal conditions ($n = 4$, $P < 0.01$) and prevented DHPG-mediated 2-AG elevation ($n = 4$, $P < 0.01$), thus mimicking the effects of FAAH inactivation. Blockade of CB1 receptors failed to reverse the effects of Met-AEA on 2-AG levels ($n = 4$, $P < 0.01$; **Fig. 5a**).

AEA and 2-AG both interact with CB receptors, whereas only AEA activates TRPV1 channels[28]. High expression of TRPV1 protein has been recently reported in the striatum[29], and we confirmed this (**Fig. 5b**). Thus, to explore the possibility that AEA inhibits 2-AG actions by targeting AEA-specific receptor sites, we tried to mimic the effects of AEA level manipulations with the selective TRPV1 channel agonist capsaicin. As observed after treatment with URB597 and in FAAH knockout mice, capsaicin (1 μM) did not affect GABAergic mIPSCs, but fully prevented the DHPG-mediated suppression of these synaptic currents ($n = 12$, $P > 0.05$; **Fig. 5c**). Capsaicin (1 μM) also mimicked the effects of Met-AEA and FAAH inhibition on 2-AG metabolism, as it reduced basal 2-AG levels ($n = 4$, $P < 0.05$), and blocked the elevation of 2-AG in response to DHPG ($n = 4$, $P < 0.05$; **Fig. 5d**). Although higher concentrations of capsaicin (30 μM) increased the frequency of glutamate-mediated mEPSCs (~150% of basal mEPSC frequency, $n = 6$, $P < 0.01$), the effects of this compound on 2-AG metabolism and physiological action were probably not

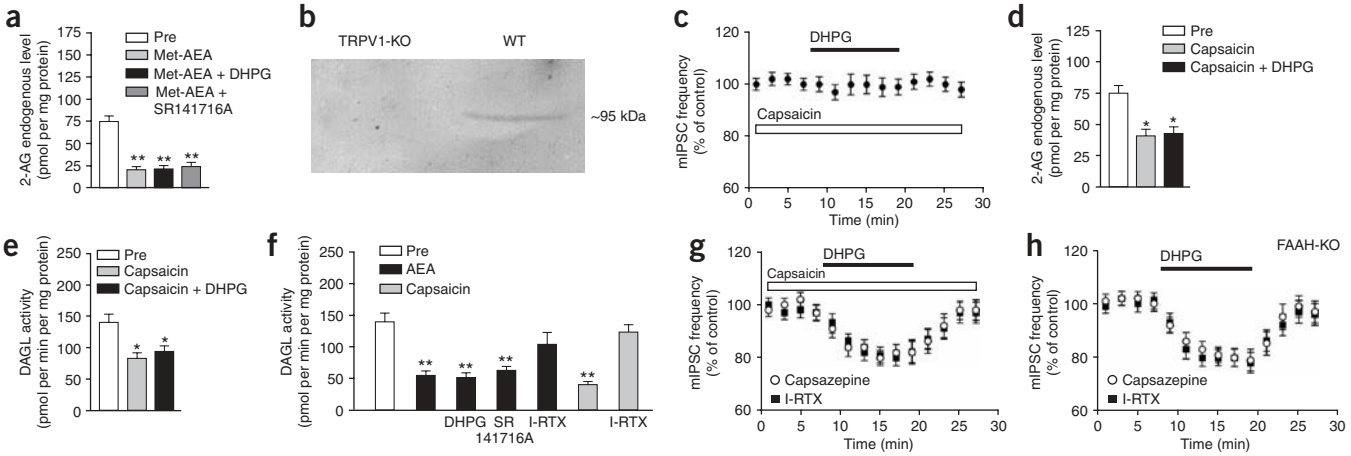

Figure 5 Role of Met-AEA and vanilloid receptors in striatal DHPG effects. (**a**) In the presence of the stable AEA derivative Met-AEA, 2-AG levels were downregulated in the absence and in the presence of DHPG or SR141716A. (**b**) Western blot showing the expression of TRPV1 protein in the striatum of WT mice and the lack of expression in TRPV1 knockout mice. (**c**) Capsaicin, an agonist of TRPV1 channels, prevented the DHPG-induced reduction of mIPSC frequency. (**d**,**e**) Capsaicin reduced 2-AG levels (**d**) in the absence and in the presence of DHPG by inhibiting DAGL activity (**e**). (**f**) AEA- and capsaicin-mediated modulation of DAGL activity was lost in the presence of the TRPV1 antagonist I-resiniferatoxin (I-RTX), whereas SR141716A and DHPG failed to affect the AEA-induced inhibition of DAGL. (**g**,**h**) The TRPV1 antagonists I-RTX and capazepine rescued the DHPG-mediated inhibition of mIPSC in the presence of capsaicin (**g**) and in FAAH knockout mice (**h**). * indicates $P < 0.05$; ** indicates $P < 0.01$.

