

Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system

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Abbreviations: 2-AG: 2-arachidonoylglycerol; FAAH: Fatty acid amide hydrolase; MAGL: Monoacylglycerol lipase; CFA: Complete Freund's adjuvant; SNL: Spinal nerve ligation

Abstract

Endocannabinoids are lipid molecules that serve as natural ligands for the cannabinoid receptors CB1 and CB2. They modulate a diverse set of physiological processes such as pain, cognition, appetite and emotional states, and their levels and functions are tightly regulated by enzymatic biosynthesis and degradation. 2-arachidonoylglycerol (2-AG) is the most abundant endocannabinoid in the brain and is thought to be hydrolyzed primarily by the serine hydrolase monoacylglycerol lipase (MAGL). Although 2-AG binds and activates cannabinoid receptors *in vitro*, when administered *in vivo* it induces only transient cannabimimetic effects as a result of its rapid catabolism. Here we show using a mouse model with a targeted disruption of the MAGL gene that MAGL is the major modulator of 2-AG hydrolysis *in vivo*. Mice lacking MAGL exhibit dramatically reduced 2-AG hydrolase activity and highly elevated 2-AG levels in the nervous system. Lack of MAGL activity and subsequent chronic elevation of 2-AG levels lead to desensitization of brain CB1 receptors with a significant reduction of cannabimimetic effects of CB1 agonists. Also consistent with CB1 desensitization, MAGL deficient mice do not show alterations in neuropathic and inflammatory pain sensitivity. These findings provide the first genetic *in vivo* evidence that MAGL is the major regulator of 2-AG levels and signaling, and reveal a pivotal role for 2-AG in modulating CB1 receptor sensitization and endocannabinoid tone.

INTRODUCTION

Endocannabinoids modulate a number of physiological processes, including pain, appetite, immune functions and emotional state (Di Marzo et al., 2001; Gallily et al., 2000; Hohmann et al., 2005; Kaminski et al., 1992; Klein and Cabral, 2006; Klein et al., 2000; Lee et al., 1995; Marsicano et al., 2002; Varvel and Lichtman, 2002). They serve as natural ligands for the cannabinoid (CB) receptors and TRP channels. Endocannabinoid levels are tightly controlled by a balanced enzymatic biosynthesis and degradation *in vivo*. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are two central components of the endocannabinoid signaling networks (Ahn et al., 2008; Cravatt et al., 2001; Kogan and Mechoulam, 2006; Lambert and Fowler, 2005; Ueda, 2002). The hydrolysis of AEA is principally mediated by the membrane enzyme fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). Pharmacological inhibition of FAAH using selective inhibitors was shown to elevate AEA levels throughout the nervous system and periphery (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004). FAAH-deficient mice lack AEA- hydrolyzing activity and the endogenous brain levels of AEA are dramatically elevated in these animals resulting in a variety of CB-receptor-mediated behavioral phenotypes (Ahn et al., 2008). These results suggest that FAAH is responsible for AEA degradation in the brain.

Although several enzymes have been implicated in 2-AG hydrolysis, several lines of evidence suggest that the serine hydrolase monoacylglycerol

lipase (MAGL) is the main contributor to 2-AG hydrolysis *in vivo* (Blankman et al., 2007; Kinsey et al., 2009). MAGL is abundant in brain tissues, where it localizes to presynaptic terminals (Dinh et al., 2002). A comprehensive profile of brain serine hydrolases revealed that 85% of brain 2-AG hydrolase activity may be attributed to MAGL, with the newly identified enzymes ABHD6 and ABHD12 hydrolyzing the remaining 15% (Blankman et al., 2007). The newly identified MAGL selective inhibitor JZL184 was shown to raise the levels of brain 2-AG by eight-fold and reduce 2-AG hydrolysis by 85% (Long et al., 2009).

2-AG acts as a full agonist at both CB1 and CB2 receptors whereas AEA was shown to act only as a partial agonist at these receptors (Sugiura et al., 2002). Lack of many CB1-mediated behavioral effects in FAAH-deficient mice (Cravatt et al., 2001) suggests that CB1-mediated signaling may be predominantly mediated by 2-AG. Similarly, progenitor cell proliferation in the brain subventricular zone (SVZ) was shown to be modulated both by CB2 and 2-AG levels (Goncalves et al., 2008), suggesting that 2-AG is also a bonafide CB2 agonist. The new role of endocannabinoids, particularly 2-AG, in adult brain neurogenesis underlies the pleiotropic nature of endocannabinoid functions and the therapeutic potential of these molecules in neurodegenerative diseases.

Here, we have tested the role of MAGL in controlling 2-AG levels *in vivo*, by generating and characterizing a MAGL-deficient mouse. Lack of MAGL activity leads to an altered profile of endogenous 2-AG hydrolase activity and dramatic increase of 2-AG levels in the nervous system. Chronic elevation of 2-

AG levels leads to desensitization of brain CB1 receptors with a significant reduction of cannabimimetic effects of 2-AG and CB1 agonists.

These findings provide the first genetic *in vivo* evidence that MAGL is the major regulator of 2-AG levels and signaling, and reveal a pivotal role for 2-AG in modulating CB1 receptor sensitization and endocannabinoid tone.

METHODS

Generation of MAGL^{-/-} mice. Approximately 13kb of genomic DNA surrounding mouse MAGL exons 1 and 2 were subcloned from a mouse BAC. Exons 1 and 2 were replaced by a loxP flanked PGK-neo cassette to allow for selection of homologous integration in C57Bl6/NTac ES cells. The final targeting vector contained approximately 5.1kb and 7.5 kb of MAGL genomic DNA on the 5' and 3' sides, respectively. Homologous integrants were identified by Southern blotting of KpnI digested ES cell genomic DNA. All animals analyzed in this study lacked the selection cassette and were maintained on a C57Bl6/NTac background.

