JPET #243808

Journal of Pharmacology and Experimental Therapeutics

Title: Investigation of Diacylglycerol Lipase Alpha Inhibition in the Mouse Lipopolysaccharide Inflammatory Pain Model.

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JPET Fast Forward. Published on October 2, 2017 as DOI: 10.1124/jpet.117.243808
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Running Title: Diacylglycerol lipase in inflammatory pain

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Number of text pages: 41

Number of figures: 6, Supplemental Figure: 1

Number of references: 74

Number of words in Abstract: 231

Number of words in Introduction: 750

Number of words in Discussion: 1,243

Abbreviations: 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ABHD6, alpha beta hydrolase domain-containing protein 6; AEA, anandamide; CNS, central nervous system; COX2, cycloxygenase-2; DAGL, diacylglycerol lipase; DSE, depolarization-induced suppression of excitation; DSI, depolarization-induced suppression of inhibition; FAAH, fatty acid amide hydrolase; LPS, lipopolysaccharide; MAGL, monoacylglycerol lipase; PGs, prostaglandins

Section: Behavioral Pharmacology

Abstract

Diacylglycerol lipase (DAGL) α and β, the major biosynthetic enzymes of the endogenous cannabinoid (endocannabinoid) 2-arachidonylglycerol (2-AG), are highly expressed in the nervous system and immune system, respectively. Genetic deletion or pharmacological inhibition of DAGL-β protects against lipopolysaccharide (LPS)-induced inflammatory responses in mouse peritoneal macrophages, and reverses LPS-induced allodynia in mice. In order to gain insight into the contribution of DAGL-α in LPS-induced allodynia, we tested global knockout mice as well as DO34, a dual DAGL-α/-β inhibitor. Intraperitoneal administration of DO34 (30 mg/kg) significantly decreased whole brain levels of 2-AG (~83%), anandamide (~42%), and arachidonic acid (~58%). DO34 dose-dependently reversed mechanical and cold allodynia, and these antinociceptive effects did not undergo tolerance after six days of repeated administration. In contrast, DO34 lacked acute thermal antinociceptive, motor, and hypothermal pharmacological effects in naïve mice. As previously reported, DAGL-β (-/-) mice displayed a protective phenotype from LPS-induced allodynia. However, DAGL-α (-/-) mice showed full allodynic responses, similar to their wildtype littermates. Interestingly, DO34 (30 mg/kg) fully reversed LPS-induced allodynia in DAGL-α (+/+) and (-/-) mice, but did not affect the antinociceptive phenotype of DAGL-β (-/-) mice in this model, indicating a DAGL-α independent site of action. These findings suggest that DAGL-α and -β play distinct roles in LPS-induced nociception. Whereas DAGL-α appears to be dispensable for the development and expression of LPS-induced nociception, DAGL-β inhibition represents a promising strategy to treat inflammatory pain.

Introduction

Diacylglycerol lipase (DAGL)-α and -β (Bisogno *et al.*, 2003; Gao *et al.*, 2010; Tanimura *et al.*, 2010) transform diacylglycerols to 2-arachidonoylglycerol (2-AG), the most highly expressed endocannabinoid in the central nervous system (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). 2-AG plays critical roles in maintaining proper neuronal function (Goncalves *et al.*, 2008; Tanimura *et al.*, 2010), mediating neuronal axonal growth (Williams *et al.*, 2003) and retrograde suppression of synaptic transmission (Kreitzer and Regehr, 2001; Ohno-Shosaku *et al.*, 2001; Wilson and Nicoll, 2001; Pan *et al.*, 2009). These enzymes are differentially expressed within cells in the nervous system and peripheral tissue (Hsu *et al.*, 2012). DAGL-α is expressed on postsynaptic neurons within various brain regions (Katona *et al.*, 2006; Yoshida *et al.*, 2006; Lafourcade *et al.*, 2007; Uchigashima *et al.*, 2007), and its genetic deletion results in marked decreases of 2-AG, anandamide (AEA), and arachidonic acid (AA) in brain (Gao *et al.*, 2010; Tanimura *et al.*, 2010; Shonesy *et al.*, 2014) and spinal cord (Gao *et al.*, 2010). Accordingly, DAGL-α (-/-) mice display impaired depolarization-induced suppression of inhibition (DSI) and excitation (DSE) in the brain (Gao *et al.*, 2010; Tanimura *et al.*, 2010; Yoshino *et al.*, 2011). These mice also show an increased mortality rate (Sugaya *et al.*, 2016), display increased spontaneous seizures in the kainate model of status epilepticus (Sugaya *et al.*, 2016), and exhibit an anxiogenic phenotype (Shonesy *et al.*, 2014). In contrast, DAGL-β is most highly expressed on macrophages and although its relative brain expression is sparse, it is highly expressed on microglia (Hsu *et al.*, 2012). This distribution pattern suggests that DAGL-β activity contributes to inflammatory responses. Importantly, DAGL-β deletion does not affect endocannabinoid-mediated forms of retrograde synaptic suppression (Gao *et al.*, 2010). However, DAGL-β blockade reduces lipopolysaccharide (LPS)-induced inflammatory responses in peritoneal