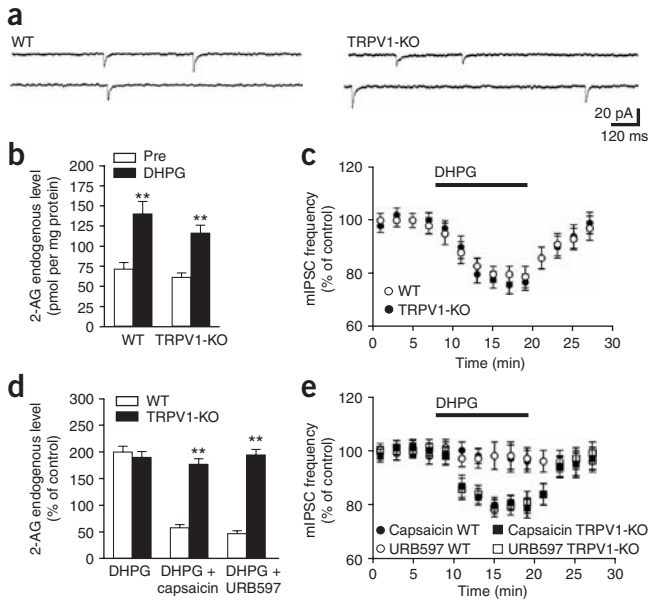

Figure 6 GABA transmission, mGlu receptor modulation and endocannabinoid metabolism in striatal slices of TRPV1 knockout mice. (**a**) The electrophysiological traces are examples of mIPSC voltage clamp recordings in wild-type and TRPV1 knockout mice. (**b**) DHPG-induced increase of 2-AG levels was similar in wild-type and TRPV1 knockout mice. (**c**) The inhibitory effect of DHPG on mIPSC frequency was maintained in TRPV1 knockout mice. (**d**) The graph shows 2-AG endogenous levels in TRPV1 knockout and wild-type mice in the presence of DHPG, DHPG and capsaicin, and DHPG and URB597. (**e**) In TRPV1 knockout mice, the DHPG-induced reduction of mIPSC frequency was present even in the presence of URB597 and capsaicin. ** indicates $P < 0.01$.

mediated by increased glutamate release. Accordingly, 1 μM capsaicin did not enhance striatal mEPSC frequency ($n = 8$, $P > 0.05$), and pharmacological blockade of NMDA and AMPA glutamate receptors with MK-801 and CNQX, respectively, and of mGlu 1 receptors, group II and III mGlu receptors with LY367385, EGLU or CPPG, respectively, failed to prevent the effects of capsaicin (1 μM) on DHPG-mediated inhibition of mIPSCs ($n = 6$, $P > 0.05$; data not shown). In all of the recorded neurons, capsaicin (1 and 30 μM) did not induce detectable inward currents.

Inhibition of DAGL appeared to mediate the effects of capsaicin on basal and DHPG-stimulated 2-AG levels ($n = 4$, $P < 0.05$), as observed following FAAH blockade (**Fig. 5e**). DAGL activity was also markedly downregulated by exogenously applied AEA, either in the absence ($n = 4$, $P < 0.01$) or in the presence of DHPG ($n = 4$, $P < 0.01$) or SR141716A ($n = 4$, $P < 0.01$), whereas both capsaicin-mediated (1 μM, $n = 4$, $P > 0.05$) and AEA-mediated inhibition of DAGL activity ($n = 4$, $P > 0.05$) was prevented by pharmacological inhibition of TRPV1 channels with I-resiniferatoxin (**Fig. 5f**). Furthermore, both I-resiniferatoxin and capsazepine, another antagonist of TRPV1 channels, rescued the DHPG-mediated inhibition of mIPSCs in the presence of capsaicin (1 μM, $n = 8$, $P < 0.01$) and in FAAH knockout mice ($n = 7$, $P < 0.01$) (**Fig. 5g**,**h**).

We also examined 2-AG metabolism and its physiological effects in TRPV1 knockout mice. In these mutants, basal ($n = 4$) and DHPG-stimulated 2-AG levels ($n = 4$) were similar to those observed in wild-type control mice ($P < 0.01$ compared with pre-DHPG conditions in both genotypes), as were mIPSC frequencies (wild type, 0.95 ± 0.1 Hz; knockout,1.05 ± 0.1 Hz) and amplitudes (wild type, 27 ± 3 pA; knockout, 28 ± 3 pA) recorded in the basal condition ($n = 12$, $P > 0.05$) and after the application of DHPG ($n = 11$, $P < 0.01$) (**Fig. 6a–c**). In TRPV1 knockout mice, however, URB597 and capsaicin (1 μM) failed to prevent both DHPG-mediated elevation of 2-AG levels ($n = 4$, $P < 0.01$) (**Fig. 6d**) and DHPG-mediated inhibition of mIPSC frequency ($n = 10$, $P < 0.01$) (**Fig. 6e**). Notably, when we compared the amount of AEA (pmol mg^{-1} protein) in striatal slices with the amount of cells needed to yield 1 mg of protein, using neuronal cell lines of known diameter and purported spherical shape such as CHP100 (diameter, ~40 μm; ~1 × 10^6 cells mg^{-1} protein) or SH-SY5Y (diameter, ~20 μm; ~2.5 × 10^6 cells mg^{-1} protein), we estimated that the intracellular concentration of AEA in striatal slices is ~1–2 μM. This amount is about tenfold higher than what is necessary to fully activate vanilloid receptors[30].