Preparation of membrane-bound MAGL. Brains and other tissues were harvested from MAGL^{+/+}, MAGL^{+/-}, and MAGL^{-/-} mice and immediately frozen on dry ice and kept at -80°C until use. The membrane-bound MAGL was prepared using high speed centrifugation as described previously (Blankman et al., 2007).

Recombinant MAGL protein expression. The coding sequence of mouse MAGL was cloned into pColdIII expression vector (Takara-Mirus, Madison, WI) and expressed in *E. coli* as described previously (Wang et al., 2008).

MAGL activity assay. MAGL activity was evaluated using its natural substrate 2-AG, by incubation of recombinant MAGL protein or endogenous tissue extracts (20µg) at room temperature followed by measurement of 2-AG hydrolysis products (arachidonic acid, AA) by LC/MS/MS method as described previously (Mei-Yi zhang, 2009).

Western blot analysis. MAGL^{+/+} or MAGL^{-/-} mouse brain extracts were loaded onto a 4-12% SDS NuPAGE Bis-Tris polyacrylamide gel (Invitrogen) under reducing condition and was blotted onto a PVDF membrane. The membrane was probed using a rabbit polyclonal antibody (Cayman Chemicals) and the protein bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) as previously described (Wang et al., 2008).

Measurement of brain and spinal cord Levels of 2-AG and AEA. 2-AG and AEA levels in the brains of MAGL^{-/-} and MAGL^{+/+} mice were quantified using a previously described procedure (Mei-Yi zhang, 2009).

CB1 receptor binding assay. MAGL^{+/+} and MAGL^{-/-} mouse brains were dissected and homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.4), followed by centrifugation twice at 3,800 x g for 15 min at 4 °C. The resulting pellets were resuspended in 50 mM Tris-HCl (pH 7.4), 3 mM MgCl₂, and 1 mM EGTA. For *in vitro* binding assays, 72 µg of mouse brain protein was incubated at room temperature for 1 h with [³H]-CP55940 in 0.05 ml of assay buffer (50 mM Tris-HCl, 5 mM MgCl₂, 2.5 mM EDTA, and 2.5 mg/ml BSA) in the presence of 2 µM unlabeled CP55,940. Reactions were terminated by rapid filtration using a 96-well filtration apparatus (Brandel) onto GF/B filter mats followed by washing (6X) with cold assay buffer without MgCl₂. Filter circles were placed in 7 ml scintillation vials with 3 ml of Opti Flour (Perkin Elmer) and the vials were counted on a Perkin Elmer scintillation counter. Statistical significance was determined using Student's t test.

Behavioral studies. For the hot plate, mice (*n* =8-10 /group) were placed on a metal plate maintained at 52° C (Columbus Instruments, Columbus, OH). The latency to thermal nociceptive response (hind paw lift, flutter, licking or escape behavior), was measured. Cut-off was set at 30 s. For the tail flick, mice (*n* =4-5 /group) were placed on the Tail-Flick Unit (Ugo Basile, Varese, Italy) and an infrared beam was focused onto the tail, 5 cm from the tip. The latency for the animal to withdraw its tail was measured. Cut-off was set at 20 s and the intensity was set at 250 mb. WIN5512-2 was administered intraperitoneally (IP) as a solution in 2% Tween 80/0.5% Methyl cellulose. Latencies were determined at 1,

3 and 5 h post drug administration. Catalepsy was evaluated using the bar test in which the animal's front paws were placed over a thin horizontal rod 1 3/4" high ($n = 8-10$ /group). The amount of time (in sec) for which the animal remains motionless was recorded (60 second maximum). Locomotor activity was assessed by placing each mouse separately in a 9 x 9 inch open field cage that is surrounded by clear Plexiglas walls ($n = 8-10$ /group). The position of the animal in the cage was monitored by infrared sensors placed outside the cage. In the rotarod ataxia assay, animals were placed on rotarod (Ugo, Basile, Varese, Italy) and the speed of rotarod was set to accelerate from 4 to 40 rpm over a period of 300s with the maximum time spent on the rotarod set at 300s ($n = 8-10$ /group). The amount of time spent on rotarod was recorded and data was presented as percentage of baseline latency. Thermal (hot plate) and mechanical (von Frey) hypersensitivity assessments in inflammatory [complete Freund's adjuvant (CFA)] and neuropathic [spinal nerve ligation (SNL)] pain models ($n = 8-10$ /group) were conducted as described previously (Whiteside et al., 2005). Statistical significance was determined using a one-way ANOVA followed by least squared differences post hoc analysis (SAS Institute, Cary, NC). The criterion for significant differences was set at $p < 0.05$. Male mice were used for behavioral experiments. Asterisks denote statistical significance as compared to MAGL^{+/+} controls.

RESULTS

MAGL hydrolyzes 2-AG and maintains its levels *in vivo*. The most abundant endocannabinoid in the brain, 2-AG, is primarily catabolized by a serine hydrolase MAGL that is thought to contribute to the maintenance of 2-AG levels (Ahn et al., 2008; Kinsey et al., 2009). An expression profiling study of mouse MAGL mRNA analysis reveals a relatively even distribution of MAGL expression across central and peripheral nervous system tissues (supplemental data, Fig. S1).