macrophages from C57BL/6 mice by decreasing levels of 2-AG, arachidonic acid, prostanoids, and proinflammatory cytokines (Hsu *et al.,* 2012). Similarly, DAGL-α inhibition leads to protection from the neuroinflammatory effects of 20 mg/kg systemic LPS (Ogasawara *et al.,* 2016).

A wide scope of evidence supports inflammatory as well as neuronal signaling contributions to many forms of pathological pain. Immune cell signaling plays a critical role in the development and maintence of neuropathic pain (Watkins *et al.,* 2001; De Leo *et al.,* 2006; Beggs and Salter, 2013). Likewise, increased neuronal signaling underlies pathological inflammatory pain, and can contribute to a positive pain feedback loop (De Leo *et al.,* 2006; Chen *et al,* 2015). For example, in LPS-stimulated neurons, neuronal signaling leads to further inflammatory signaling and immune cell activation (Chen *et al,* 2015). Determing the antecedents of pathological pain and the subsequent identification of potential therapeutic targets remain important areas of research. Accordingly, DAGL-α and DAGL-β represent provocative targets to treat pathological pain conditions.

Complementary approaches of pharmacological agents and genetically modified mice demonstrate that DAGL-β blockade reduces nociceptive behavior in the LPS model of inflammatory pain (Wilkerson *et al.,* 2016). The DAGL-β inhibitor KT109 reverses nociceptive behavior in models of neuropathic pain (Wilkerson *et al.,* 2016). These findings strongly implicate inhibition of this enzyme as a viable approach to treat inflammatory and neuropathic pain. However, it remains to be determined whether DAGL-α inhibition or deletion produces antinociceptive effects in pathological pain models. The present study attempted to investigate the role of this enzyme in LPS-induced allodynia, using the DAGL inhibitor DO34, which disrupts DSE and DSI in the cerebellum and hippocampus and reduces LPS-induced

anapyrexia *in vitro* responses (Ogasawara *et al.,* 2016), provides a useful tool for *in vitro* and *in vivo* studies. Thus, the present study examined DAGL-α (-/-) and DAGL-β (-/-) mice in the LPS model of inflammatory pain

In initial experiments, we quantified brain levels of endogenous cannabinoids and arachidonic acid in mice administered vehicle or DO34 (30 mg/kg), as well as tested DO34 in assays of locomotor behavior, catalepsy, body temperature, and acute thermal antinociceptive responses. We then evaluated the dose-response relationship and time course of acute DO34 administration in attenutating LPS-induced mechanical and cold allodynia. In addition, and we examined whether the anti-allodynic effects would undergo tolerance following repeated DO34 administration. Finally, we tested DO34 in DAGL-α (-/-) and –β (-/-) mice, and respective wild type littermates in the LPS model of inflammatory pain. Because DO34 also inhibits the serine hydrolase ABHD6 (Ogasawara *et al.,* 2016), a 2-AG hydrolytic enzyme expressed on postsynaptic neurons (Blankman *et al.,* 2007; Marrs *et al.,* 2010), we employed the selective ABHD6 inhibitor DO53, which lacks DAGL activity (Ogasawara *et al.,* 2016), for comparison.

Methods

Animals

Adult male C57BL/6J and ICR mice (23-40 gram, Jackson Laboratory, Bar Harbor, ME) served as subjects in these experiments. DAGL-α (-/-) and DAGL-β (-/-) mice were generated in the Cravatt laboratory on a mixed C57BL/6J and 129/SvEv background, as previously described (Hsu *et al.*, 2012), and breeding pairs were transferred to Virginia Commonwealth University. A total of 84 DAGL-α (-/-) and 36 DAGL-β (-/-) mice were used in these studies. Mice were housed four per cage in a temperature (20–22 °C), humidity (55 ± 10 %), and light-controlled (12 hour light/dark; lights on at 0600) AAALAC-approved facility, with standard rodent chow and water available *ad libitum*.