Glutathione controls 2-AG–mediated physiological effects

Capsaicin reduced glutathione content in striatal slices by 50% (from 1.8 ± 0.2 to 0.9 ± 0.1 mmol g^{-1} tissue, $n = 4$, $P < 0.01$) and I-resiniferatoxin fully reversed this effect (2.0 ± 0.2 mmol g^{-1} tissue). In addition, capsaicin (1 μM) prevented DHPG-mediated elevation of glutathione content in striatal slices ($n = 4$, $P < 0.01$). These observations suggest a mechanism by which TRPV1 agonists, such as capsaicin and AEA, inhibit 2-AG synthesis. Glutathione, in fact, stimulates DAGL activity at physiological concentrations[18] and is upregulated by DHPG (ref. 31 and **Fig. 7a**). Notably, pretreatment with buthionine sulfoximine (BSO), an irreversible inhibitor of glutathione synthesis, prevented DHPG-mediated inhibition of GABA transmission ($n = 9$, $P > 0.05$), confirming the physiological relevance

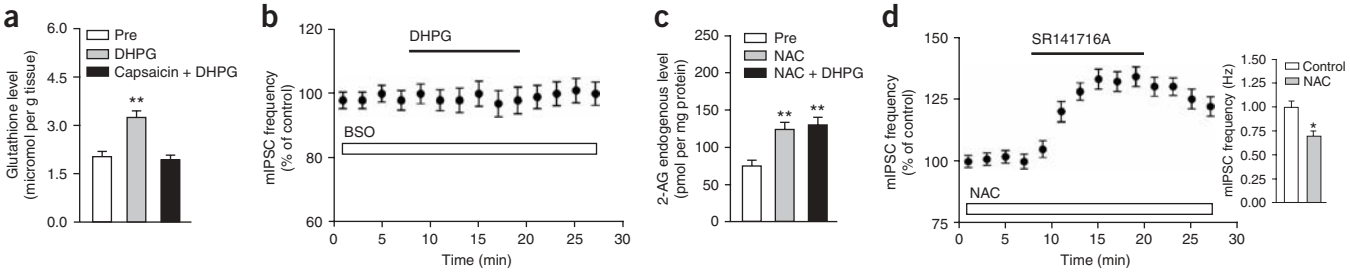

Figure 7 Glutathione levels control 2-AG levels and its physiological effects. (**a**) DHPG enhanced glutathione contents in striatal slices, an effect that was prevented by capsaicin. (**b**) BSO, an irreversible inhibitor of glutathione synthesis, blocked the DHPG-mediated reduction of mIPSC frequency. (**c**) NAC, a precursor of glutathione synthesis, mimicked and occluded the DHPG-induced increase of 2-AG levels. (**d**) Bath application of SR141716A increased mIPSC frequency in slices pretreated with NAC. Inset, NAC induced *per se* inhibition of mIPSC frequency . ** *P* indicates < 0.01.

of glutathione in DHPG- and 2-AG–mediated synaptic effects (**Fig. 7b**). Consistent with this conclusion, elevation of glutathione contents with *N*-acetylcysteine (NAC), a precursor of glutathione synthesis, mimicked ($n = 4$, $P < 0.01$) and occluded the effects of DHPG on 2-AG levels ($n = 4$, $P > 0.05$ compared with NAC alone) and on mIPSC frequency (NAC alone: $n = 12$, $P < 0.01$; NAC + DHPG: $n = 6$, $P > 0.05$ compared with NAC alone). Notably, bath application of SR141716A ($n = 4$) increased the mIPSC frequency recorded in slices pretreated with NAC to normal values ($P < 0.05$ compared to pre-SR141716A values), providing further support for the notion that NAC-induced inhibition of mIPSC frequency is mediated by 2-AG acting on CB1 receptors (**Fig. 7c,d**). On the other hand, further studies are necessary for addressing the details of the regulation of glutathione metabolism by TRPV1 channels, including the precise location of these receptors in striatal cellular elements and the potential role of calcium ions flowing through TRPV1 channels.

DISCUSSION

We have identified a previously unknown function for AEA in the striatum: the modulation of the metabolism of 2-AG and its physiological effects. Our data also indicate that striatal levels of 2-AG are mostly regulated by the activity of its synthesizing enzyme, DAGL. Accordingly, all manipulations changing 2-AG levels were associated with parallel changes of DAGL activity. In contrast, the activity of MAGL was less consistently associated with 2-AG levels, and changed unpredictably in our experiments.