We first analyzed 2-AG hydrolytic activity in brain extracts, using the purified recombinant murine MAGL protein as control (Fig. 1a). Incubation with mouse brain extracts led to a rapid and time-dependent hydrolysis of 2-AG, with the concomitant appearance of its hydrolytic product arachidonic acid (Dinh et al., 2002) (Fig. 1a). More than 80% of 2-AG (50 μ M) was hydrolyzed after 90 min in the presence of 20 μ g of MAGL^{+/+} brain extracts at room temperature (Fig. 1a).

In order to study the physiological role of MAGL *in vivo*, we generated mice lacking exons 1 and 2 of the MAGL genomic locus (Fig. 1b). MAGL^{-/-} mice are viable and fertile, and display the expected Mendelian frequency with no morphological defects. Lack of MAGL expression in brain of MAGL^{-/-} mice was confirmed by *in situ* hybridization and Western blot analyses (Fig. 1c and d). To determine whether deletion of MAGL resulted in compensatory changes in the expression of components of the endocannabinoid signaling pathway, we assessed expression levels of FAAH, CB1, and CB2 receptors. No significant difference in mRNA levels of these genes could be observed between MAGL^{+/+} and MAGL^{-/-} mice (supplemental data, Fig. S2).

A dramatic reduction in 2-AG hydrolysis was observed after incubation of 2-AG with MAGL^{-/-} brain extracts as compared to MAGL^{+/+} (Fig. 2a) over the same time course. Only a small fraction (~10-15%) of 2-AG was hydrolyzed in the presence of MAGL^{-/-} brain extracts after 120 minutes (Fig. 2a). This residual 2-AG hydrolysis activity observed in MAGL^{-/-} brain extracts may be attributed to the recently identified hydrolases ABHD6 and ABHD12 (Blankman et al., 2007). We used the previously described MAGL selective inhibitor JZL184 (Long et al., 2009) to assess the residual 2-AG hydrolytic activity in MAGL-deficient mice. JZL-184 significantly reduces 2-AG hydrolysis in MAGL^{+/+} brain extracts but does not affect the reduced levels of 2-AG hydrolysis in the MAGL^{-/-}, further confirming the lack of MAGL activity in these mice (Fig. 2b). These data support the role of MAGL as the major 2-AG hydrolyzing enzyme in the brain.

To further assess the role of MAGL in regulating endocannabinoid levels *in vivo*, we quantified the two major endocannabinoids, 2-AG and AEA, in brain, spinal cord, thymus and spleen tissues (Fig. 2c and d and supplemental data Fig. S3) from MAGL^{+/+} and MAGL^{-/-} mice using liquid chromatography-tandem mass spectrometry (LC/MS/MS) (Mei-Yi zhang, 2009). Levels of 2-AG are dramatically elevated in MAGL^{-/-} tissues as compared to MAGL^{+/+}, with 30 and 16-fold increases in the brain and spinal cord respectively (Fig. 2c). The levels of 2-AG were also increased in thymus and spleen (supplemental data, Fig. S3). Interestingly, levels of AEA do not differ significantly between MAGL^{+/+} and MAGL^{-/-} mouse tissues (Fig. 2d). The dramatic and specific elevation of 2-AG levels in MAGL^{-/-} mice confirms the pivotal role of MAGL as a regulator of 2-AG levels. The lack of change in AEA levels in MAGL^{-/-} tissues

compared to MAGL^{+/+} controls further demonstrates that MAGL does not contribute to the maintenance of AEA levels.

2-AG mediated behavioral responses are not dramatically enhanced in MAGL^{-/-} mice. Adult male and female MAGL^{-/-} mice exhibit an average decrease in total body weight of 16.5% as compared to MAGL^{+/+} controls at three months of age (Fig. 3a). Monitoring body weight over the first six weeks after birth reveals a persistent and statistically significant difference between MAGL^{-/-} mice and MAGL^{+/+} littermates (Fig. 3b). Surprisingly, the mutant mice do not display the typical behavioral features of cannabinoid receptor-mediated signaling despite a 30-fold increase in brain 2-AG levels compared to MAGL^{+/+} (Fig. 3c-g). No significant differences were observed in core body temperature (Fig. 3c), locomotion (Fig. 3d) and thermal nociception (Fig. 3f and g) between MAGL^{+/+} and mutant mice. MAGL-deficient mice displayed no significant ataxia (Fig. 3e) or catalepsy.

In the CFA model of peripheral inflammation, thermal and mechanical hypersensitivities developed at 24 hrs post CFA injection in MAGL^{+/+} and MAGL^{-/-} mice and were monitored for two weeks (Fig. 4a). MAGL^{-/-} mice and MAGL^{+/+} controls exhibit a comparable magnitude of thermal and mechanical hypersensitivity with a small but significant increase in thermal latency in the mutants only at 48 hrs post CFA (Fig. 4a). In the spinal nerve ligation (SNL) model of neuropathic pain, no significant difference in the development of thermal and mechanical hypersensitivity could be observed between MAGL^{-/-} and

MAGL^{+/+} controls (Fig. 4b). Our data indicate that elevated levels of 2-AG seen in MAGL^{-/-} mice do not attenuate the development of inflammatory and neuropathic pain, in contrast with acute pharmacological inhibition of MAGL which was shown to reduce pain hypersensitivity (Kinsey et al., 2009). Therefore, these findings suggest that cannabinoid signaling might be altered in the MAGL^{-/-} mice, and this alteration can be evaluated by assessing exogenous and endogenous cannabinoid signaling in these mice.