All tests were conducted during the light phase. The sample size selected for each treatment group in each experiment was based on previous studies from our laboratory and complied with power analyses.

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). After testing was completed, mice were euthanized via CO_2 asphyxia, followed by rapid cervical dislocation.

Drugs

The DAGL inhibitor DO34 and the selective ABHD6 inhibitor DO53 were synthesized by the Cravatt laboratory according to previous methods (Ogasawara *et al.*, 2016). All drugs were dissolved in a vehicle solution consisting of a mixture of ethanol, alkamuls-620 (Sanofi-

Aventis, Bridgewater, NJ), and saline (0.9 % NaCl) in a 1:1:18 ratio. All drugs were administered in an injection volume of 10 µl/g body mass. Each drug was given via the intraperitoneal (i.p.) route of administration. The dose range of DO34 was selected based on results reported by Ogasawara and colleagues, indicating that acute administration of 30 mg/kg DO34 in mice treated with LPS was sufficient to produce inhibition of DAGL-α, as well as measured decreases in peritoneal macrophages of arachidonic acid and proinflammatory cytokines (Ogasawara *et al.*, 2016).

Extraction and quantification of endocannabinoids by liquid chromatography-tandem mass spectrometry

2-AG, arachidonic acid (AA), and AEA levels were quantified from the whole brain of ICR mice, after acute i.p. administration of DO34 (30 mg/kg) or 1:1:18 vehicle. Brains were collected and processed for quantification of 2-AG, AA, and AEA. Because equivalent doses of DO34 significantly attenuated allodynia associated with LPS at 2 h after injection, mice were euthanized via rapid decapitation at this time point. Brains were rapidly harvested, snap-frozen in dry ice, and stored at -80°C until the time of processing. Tissues were further processed according to methods described previously (Ramesh *et al.*, 2011; Ignatowska-Jankowska *et al.*, 2014). See supplementary methods for details.

Evaluation of acute pharmacological effects of DO34

Mice (counterbalanced Latin square within subject design) were housed individually overnight. The behavioral testing was conducted in the following order: bar test (catalepsy), tail withdrawal test, rectal temperature, locomotor activity. Testing was performed according to

previously described procedures (Long *et al.*, 2009; Schlosburg *et al.*, 2010). Catalepsy was assessed on a bar 0.7 cm in diameter placed 4.5 cm off of the ground. The mouse was placed with its front paws on the bar and a timer (Timer #1) was started. A second timer (Timer #2) was turned on only when the mouse was immobile on the bar, with the exception of respiratory movements. If the mouse moved off the bar, it was placed back on in the original position. The assay was stopped when either Timer #1 reached 60 s, or after the fourth time the mouse moved off the bar, and the cataleptic time was scored as the amount of time on Timer #2. Nociception was then assessed in the tail immersion assay. The mouse was placed head first into a small bag fabricated from absorbent under pads (VWR Scientific Products; 4 cm diameter, 11 cm length) with the tail out of the bag. Each mouse was hand-held and 1 cm of the tail was submerged into a 52 °C water bath. The latency for the mouse to withdraw its tail within a 10 s cut off time was scored. Rectal temperature was assessed by inserting a thermocouple probe 2 cm into the rectum, and temperature was determined by thermometer (BAT-10 Multipurpose Thermometer, Clifton, NJ, USA). Locomotor activity was assessed 120 min after treatment, for a 60 min period in a Plexiglas cage (42.7 x 21.0 x 20.4 cm) and Anymaze (Stoelting, Wood Dale, Illinois) software was used to determine the percentage of time spent immobile, mean speed and distance traveled.

Lipopolysaccharide (LPS) inflammatory pain model

Mice were given an injection of 2.5 μg LPS from Escherichia coli 026:B6 Sigma (St. Louis, MO, USA) in 20 μl of physiological sterile saline (Hospira Inc, Lake Forest, IL) into the plantar surface of the right hind paw. As previously reported, this is the minimally effective dose of LPS that elicits mechanical allodynia, but not measurable increases in paw thickness (Booker *et al.*, 2012). Following LPS administration, mice were returned to their home cages. At 22 h,

mice were given the appropriate injection of drug or vehicle and tested at 24 h for allodynia. In the time course study, allodynia was assessed at 40 min, and 1, 3, 5, 8, and 24 h after the i.p. injection.