Stimulation of TRPV1 channels mediates the effects of AEA by interfering with glutathione-dependent 2-AG synthesis (**Supplementary Fig. 1** online). However, the general validity of this mechanism throughout the brain is not guaranteed. Evidence exists, in fact, that 2-AG levels remain unaltered in the whole brain of FAAH knockout mice and that acute administration of FAAH inhibitors, including URB597, does not affect 2-AG levels in most brain areas, whereas treatment with URB597 increases 2-AG levels in some brainstem areas and in the spinal cord[32,33]. Furthermore, other FAAH inhibitors, also elevate 2-AG levels in whole brain[34], particularly if administered subchronically, whereas previous physiological studies have failed to observe FAAH inhibition interfering with 2-AG retrograde signaling[35–38]. Therefore, it seems likely that the mechanism described herein applies only under certain conditions and in select brain areas. One example could be the opposing changes of AEA and 2-AG during dark and light phases of the day in some brain regions, including the striatum[39].

The dendritic region of striatal projection neurons is a possible site for the interaction between TRPV1 channels and DAGL, as DAGL is particularly enriched in the spines of striatal neurons[10]. However, we failed to record ion currents induced by capsaicin in the recorded cells, as one would expect to see when assuming that vanilloid receptors contain an intrinsic ion channel. Alternatively, TRPV1 channels that are important for the control of 2-AG synthesis might be located intracellularly in striatal neurons, where they seem to regulate calcium release from intracellular stores[29].

At any rate, our results might suggest a reconceptualization of the eCB system, which has so far been considered to be made of interchangeable molecules with similar physiological roles. Although differentially involved in different brain areas, endogenous AEA and 2-AG mediate identical synaptic phenomena throughout the brain via CB1 receptors[2], giving rise to the idea that the two molecules may cooperate to modulate synaptic transmission. Here, we have shown that AEA elevation in the striatum did not potentiate the physiological effects of 2-AG on GABAergic transmission, but rather opposed this action by reducing basal and DHPG-stimulated production of 2-AG. Incidentally, the levels of 2-AG found here in striatal slices were about threefold higher than those of AEA, a value that is at variance with previous reports that have suggested a much higher difference[40]. For instance, ratios of 2-AG to AEA of ~200 have been observed in the striatum of rats[41]. However, the same group subsequently reported 2-AG to AEA ratios in rat striatum (~10), substantia nigra (~3) and globus pallidus (~4) that are comparable with our present findings[42]. These differences may arise from different methodologies; for example, killing the animals by decapitation without immediate freezing, rather than soaking them in liquid nitrogen, can increase 2-AG levels by ~15-fold[40]. Discrepancies may also be a consequence of the high sensitivity of eCBs to environmental factors such as animal diets, caging and bedding systems, viral load, water quality, and pathogen infections. An example of the notable effect of these factors on eCB levels has been recently reported[43]. On the other hand, the pmol amounts of AEA found here in striatal slices are consistent with previous data in different brain areas[40].

A recent biochemical study showed that pharmacological stimulation of mGlu 5 receptors increases 2-AG, but not AEA, levels in cultured hippocampal and striatal slices[44]. Here we extend and provide a functional correlate for these findings. Our data show that mGlu 5–driven eCB effects are selectively expressed at GABAergic synapses, although functional CB1 receptors have also been found at glutamatergic terminals[9,16]. These results support the idea that eCBs can select specific synaptic inputs, thereby finely tuning synaptic integration.

The interference of AEA with 2-AG levels and physiological effects might be a mechanism for synaptic crosstalk among excitatory and inhibitory inputs to striatal neurons. Accordingly, pharmacological modulation of AEA transport and degradation have implicated AEA as the primary eCB for reducing glutamate inputs to striatal neurons[9,45]. Thus, AEA might be critically involved in the control of the excitability of striatal neurons through a direct depressant action at excitatory synapses and an indirect interference with inhibitory inputs regulated by 2-AG. This mechanism might be important for the control of motor activity, which requires coordinated activity of both TRPV1 channels and CB1 receptors in the striatum[28].

METHODS

Animals. Corticostriatal coronal slices (200 μm) were prepared from brain blocks of 6–7-week-old C57BL/6 mice with a vibratome[16]. All efforts were made to minimize animal suffering and to reduce the number of mice used, in accordance with the European Communities Council Directive of 24 November, 1986 (86/609/EEC).

Electrophysiology. A single slice was transferred to a recording chamber and submerged in continuously flowing artificial cerebrospinal fluid (33 °C, 2–3 ml min^{-1}) gassed with 95% O_2 and 5% CO_2. The composition of the bathing solution was 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 11 mM glucose and 25 mM $NaHCO_3$.