CB1 signaling is altered in MAGL^{-/-} mice. To assess whether the CB1 signaling pathway is still functional in the mutant mice, we looked at the effects of the prototypic CB1 agonist WIN55212 (Felder et al., 1995) in MAGL^{-/-} and MAGL^{+/+} mice. Administration of WIN55212 (10 mg/kg) led to an increase in thermal nociception threshold in MAGL^{+/+} mice as assessed in the hot plate and tail flick assays. In the MAGL^{-/-} mice, WIN55212 had no effect on the thermal threshold in the tail flick assay and a small but statistically significant effect was observed in the hot plate test (Fig. 5a and b). Moreover, MAGL^{+/-} and MAGL^{-/-} mice exhibited significantly less catalepsy (20 to 40 seconds less than the MAGL^{+/+}) at 1, 3 and 5 hrs following WIN55212 administration (Fig. 5c). These results indicate that CB1 receptor-mediated behavioral responses are altered in MAGL^{-/-}. This alteration in CB1 activity may be responsible for the unexpected lack of enhanced effects of 2-AG signaling in the mutant mice compared to MAGL^{+/+} controls despite the deficiency in 2-AG degradation in the mutants and subsequent elevation of 2-AG levels (Fig. 2).

The altered behavioral response to the CB1 agonist WIN55212 in MAGL^{-/-} mice led us to assess levels of CB1 receptor density and activity in samples from MAGL^{+/+} and MAGL^{-/-} brain. Ligand binding assay performed on brain membrane extracts from MAGL^{+/+} and MAGL^{-/-} mice (n=5 per group) reveals a statistically significant 30% decrease in CB1 receptor density, but not ligand binding affinity, in MAGL^{-/-} brain membrane extracts compared to MAGL^{+/+}, using the cannabinoid agonist ³H-CP55940 (Bmax ±SD: MAGL^{+/+} 2925.8 ± 529.9; MAGL^{-/-} 2066.8 ± 393.6 fmol/mg) or antagonist Rimonabant ³H-SR141716 (Bmax±SD: MAGL^{+/+} 3668.0 ± 172.1; MAGL^{-/-} 2647.3 ± 181.2 fmol/mg) (Fig. S4). In contrast, CB2 receptor density levels are not significantly changed in the mutants as assessed by ³H-CP55940 binding on spleen tissue extracts from the MAGL^{-/-} and MAGL^{+/+} mice (Bmax: MAGL^{+/+} 601.8 fmol/mg; MAGL^{-/-} 549.2 fmol/mg).

In order to assess whether the decrease in membrane CB1 receptor density in the mutants results in a decrease in ligand-induced CB1 signaling, we measured GTPγS binding activity on brain membrane extracts from MAGL^{-/-} and MAGL^{+/+} mice, in the presence or absence of the agonist CP55940 (supplemental data, Fig. S4). A statistically significant reduction in CP55940-mediated activation of CB1 signaling, as shown by a two-fold increase in EC50 (MAGL^{+/+} 4.4 ± 0.7 nM; MAGL^{-/-} 8.7 ± 0.9 nM) and approximately 25% decrease in Emax (MAGL^{+/+} 81.8 ± 1.5; MAGL^{-/-} 61.2 ± 0.9), was observed. Our data show that chronically enhanced 2-AG levels in the mutant mice lead to a reduction of CB1 receptor density and signaling in the brain.

Levels of 2-AG are tightly regulated by enzymatic biosynthesis and rapid degradation. Therefore, pharmacological studies to assess the physiological and behavioral implications of 2-AG administration *in vivo* are compromised by MAGL activity and 2-AG rapid degradation. In MAGL^{-/-} mice, lack of MAGL activity leads to prolonged 2-AG half-life, but altered CB1 signaling attenuates 2-AG effects. Likely as a result of the combination of increased 2-AG levels and decreased CB1 signaling, the physiological and behavioral effects of 2-AG administration in the mutant mice are overall comparable to those observed in the MAGL^{+/+} controls, despite the lack of endogenous 2-AG degradation in the mutants (supplemental data, Fig. S5).

DISCUSSION

Endocannabinoids, including 2-AG, are produced on demand by neurons, rather than being stored in synaptic vesicles like classical neurotransmitters (Di Marzo, 2008). The hydrophobic nature of these lipids enables them to freely diffuse across cell membranes, and highlights the role of the degradative enzymes, including MAGL, as the major component of endocannabinoid signal termination. Biochemical and pharmacological studies suggest that 2-AG is abundant in the brain, and that it is primarily hydrolyzed by MAGL (Dinh et al., 2002). The study of the full physiological role of 2-AG *in vivo* has been limited by the activity of endogenous 2-AG degrading enzymes which quickly hydrolyze 2-AG and prevent it from signaling through the cannabinoid receptors. The recent characterization of JZL-184 as a selective MAGL

inhibitor (Kinsey et al., 2009; Long et al., 2009) provided the first tool to begin assessing the role of MAGL *in vivo* and elucidating of the physiological implications of 2-AG.

We provide here genetic evidence, using MAGL deficient mice, that MAGL is the major 2-AG catabolic enzyme and a critical modulator of 2-AG levels in the CNS. MAGL-deficient mice with their high basal 2-AG levels, in the absence of significant endogenous degradation, allow the characterization of the role of this endocannabinoid *in vivo*. Our data demonstrate that 2-AG levels and signaling *in vivo* are primarily regulated by MAGL activity. MAGL^{-/-} mice exhibit dramatically elevated endogenous brain and spinal cord levels of 2-AG (16-30-fold) with no significant alteration in AEA levels (Fig. 2c and d).