To determine whether repeated administration of DO34 would produce sustained antinociceptive effects, mice were given i.p. injections of vehicle or DO34 (30 mg/kg) once a day for five days. On day 5, each mouse received its appropriate i.p. injection of vehicle or DO34, and 2 h later all mice were given an intraplantar injection of LPS. On day 6 (22 h after LPS administration), each mouse received its final i.p. injection. The vehicle-treated mice were divided into two groups. The first group received another injection of vehicle (vehicle control group) and the second group was given 30 mg/kg DO34 (acute DO34 group). The mice that had been given repeated injections of drug received their final injection of DO34 (repeated DO34 group). All mice were tested for mechanical and cold allodynia 2 h after the final i.p. injection.

Behavioral assessment of nociception

Baseline responses to light mechanical touch were assessed using the von Frey test following habituation to the testing environment, as described elsewhere (Murphy *et al.*, 1999). In brief, mice were placed atop a wire mesh screen, with spaces 0.5 mm apart and habituated for approximately 30 min/day for four days. Mice were unrestrained, and were singly placed under an inverted wire mesh basket to allow for unrestricted air flow. The von Frey test utilizes a series of calibrated monofilaments, (2.83 – 4.31 log stimulus intensity; North Coast Medical, Morgan Hills, CA) applied randomly to the left and right plantar surface of the hind paw for 3 s. Lifting, licking, or shaking the paw was considered a response. After completion of allodynia testing for LPS experiments, cold allodynia testing was performed with the application of acetone (Decosterd and

Woolf, 2000). In this assay 10 µl of acetone (99% high-performance liquid chromatography grade; Thermo Fisher Scientific, Waltham, MA) was projected via a 100-µl pipette (Rainin Instruments, Woburn, MA) onto the plantar surface of each hind paw. Acetone was propelled from below via air burst by expressing the pipette, thereby avoiding mechanical stimulation of the paw with the pipette. Total time lifting/clutching each hind paw was recorded with an arbitrary maximum cutoff time of 60 s. For all behavioral testing, threshold assessment was performed in a blinded fashion.

Data analysis

Data were analyzed using student's T test (evalutation of endocannabinoid and AA levels), or one-way or two-way analysis of variance (ANOVA). Tukey's test was used for *post hoc* analysis following a significant one-way ANOVA. Multiple comparisons following two-way ANOVA were conducted with Bonferroni *post hoc* comparison. A *P*-value of <0.05 was considered statistically significant. The computer program GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA) was used in all statistical analyses. All data are expressed as mean +/- SEM.

Results

DO34 (30 mg/kg) significantly decreased whole brain levels of 2-AG ($p < 0.0001$, Fig. 1A), AEA ($p < 0.05$, Figure 1B), and arachidonic acid ($p < 0.0001$, Fig. 1*C*).

To examine whether DO34 produces overt pharmacological effects, we assessed whether it affects spontaneous locomotor behavior, elicits cataleptic effects in the bar test, produces antinociception in the warm water tail withdrawal assay, or alters body temperature. Naïve mice given vehicle, or 1, 3, 10, 30, 50, or 100 mg/kg DO34. DO34 did not display differences of treatment in catalepsy (Figure 2A), hypothermia ($P = 0.60$, Figure 2B), thermal antinociception ($P = 0.13$, Figure 2C), or locomotor alterations (defined as time spent immobile; $P = 0.57$, Figure 2D).

Having confirmed that the DAGL inhibitor DO34 significantly reduces endocannabinoids and arachidonic acid in whole brain, but does not affect overt motor or sensory behavior, the next set of experiments investigated this compound in the LPS model of inflammatory pain. The dose-response evaluation of the anti-allodynic effects of DO34 (1, 3, 10, 30 mg/kg) at 2 h post-injection in the von Frey and acetone-induced fliching assays are respectively shown in Figure 3A and 3B. DO34 dose-dependently reversed LPS-induced mechanical allodynia [$F(3,20) = 14.12$; $P < 0.0001$], and cold allodynia, [$F(3,20) = 15.99$; $P < 0.0001$]. The respective ED_{50} values (95% C.I.) of DO34 in reversing LPS-induced mechanical allodynia and cold allodynia were 3.8 (2.8-5.3) mg/kg and 6.0 (4.0-9.0) mg/kg. The potency ratio (95% C.I.) of DO34 for mechanical vs. cold allodynia was 1.6 (1.0-2.6), indicating equipotence in the two nociceptive assays. As depicted in Figure 3C, DO34 significantly reversed allodynia within 30 min of i.p.

administration, and the antinociceptive effect of 30 mg/kg DO34 persisted for at least 8 h [interaction between treatment and time $F(20,125) = 3.58$; $P < 0.0001$].