Individual neurons were visualized *in situ* using a differential interference contrast (Nomarski) optical system. We employed an Olympus BX50WI upright microscope with 40× water-immersion objective combined with an infrared filter, a monochrome CCD camera (COHU 4912) and a PC-compatible system for analysis of images and contrast enhancement (WinVision 2000, Delta Sistemi). Recordings were made with borosilicate glass pipettes (1.8-mm outer diameter, 2–4 MΩ) by using an Axopatch 1D patch-clamp amplifier (Axon Instruments). Whole-cell access resistances were in the range of 5–20 MΩ.

To study GABA transmission, recording pipettes were filled with internal solution containing 110 mM CsCl, 30 mM potassium gluconate, 1.1 mM EGTA, 10 mM HEPES, 0.1 mM $CaCl_2$, 4 mM Mg-ATP and 0.3 mM Na-GTP. MK-801 and CNQX were added to the external solution to block NMDA and nonNMDA glutamate receptors, respectively. The identification of putative

spiny neurons was achieved immediately after rupture of the GΩ seal by evaluating the firing response to the injection of depolarizing current and/or pharmacologically, as DHPG does not alter the membrane properties of these cells, but causes membrane excitation in interneurons[46].

To study eEPSCs and mEPSCs, the recording pipettes were filled with internal solution containing 125 mM potassium gluconate, 10 mM NaCl, 1.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 0.5 mM BAPTA, 19 mM HEPES, 0.3 mM GTP and 1.0 mM Mg-ATP, adjusted to pH 7.3 with KOH. Bicuculline was added to the perfusing solution to block $GABA_A$-mediated transmission.

All synaptic events were recorded at the holding potential of –80 mV and stored using P-CLAMP 9 (Axon Instruments). Bipolar electrodes were used for synaptic stimulation. We placed these stimulating in the white matter between the cortex and the striatum to evoke corticostriatal eEPSCs or intrastriatally to induce GABA-mediated eIPSCs. Both eEPSCs and eIPSCs were evoked at a frequency of 0.1 Hz. The pulse interval was 70 ms for PPR experiments on eIPSCs.

Miniature excitatory and inhibitory currents were analyzed off-line on a personal computer with Mini Analysis 5.1 (Synaptosoft). The detection threshold of these events was set at twice the baseline noise. Off-line analysis was carried out on spontaneous and miniature synaptic events recorded during a fixed time epoch (1 to 3 min), sampled every 2 or 3 min before (2–5 samplings) and after (10–15 samplings) the application of the drugs. Only cells that showed stable frequencies in control (less than 20% changes during the control samplings) were taken into account. Events with complex peaks were eliminated.

Unless otherwise specified, DHPG was applied for 12 min and statistical significance was calculated by comparing the predrug value with that recorded immediately before DHPG wash out. Drugs were applied by dissolving them to the desired final concentration in the bathing artificial cerebrospinal fluid. The concentrations of the drugs were chosen on the basis of previous studies in brain slices, and were as follows: 1 or 30 μM capsaicin, 10 μM capsazepine, 10 μM CNQX, 1 mM CPPG, 50 μM 3,5-DHPG, 200 μM EGLU, 1 μM HU210, 1 μM I-resiniferatoxin, 50 μM LY367385, 30 μM MK-801, 30 μM MPEP, 1 μM tetrodotoxin (from T. Cookson, Bristol, UK), 10 μM bicuculline (from Sigma-RBI) and 1 μM URB597 (Alexis Biochemicals). SR141716A (1 μM) and SR144528 (1 μM) were kind gifts from Sanofi-Aventis. O-3841 (500 nM) was a kind gift from T. Bisogno (Istituto di Chimica Biomolecolare, CNR). To deplete or increase striatal glutathione, slices were incubated with 5 mM BSO or with 5 mM NAC (both from Sigma-RBI) for 2 h before the electrophysiological experiments[47]. Capsazepine, HU210, I-resiniferatoxin, O-3841, SR141716A, SR144528 and URB597 were dissolved in DMSO. The remaining compounds were dissolved in water.

Biochemistry. [^{3}H]AEA (223 Ci mmol^{-1}), [^{3}H]CP55.940 (126 Ci mmol^{-1}) and *sn*-1-stearoyl-2-[^{14}C]arachidonoyl-glycerol (56 mCi mmol^{-1}) were purchased from Perkin Elmer Life Sciences. Met-AEA (1 μM) was from Calbiochem and AEA (10 μM) was from Sigma-RBI. *N*-[^{3}H]arachidonoyl-phosphatidylethanolamine ([^{3}H]NArPE, 200 Ci mmol^{-1}) and 2-oleoyl-[^{3}H]glycerol (20 Ci mmol^{-1}) were from ARC. 2-[^{3}H]arachidonoylglycerol ([^{3}H]2-AG) was synthesized from 1,3-dibenzyloxy-2-propanol and [^{3}H]arachidonic acid (200 Ci mmol^{-1}; ARC), as reported[48].