Our data also provide *in vivo* evidence that chronic elevation in levels of endogenous 2-AG leads to downregulation of CB1 signaling as assessed by the reduced response to CB1 agonist in MAGL^{-/-} mice (Fig. 5). Interestingly, a decrease in brain CB1 but not spleen CB2 receptor density was observed, suggesting that only the brain CB1 receptor expression is altered in response to high 2-AG levels. The differential down-regulation between CB1 and CB2 receptors may be based on a distinction between CNS and peripheral tissues. The very low levels of CB2 expression in the CNS, and the low levels of CB1 in peripheral tissues make quantification of receptor activity and binding virtually impossible, therefore, we cannot exclude that levels of both receptors are altered in the CNS, nor that neither receptor levels are altered in the periphery.

MAGL^{-/-} mice do not exhibit the classical tetrad of CB1-mediated behavioral and physiological effects despite the dramatic increase in 2-AG levels. No significant effect on locomotor activity, rotarod ataxia, and hypothermia was detected (Fig. 3c-e). In addition, no difference in thermal nociception was observed between MAGL^{+/+} and MAGL^{-/-} mice (Fig. 3f and g). Similarly, FAAH^{-/-} mice do not exhibit the classical tetrad of CB1-mediated behaviors, despite large increases in anandamide levels. However, acute pharmacological blockade of MAGL produces significant hypothermic and hypomotility (Long et al., 2009), while no such effects are elicited by FAAH inhibitors. MAGL^{-/-} mice do not exhibit a significant reduction in inflammatory or neuropathic pain hypersensitivity, despite the dramatic increase in brain 2-AG levels, suggesting that endocannabinoid signaling is altered in these mice. CB1 antagonism was also shown to modulate food intake and body weight (Di Marzo, 2009), therefore, the partial desensitization of CB1 receptor observed in MAGL^{-/-} mice may be responsible for the apparent reduction in body weight in the mutant mice.

The combined effects of the lack of 2-AG hydrolytic activity and the decrease in CB1 signaling in MAGL^{-/-} mice precluded a full interpretation of the behavioral effects of 2-AG administration in MAGL^{-/-} mice. Unlike the response of FAAH-deficient mice to AEA administration (Cravatt et al., 2001), the decrease in CB1 signaling in MAGL^{-/-} mice prevented the manifestation of dramatically enhanced 2-AG behavioral effects in these mice. The overall behavioral response to 2-AG administration in the mutant mice is the combined outcome of increased 2-AG half life in the absence of MAGL activity, and partial desensitization of CB1 receptors.

These results, together with the reduced response to the CB1 agonist WIN55212, suggest that 2-AG signaling is attenuated in the mutant mice through a downregulation of CB1 receptor density and signaling in the brain. The small hypoalgesic response to WIN55212, observed in the mutants using the hotplate test at 1 h could be the result of motor impairment, as there is an apparent small degree of catalepsy observed at the same time point. A recent study reported that upon administration of the selective MAGL inhibitor JZL 184, brain levels of 2-AG rose by eight-fold and JZL 184-treated mice exhibited a wide array of CB1-dependent behavioral effects, including analgesia, hypomotility and hypothermia (Kinsey et al., 2009). However, chronic administration of JZL184 may lead to alteration in CB1 signaling similar to the alteration observed in MAGL-deficient mice. Taken together, our findings suggest that the CB1 receptor acts more as an “off switch” than a negative feedback loop in response to chronic elevation of 2-AG levels leading to profound alterations in CB1-mediated functions in MAGL^{-/-} mice. Modulation of brain CB1 and not spleen CB2 levels was observed in the mutant mice, suggesting that the CB1 signaling cascade is more sensitive to 2-AG levels or that brain CB1 and CB2 signalings are more sensitive to 2-AG levels than peripheral cannabinoid receptors.

In summary, we provide data showing that MAGL is a critical modulator of 2-AG levels and functions, and that the endocannabinoid system adapts to chronic elevation of 2-AG levels by downregulating CB1 receptor density and signaling. Moreover, our gain of function *in vivo* model of 2-AG signaling sheds light on the physiological and pathophysiological consequences of chronic inhibition of MAGL, a pharmacological target with therapeutic potential for neurologic and metabolic diseases.

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Footnotes:

These authors (P.K.C., Y.G.) contributed equally to this work.

Figure Legends

Fig. 1. Characterization of the enzymatic activity of MAGL and targeted disruption of the MAGL gene in MAGL^{-/-} mice. (a) Conversion of 2-AG into 1-AG or AA in the presence of recombinant MAGL protein or 20 µg of mouse membrane-enriched brain extract was compared in a 120-min time course. Data presented as mean percentage of 2-AG, 1-AG, and AA to the sum of all three ± s.e.m. (b) Schematic diagram of the targeting vector and the mouse MAGL genomic locus surrounding the deleted MAGL exons. (c) *In situ* hybridization of brain hippocampus from MAGL^{+/+} (+/+) and MAGL^{-/-} (-/-) mice. (d) Western blot analysis of recombinant MAGL protein (Ctrl), 3 sets of brain lysates from MAGL^{+/+} and MAGL^{-/-} mice, respectively. Both mRNA and protein expression of MAGL are selectively absent in the MAGL^{-/-} (-/-) mice.