In order to evaluate whether the antinociceptive effects of DO34 undergo tolerance, we evaluated von Frey thresholds and acetone-induced flinching in mice that received vehicle or DO34 (30 mg/kg) following one injection or six days of repeated administration. As shown in Figure 4, DO34 retained its anti-allodynic effects after six days of repeated administration in the von Frey assay [$F(1,20) = 22.55$; $P < 0.0001$; Panel A], as well as in the acetone-induced flinching assay [$F(1,20) = 46.34$; $P < 0.0001$; Panel B].

In the next series of experiments, we evaluated LPS-induced allodynia in DAGL-α (-/-) mice or DAGL-β (-/-) mice given an i.p. injection of vehicle, DO34 (30 mg/kg), or DO53 (30 mg/kg), a selective ABHD6 inhibitor that served as a control for this off-target of DO34. As previously reported (Wilkerson *et al.,* 2016), DAGL-β (-/-) mice displayed an anti-allodynic phenotype in the von Frey assay [$F(1,10) = 27.9$; $P < 0.001$; Figure 5A]. In addition, these mice showed a reduction in acetone-induced flinching [$F(1,10) = 225$; $P < 0.0001$; Figure 5B] 24 h following intraplantar LPS administration. DO34 (30 mg/kg, i.p.) administered at 22 h post LPS injection reversed mechanical ($P < 0.001$; Figure 5A) and cold ($P < 0.0001$; Figure 5B) allodynia in DAGL-β (+/+) mice, but did not alter the anti-allodynic phenotypes of the DAGL-β (-/-) mice. DO53 (30 mg/kg i.p.) did not produce significant effects in either mechanical ($P = 0.97$) or cold ($P = 0.49$) allodynia, and did not alter the DAGL-β (-/-) anti-allodynic phenotype.

JPET Fast Forward. Published on October 2, 2017 as DOI: 10.1124/jpet.117.243808
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In the final experiment, we examined the dose-response relationship of DO34 (0, 1, 3, 10, or 30 mg/kg) in LPS-treated DAGL-α (-/-) and (+/+) mice. LPS elicited similar magnitudes of mechanical (Figure 6A) and cold (Figure 6B) allodynia regardless of genotype. DO34 dose-relatedly reversed LPS-induced mechanical allodynia in DAGL-α (+/+) mice [$F(3,20) = 29.42$; $P < 0.0001$] and DAGL-α (-/-) mice [$F(3,20) = 4.45$; $P < 0.05$; Figure 6A]. The respective ED_{50} (95% C.I.) values of DO34 in reversing LPS-induced mechanical allodynia in DAGL-α (+/+) and (-/-) mice were 8.6 (6.4-11.5) mg/kg and 5.9 (3.9-8.8) mg/kg. The potency ratio for DAGL-α (+/+) vs. DAGL-α (-/-) for mechanical allodynia was 1.4 (0.8-2.5). Likewise, DO34 reversed cold allodynia in DAGL-α (+/+), [$F(3,20) = 48.47$; $P < 0.0001$] and DAGL-α (-/-) mice [$F(3,20) = 30.99$; $P < 0.0001$; Figure 6B]. The respective ED_{50} values (95% C.I.) of DO34 in reversing LPS-induced cold allodynia in DAGL-α (+/+) and (-/-) mice were 6.1 (4.7-7.9) mg/kg and 4.5 (3.4-6.0) mg/kg. The potency ratio for DAGL-α (+/+) vs. DAGL-α (-/-) for cold allodynia was 1.35 (0.9-1.9). Finally, DO53 (30 mg/kg) administered at 22 h post LPS injection did not produce antinociceptive effects in either genotype (see Supplemental Figure 1).