Intact slices were treated as described above for the electrophysiological recordings and were subjected to biochemical measurements after tissue processing. Controls were treated, and the proper solvents were used as vehicles for the different drugs. In a separate set of experiments, the possible direct effect of URB597 on DAGL activity was ascertained on striatal slice homogenates by adding URB597 directly to the assay mixture.

The transport of 1 μM [^{3}H]AEA by AMT in synaptosomes prepared from striatal slices (100 μg per test), and the hydrolysis of 15 μM [^{3}H]AEA by FAAH (E.C. 3.5.1.4) in slice homogenates (20 μg per test) were carried out as reported[45]. AMT activity was expressed as pmol of AEA taken up per min per mg of protein, whereas FAAH activity was expressed as pmol of arachidonate released per min per mg of protein. The binding of 800 pmol [^{3}H]CP55,940 to CB1 receptors in membrane fractions (50 μg per test) prepared from mouse striatal slices was determined through rapid filtration assays under saturating conditions[45]. CB1 receptor binding was expressed as fmol of CP55940 bound per mg of protein. The synthesis of AEA through NAPE-PLD (E.C. 3.1.4.4) was assayed in slice homogenates (50 μg per test), using 100 μM [^{3}H]NArPE as reported[49]. NAPE-PLD activity was expressed as pmol of AEA released per min per mg of protein. The activity of DAGL was assayed with 10 μM *sn*-1-stearoyl-2-[^{14}C]arachidonoyl-glycerol as substrate[18], and that of MAGL was determined using 10 μM 2-oleoyl-[^{3}H]-glycerol as substrate, as reported[19]. Both DAGL and MAGL activities were expressed as pmol of product per min per mg of protein. The uptake of 2-AG through 2-AGMT was assayed as described above for AMT, using 1 μM [^{3}H]2-AG as the substrate.

For the evaluation of endogenous levels of AEA and 2-AG, mouse striatal slices were homogenized with an Ultra Turrax T25 in 50 mM Tris-HCl, 1 mM EDTA pH 7.4 and 1 mM phenylmethanesulfonyl fluoride buffer, at a 1:10 (wt/vol) homogenization ratio. Lipids were then extracted and the organic phase was dried under nitrogen. The dry pellet was resuspended in 20 μl of methanol, and was processed and analyzed by high-performance liquid chromatography with fluorimetric detection[50]. Also, 1-AG was included in 2-AG measurements, to account for isomerization in solution[50].

The levels of glutathione in striatal slices were quantified by means of the GSH assay kit (Cayman Chemical Company), following the manufacturer's instructions.

To assess the expression of TRPV1, we ran striatal extracts on 12% SDS-PAGE (200 μg per lane) under reducing conditions. For immunochemical analysis, gels were electroblotted onto 0.45-μm nitrocellulose filters (Bio-Rad) and were immunoreacted with TRPV1 (1: 500) polyclonal antibodies, and GAR-AP (diluted 1: 2000) was used as a secondary antibody. Rabbit antibodies to TRPV1 were from Santa Cruz Biotechnologies. Goat antibodies to rabbit, conjugated to alkaline phosphatase (GAR-AP), were from Bio-Rad.

Statistical analysis. Electrophysiological data are presented as mean ± s.e.m. Statistical analysis was carried out using a paired or unpaired Student's *t*-test or Wilcoxon's test. Biochemical data are presented as mean ± s.d. of at least three independent experiments, each performed in duplicate. Statistical analysis was performed by the nonparametric Mann-Whitney U test, elaborating experimental data by means of the InStat 3 program (GraphPAD Software for Science). The significance level was established at $P < 0.05$.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

M.M. planned the biochemical experiments, coordinated the study and revised the draft manuscript. S.R. carried out the electrophysiological recordings, analyzed the data and prepared the figures. M.B., F.F. and V.G. performed the biochemical experiments and analyzed the data. V.D.C., C.P. and A.M. carried out electrophysiological recordings, G.B. and A.F.-A. participated in the study design and revised the draft manuscript, B.F.C. participated in the study design and provided the FAAH knockout mice, and D.C. planned the electrophysiological experiments, coordinated the study and drafted the manuscript. All authors contributed to the discussion and interpretation of the results.

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1. Piomelli, D. The molecular logic of endocannabinoid signaling. *Nat. Rev. Neurosci.* **4**, 873–884 (2003).
2. Chevaleyre, V., Takahashi, K.A. & Castello, P.E. Endocannabinoid-mediated synaptic plasticity in the CNS. *Annu. Rev. Neurosci.* **29**, 37–76 (2006).
3. Freund, T.F., Katona, I. & Piomelli, D. Role of endogenous cannabinoids in synaptic signaling. *Physiol. Rev.* **83**, 1017–1066 (2003).
4. Barinaga, M. How cannabinoids work in the brain. *Science* **291**, 2530–2531 (2001).