Fig. 2. 2-AG hydrolytic activity is altered and 2-AG levels are increased in MAGL^{-/-} mice. (a) Brain extracts from MAGL^{+/+} and MAGL^{-/-} mice were incubated with 2-AG for various periods of time as indicated, at room temperature. 2-AG hydrolytic activity was measured as the percentage of 2-AG and its isomer 1-AG. (b) Brain 2-AG hydrolytic activities from MAGL^{+/+} and MAGL^{-/-} brain extracts in the absence or presence of the MAGL selective inhibitor JZL184 (0.5 µM) confirm the lack of MAGL activity in MAGL^{-/-} mice. (c) Levels of 2-AG in the brain and spinal cord of MAGL^{+/+} mice as compared to heterozygous (+/-) and

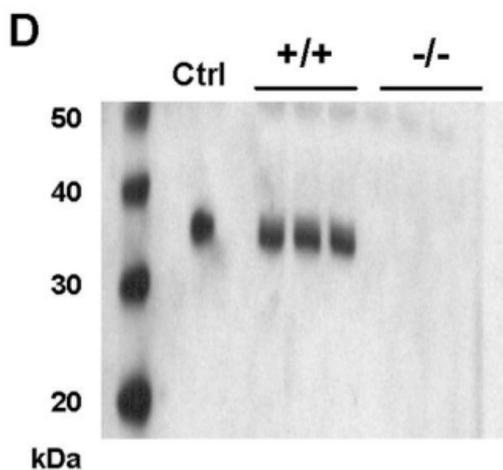
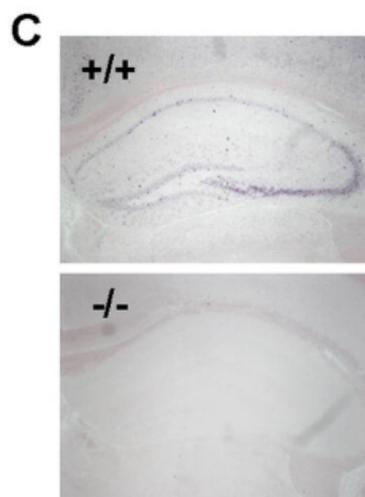
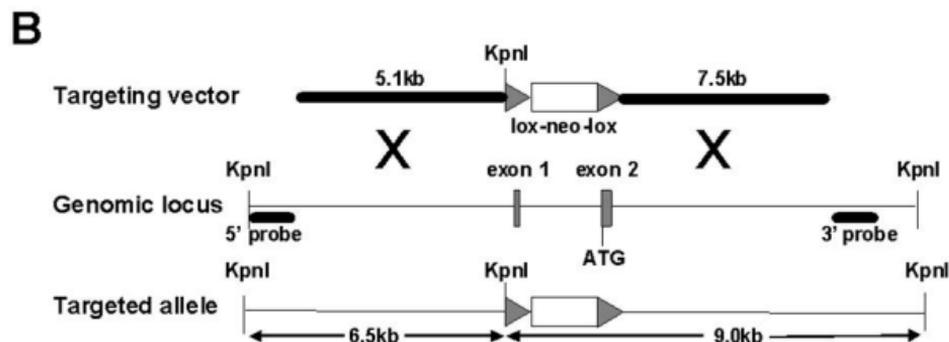
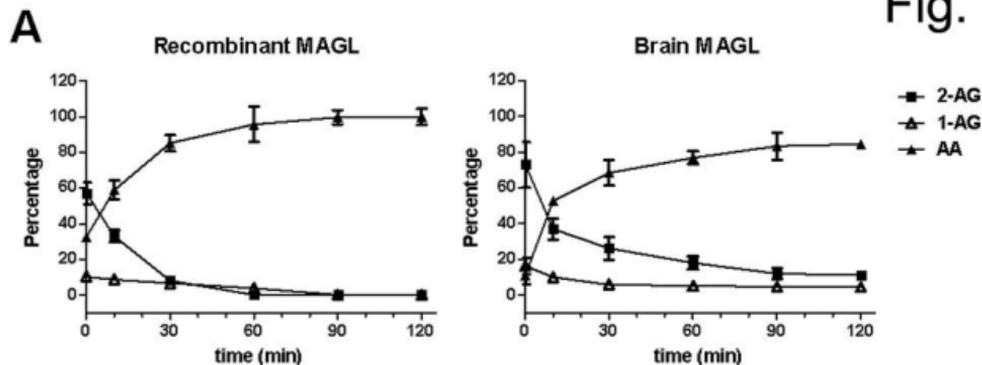
homozygous (-/-) mice. ** $p < 0.01$ versus WT brain, # $p < 0.05$ versus WT spinal cord, t-test. (d) Levels of AEA in the brain of MAGL^{+/+} and MAGL^{-/-} mice. 2-AG and AEA were quantified by LC/MS/MS using d8-1-AG and d8-AEA as respective standards. The results are presented as mean \pm s.e.m., 5 mice per genotype.