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Discussion

The present study employed complementary pharmacological and genetic approaches to test whether blockade of the 2-AG biosynthetic enzymes DAGL-β and DAGL-α produces antinociceptive effects in the LPS model of inflammatory pain. As previously reported (Wilkerson *et al.,* 2016), DAGL-β (-/-) mice were resistant to the development of LPS-induced mechanical allodynia. Moreover these mice were also resistant to the development of LPS-induced cold allodynia. In contrast, DAGL-α (-/-) mice displayed full development of LPS-induced mechanical and cold allodynia. These findings suggest that DAGL-α and DAGL-β play differential roles in the development of LPS-induced hyperalgesic states. Thus, whereas DAGL-α is dispensible for the development of LPS-induced allodynia, DAGL-β plays a nececessary role in the increased nociceptive behavior following endotoxin treatment.

The disparate roles that DAGL-α and DAGL-β play in LPS-induced allodynia are consistent with the differential expression of these enzymes on cells in the nervous system and peripheral tissue (Hsu *et al.,* 2012). Specifically, DAGL-α is expressed on postsynaptic neurons within the hippocampus, cerebellum, prefrontal cortex and the striatum (Katona *et al.,* 2006; Yoshida *et al.,* 2006; Lafourcade *et al.,* 2007; Uchigashima *et al.,* 2007), and its genetic deletion results in marked decreases in 2-AG, AEA, and AA in brain (Gao *et al.,* 2010; Tanimura *et al.,* 2010; Shonesy *et al.,* 2014) and spinal cord (Gao *et al.,* 2010). In contrast, the relative expression of DAGL-β throughout the brain is generally sparse. DAGL-β (-/-) mice express wild type levels of 2-AG in whole brain (Hsu *et al.,* 2012) and endocannabinoid-mediated forms of retrograde synaptic suppression in these mice are spared (Gao *et al.,* 2010). However, the high expression of DAGL-β on microglia in the CNS (Viader *et al.,* 2016) and on macrophages in the periphery (Hsu *et al.,* 2012) is consistent with its role within the innate immune system. Specifically,

pharmacological inhibition or genetic deletion of DAGL-β leads to decreased levels of endocannabinoids, AA, prostanoids, and proinflammatory cytokines in LPS-treated peritoneal macrophage cell cultures from C57Bl/6 mice (Hsu *et al.,* 2012).

Here we show that acute administration of DO34, at a dose which produced reversal of mechanical and cold allodynia (30 mg/kg), produced significant reductions of 2-AG (~83%), AEA (~42%), and AA (~58%) in naïve mice. These findings are in agreement with previous work showing that DO34 reduces 2-AG, AEA, and AA in mouse whole brains (Ogasawara *et al.,* 2016). However, the use of whole brain precludes insight of whether DO34 differentially affects lipid levels in discrete brain regions.

Here, we also report that the DAGL inhibitor DO34 dose-dependently reversed LPS-induced mechanical allodynia and cold alldynia. These antinociceptive effects are DAGL-α dispensible, as genetic deletion of this enzyme did not alter the dose-response curves of DO34 for both measures. DO34 also did not alter the antinociceptive phenotype DAGL-β (-/-) mice response, but completely reversed the LPS-induced allodynic responses in DAGL-β (+/+) mice. Additionally, the DO34 time course experiment demonstrates that its anti-allodynic effects persist for at least 8 hours, which is consistent with its long duration of action in inhibiting DAGL-β activity (Ogasawara *et al.,* 2016). Besides its actions at DAGL-α and –β, DO34 also inhibits ABHD6 (Ogasawara *et al.,* 2016), a serine hydrolase that hydrolyzes 2-AG, but to a much lesser extent than MAGL (Blackman *et al.*, 2007; Marrs *et al.*, 2010). Thus, we tested DO53, a structurally similar compound that inhibits ABHD6 without actions at either DAGL-β or DAGL-α (Ogasawara *et al.,* 2016), in the LPS model of inflammatory pain. DO53 did not reverse LPS-induced allodynia in either DAGL-α (-/-) or (+/+) mice, suggesting that ABHD6 inhibition alone or in combination with DAGL-α inhibition does not elicit antinociceptive effects

in this assay. The observation that another ABHD6 inhibitor, KT195, lacked efficacy in reversing LPS-induced mechanical allodynia (Wilkerson *et al.,* 2016) further excludes consideration the involvement of this enzyme in the results reported here. Although the indirect evidence offered here is consistent with the idea that DAGL-β mediates the anti-allodynic effects of DO34, it does not rule out the possibility of another target.