5. Kreitzer, A.C. & Regehr, W.G. Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron* **29**, 717–727 (2001).
6. Wilson, R.I. & Nicoll, R.A. Endogenous cannabinoids mediate retrograde signaling at hippocampal synapses. *Nature* **410**, 588–592 (2001).
7. Maejima, T., Hashimoto, K., Yoshida, T., Aiba, A. & Kano, M. Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. *Neuron* **31**, 463–475 (2001).
8. Melis, M. *et al.* Prefrontal cortex stimulation induces 2-arachidonoyl glycerol–mediated suppression of excitation in dopamine neurons. *J. Neurosci.* **24**, 10707–10715 (2004).
9. Gerdeman, G.L., Ronesi, J. & Lovinger, D.M. Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. *Nat. Neurosci.* **5**, 446–451 (2002).
10. Uchigashima, M. *et al.* Subcellular arrangement of molecules for 2-arachidonoyl glycerol–mediated retrograde signaling and its physiological contribution to synaptic modulation in the striatum. *J. Neurosci.* **27**, 3663–3676 (2007).
11. Soler-Llavina, G.J. & Sabatini, B.L. Synapse-specific plasticity and compartmentalized signaling in cerebellar stellate cells. *Nat. Neurosci.* **9**, 798–806 (2006).
12. Ohno-Shosaku, T., Shosaku, J., Tsubokawa, H. & Kano, M. Cooperative endocannabinoid production by neuronal depolarization and group I metabotropic glutamate receptor activation. *Eur. J. Neurosci.* **15**, 953–961 (2002).
13. Rouach, N. & Nicoll, R.A. Endocannabinoids contribute to short-term, but not long-term, mGluR-induced depression in the hippocampus. *Eur. J. Neurosci.* **18**, 1017–1020 (2003).
14. Kushmerick, C. *et al.* Retroinhibition of presynaptic Ca^{2+} currents by endocannabinoids released via postsynaptic mGluR activation at a calyx synapse. *J. Neurosci.* **24**, 5955–5965 (2004).
15. Kreitzer, A.C. & Malenka, R.C. Dopamine modulation of state-dependent endocannabinoid release and long-term depression in the striatum. *J. Neurosci.* **25**, 10537–10545 (2005).
16. Centonze, D. *et al.* Abnormal sensitivity to cannabinoid receptor stimulation might contribute to altered gamma-aminobutyric acid transmission in the striatum of R6/2 Huntington's disease mice. *Biol. Psychiatry* **57**, 1583–1589 (2005).
17. Sarnataro, D. *et al.* Plasma membrane and lysosomal localization of CB1 cannabinoid receptor are dependent on lipid rafts and regulated by anandamide in human breast cancer cells. *FEBS Lett.* **579**, 6343–6349 (2005).
18. Bisogno, T. *et al.* Cloning of the first *sn*1-DAG lipases points to the spatial and temporal regulation of endocannabinoid knockout in the brain. *J. Cell Biol.* **163**, 463–468 (2003).
19. Dinh, T.P. *et al.* Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. USA* **99**, 10819–10824 (2002).
20. Hermann, A., Kaczocha, M. & Deutsch, D.G. 2-Arachidonoylglycerol (2-AG) membrane transport: history and outlook. *AAPS J.* **8**, E409–412 (2006).
21. Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T. & Ueda, N. Molecular characterization of a phospholipase D generating anandamide and its congeners. *J. Biol. Chem.* **279**, 5298–5305 (2004).
22. Glaser, S.T., Kaczocha, M. & Deutsch, D.G. Anandamide transport: a critical review. *Life Sci.* **77**, 1584–1604 (2005).
23. Bari, M., Battista, N., Fezza, F., Gasperi, V. & Maccarrone, M. New insights into endocannabinoid degradation and its therapeutic potential. *Mini Rev. Med. Chem.* **6**, 275–268 (2006).
24. McKinney, M.K. & Cravatt, B.F. Structure and function of fatty acid amide hydrolase. *Annu. Rev. Biochem.* **74**, 411–432 (2005).
25. Bisogno, T. *et al.* Development of the first potent and specific inhibitors of endocannabinoid biosynthesis. *Biochim. Biophys. Acta* **1761**, 205–212 (2006).
26. Kathuria, S. *et al.* Modulation of anxiety through blockade of anandamide hydrolysis. *Nat. Med.* **9**, 76–81 (2003).
27. Cravatt, B.F. *et al.* Supersensitivity to anandamide and enhanced endogenous cannabinoid signalling in mice lacking fatty acid amide hydrolase. *Proc. Natl. Acad. Sci. USA* **98**, 9371–9376 (2001).
28. Starowicz, K., Nigam, S. & Di Marzo, V. Biochemistry and pharmacology of endovanilloids. *Pharmacol. Ther.* **114**, 13–33 (2007).
29. Cristino, L. *et al.* Immunohistochemical localization of cannabinoid type 1 and vanilloid transient receptor potential vanilloid type 1 receptors in the mouse brain. *Neuroscience* **139**, 1405–1415 (2006).