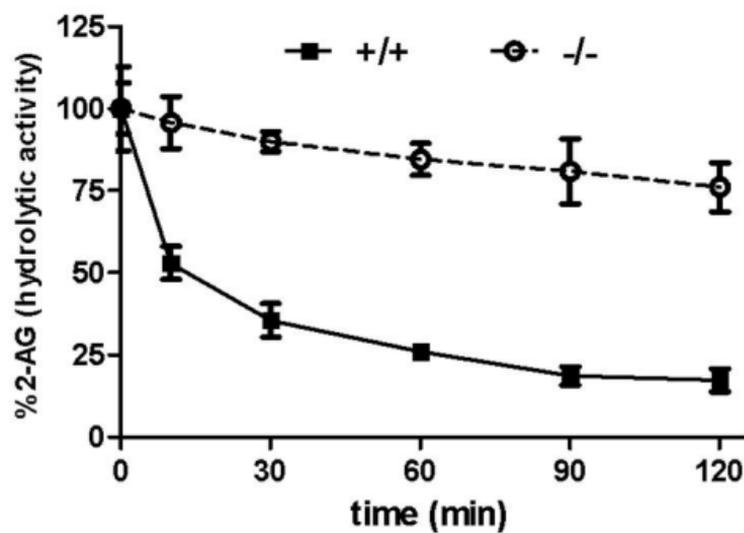
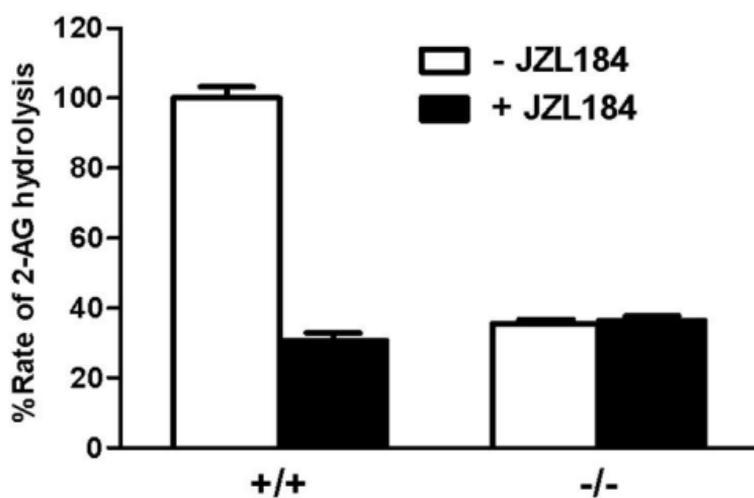
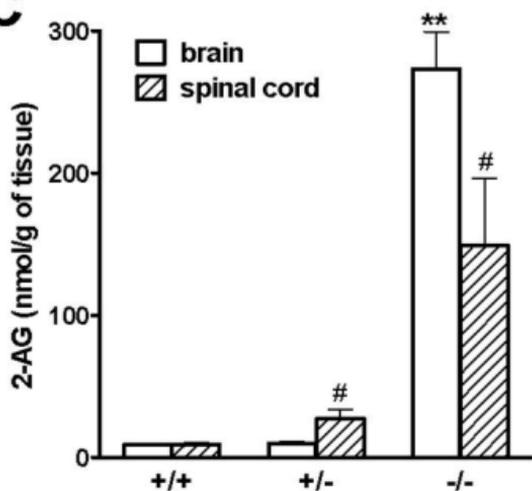
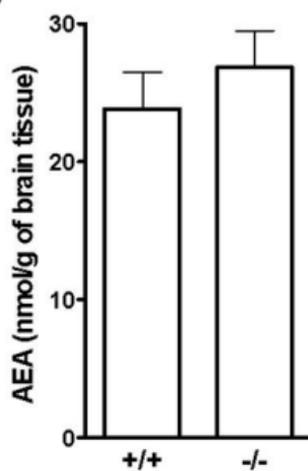
Fig. 3. MAGL^{-/-} mice weigh less and display normal physiological and behavioral responses. Body weight of adult (3 months old) (a) and newly born (b), adult body temperature (c), locomotion (d), motor coordination (e), and thermal nociceptive thresholds in the tail flick (f) and hot plate (g) tests have been examined for MAGL^{-/-} and MAGL^{+/-} compared with age- and sex- matched MAGL^{+/+} mice. The results are presented as mean \pm s.e.m., 10-17 mice per genotype. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus sex-matched MAGL^{+/+} mice, t-test.