30. Evans, R.M., Scott, R.H. & Ross, R.A. Multiple actions of anandamide on neonatal rat cultured sensory neurones. *Br. J. Pharmacol.* **141**, 1223–1233 (2004).
31. Sagara, Y. & Schubert, D. The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. *J. Neurosci.* **18**, 6662–6671 (1998).
32. Jhaveri, M.D., Richardson, D., Kendall, D.A., Barrett, D.A. & Chapman, V. Analgesic effects of fatty acid amide hydrolase inhibition in a rat model of neuropathic pain. *J. Neurosci.* **26**, 13318–13327 (2006).
33. Maione, S. *et al.* Elevation of endocannabinoid levels in the ventrolateral periaqueductal grey through inhibition of fatty acid amide hydrolase affects descending nociceptive pathways via both cannabinoid receptor type 1 and transient receptor potential vanilloid type-1 receptors. *J. Pharmacol. Exp. Ther.* **316**, 969–982 (2006).
34. de Lago, E. *et al.* Effect of repeated systemic administration of selective inhibitors of endocannabinoid inactivation on rat brain endocannabinoid levels. *Biochem. Pharmacol.* **70**, 446–452 (2005).
35. Kim, J. & Alger, B.E. Inhibition of cyclooxygenase-2 potentiates retrograde endocannabinoid effects in hippocampus. *Nat. Neurosci.* **7**, 697–698 (2004).
36. Makara, J.K. *et al.* Selective inhibition of 2-AG hydrolysis enhances endocannabinoid signaling in hippocampus. *Nat. Neurosci.* **8**, 1139–1141 (2005).
37. Melis, M. *et al.* Protective activation of the endocannabinoid system during ischemia in dopamine neurons. *Neurobiol. Dis.* **24**, 15–27 (2006).
38. Szabo, B. *et al.* Depolarization-induced retrograde synaptic inhibition in the mouse cerebellar cortex is mediated by 2-arachidonoylglycerol. *J. Physiol. (Lond.)* **577**, 263–280 (2006).
39. Valenti, M. *et al.* Differential diurnal variations of anandamide and 2-arachidonoylglycerol levels in rat brain. *Cell. Mol. Life Sci.* **61**, 945–950 (2004).
40. Sugiura, T., Kobayashi, Y., Oka, S. & Waku, K. Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukot. Essent. Fatty Acids* **66**, 173–192 (2002).
41. Bisogno, T. *et al.* Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. *Biochem. Biophys. Res. Commun.* **256**, 377–380 (1999).
42. Di Marzo, V., Hill, M.P., Bisogno, T., Crossman, A.R. & Brotchie, J.M. Enhanced levels of endogenous cannabinoids in the globus pallidus are associated with a reduction in movement in an animal model of Parkinson's disease. *FASEB J.* **14**, 1432–1438 (2000).
43. Guo, Y. *et al. N*-acylphosphatidylethanolamine–hydrolyzing phospholipase D is an important determinant of uterine anandamide levels during implantation. *J. Biol. Chem.* **280**, 23429–23432 (2005).
44. Jung, K.M. *et al.* Stimulation of endocannabinoid formation in brain slice cultures through activation of group I metabotropic glutamate receptors. *Mol. Pharmacol.* **68**, 1196–1202 (2005).
45. Gubellini, P. *et al.* Experimental parkinsonism alters endocannabinoid degradation: implications for striatal glutamatergic transmission. *J. Neurosci.* **22**, 6900–6907 (2002).
46. Pisani, A., Bonsi, P., Centonze, D., Bernardi, G. & Calabresi, P. Functional coexpression of excitatory mGluR1 and mGluR5 on striatal cholinergic interneurons. *Neuropharmacology* **40**, 460–463 (2001).
47. Pellmar, T.C., Roney, D. & Lepinski, D.L. Role of glutathione in repair of free radical damage in hippocampus *in vitro*. *Brain Res.* **583**, 194–200 (1992).
48. Cartoni, A., Margonelli, A., Angelini, G., Finazzi Agrò, A. & Maccarrone, M. Simplified chemical and radiochemical synthesis of 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Tetrahedr. Lett.* **45**, 2723–2726 (2004).
49. Fezza, F., Gasperi, V., Mazzei, C. & Maccarrone, M. Radiochromatographic assay of *N*-acyl-phosphatidylethanolamine–specific phospholipase D (NAPE-PLD) activity. *Anal. Biochem.* **339**, 113–120 (2005).
50. Wang, Y. *et al.* Simultaneous measurement of anandamide and 2-arachidonoylglycerol by polymixin B–selective adsorption and subsequent high-performance liquid chromatography analysis: increase in endogenous cannabinoids in the sera of patients with endotoxic shock. *Anal. Biochem.* **294**, 73–82 (2001).

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