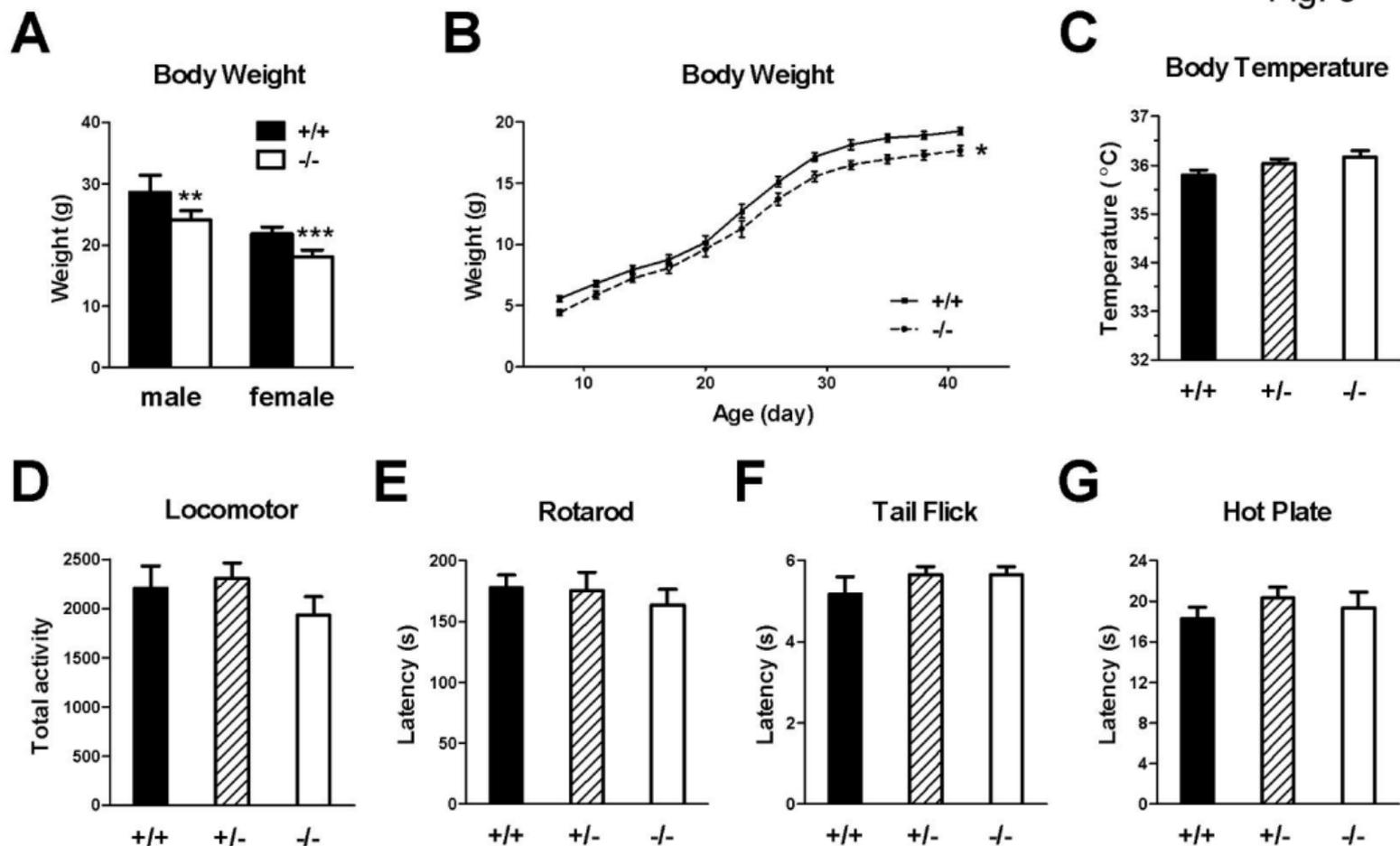
Fig. 4. Thermal and mechanical hypersensitivity after inflammatory and neuropathic injuries in MAGL^{+/+} and MAGL^{-/-} mice. (a) After hind paw inflammation (CFA), thermal (left panel) and mechanical (right panel) thresholds are significantly reduced in MAGL^{-/-} mice and wild type controls over the time period indicated. (* $P < 0.05$, +/+ vs -/-, ANOVA followed by least squared differences post hoc analysis). (b) In the spinal nerve ligation model (SNL) of neuropathic pain, thermal (left) and mechanical (right) thresholds are reduced

over a three week time period as indicated, with no significant difference between MAGL^{-/-} and wild type control mice (10 mice per genotype). The results are presented as mean ± s.e.m.

Fig. 5. Cannabimimetic behavioral responses induced by CB1 agonist are diminished in MAGL^{-/-} mice. CB1 agonist WIN55122 (10 mg/kg) (WIN) was administered intraperitoneally (I.P.). Mice behavioral responses were recorded before WIN administration as baseline (BL). (a) One hour post administration, MAGL^{+/+}, MAGL^{+/-} or MAGL^{-/-} mice were placed on the Tail-Flick Unit and the latency for the animal to withdraw its tail following application of infrared beam was recorded. (b) Mice were placed on a hot (50°C) metal plate one hour post WIN treatment and the thermal nociceptive responses (hind paw lift, licking or escape behavior) were measured. (c) Catalepsy was evaluated 1, 3, and 5 hour post drug administration using the bar test. The amount of time for which the mice remains motionless was recorded. There was no catalepsy observed in mice from either genotype before drug application. The results are presented as mean ± s.e.m., 7-10 mice (20-30 g) per group. * p<0.05, ** p<0.01, versus MAGL^{+/+} mice, ANOVA.

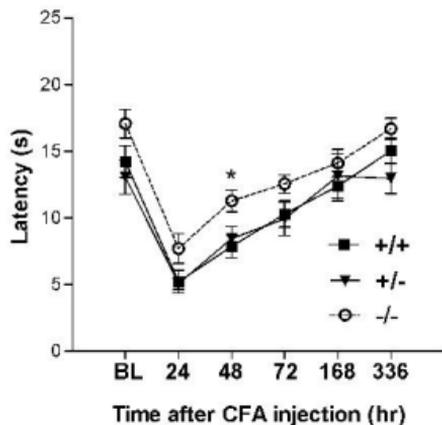


A**B****C****D**

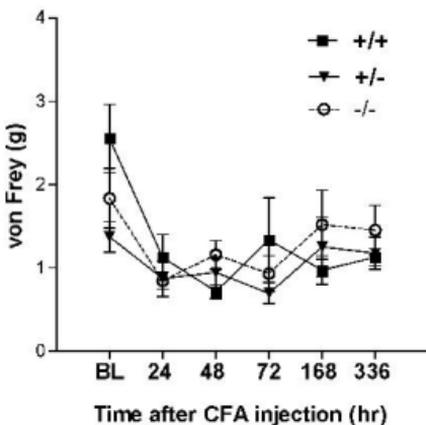


A

Thermal Hyperalgesia

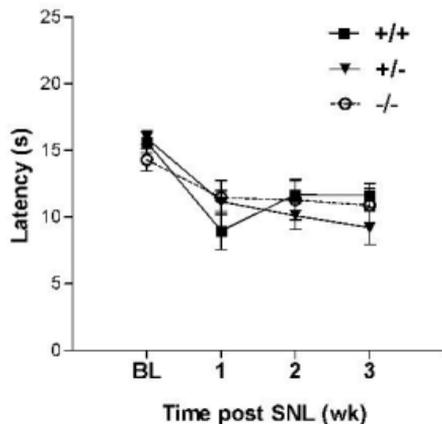


Mechanical Allodynia



B

Thermal Hyperalgesia



Mechanical Allodynia