Another relevant finding in the present study is that repeated administration of DO34 (30 mg/kg) for six days continued to prevent the expression of LPS-induced allodynia. Similarly, the anti-allodynic effects of the preferential DAGL-β inhibitor KT109 in the LPS model of inflammatory pain did not undergo tolerance. This apparent lack of tolerance is consistent with the observation that DAGL-β (-/-) mice displayed an anti-allodynic phenotype in the LPS model of inflammatory pain. It will be important in future studies to ascertain whether repeated administration DAGL inhibitors also reverses nociceptive behavior in chronic models of inflammatory or neuropathic pain. Additionally, given the previously mentioned caveats of target selectivity, there is a need for more selective inhibitors for DAGL-α and β, and further studies with these inhibitors are needed to verify our proposed mechanism of action.

The underlying mechanisms for the antinociceptive effects of DO34 remain to be determined, but may be related to a reduction of arachidonic acid and its bioactive metabolites in macrophages expressed in the LPS-treated paw. In particular, KT109 as well as DAGL-β deletion resulted in decreased levels of a variety of proinflammatory lipids and proteins in LPS-stimulated peritoneal macrophage (Hsu *et al.*, 2012). Specifically, prostaglandins are crucial for the development and maintence of inflammatory pain (Sugita *et al.,* 2016; Endo *et al.*, 2014; Ulmann *et al.,* 2010). DAGL-β inhibition is also protective from microglial activation in the brains of mice repeatedly administered LPS (Viader *et al.*, 2016), and specifically produces

reversal of LPS-stimulated proinflammatory cytokine release (Hsu *et al.*, 2012) as well as reverses allodynia and thermal sensitivity in inflammatory, chronic constriction injury of the sciatic nerve, and chemotherapy-induced peripheral neuropathy pain models (Wilkerson *et al.*, 2016). In these models of neuropathic pain, although these analgesic effects appear to be due to DAGL-β inhibition, the relative contribution of additional modulation of AA metabolites remains unclear. However, AA can act as a direct modulator of neuronal activity through its mechanosensory mediatory effects on lipid-sensitive ion channels (Meves, 2008; Brohawn *et al.*, 2014).

Given that these studies represent one of the first *in vivo* evaluations of DO34, we assessed whether it would produce overt behavioral effects. Accordingly, we examined whether DO34 would produce changes in spontaneous activity or body temperture, as well as assess whether it would elicit acute cataleptic or thermal antinociceptive effects. DO34 did not alter spontaneous activity or body temperature, and was inactive in warm water tail withdrawl test for acute thermal antinociception and in the bar test for catalepsy. Other studies have shown that DO34 decreases food intake (Deng *et al.*, 2017). It is important to note here that DAGL-α (-/-) mice display increased anxiogenic behavior in multiple assays used to infer anxiety (i.e., the open-field, light/dark box, and novelty-induced hypophagia test (Shonesy *et al.*, 2014). Thus, it will be important to assess the effects of DO34 in fear and anxiety assays in future studies.

The results of the present study provide proof of principle that DAGL-β plays a necessary role in the expression of nociceptive behavior in the LPS model of inflammatory pain. The observation that DAGL-α (-/-) mice display LPS-induced allodynia indicates that this enzyme is dispensible for these effects. Moreover, these null mice served as useful tool showing that the anti-allodynic effects of DO34 are independent of its actions on DAGL-β. Taken together, the

present study and our previous work (Wilkerson *et al.*, 2016) suggest that DAGL-β represents a potential therapeutic target to relieve pain elicited by activation of proinflammatory events.

JPET Fast Forward. Published on October 2, 2017 as DOI: 10.1124/jpet.117.243808
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Authorship Contributions

Participated in research design: Wilkerson, Donvito, Grim, Lichtman

Conducted experiments: Wilkerson, Grim, Abdullah

Contributed new reagents or analytic tools: Ogasawara, Cravatt

Performed data analysis: Wilkerson, Grim, Abdullah

Wrote or contributed to the writing of the manuscript: Wilkerson, Lichtman

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Footnotes

Research was supported by the following grants from the National Institutes of Health:

[DA009789], [DA017259], [DA032933], [DA033934-01A1], [DA035864], [DA038493-01A1].

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Figure Legends

Figure 1. Endocannabinoid levels in whole brain mouse tissue are altered 2 hr after 30 mg/kg DO34. DO34 decreases (A), 2-AG (B), AEA (C) AA compared to vehicle **** $p<0.0001$, * $p < 0.05$ vs. vehicle. Data reflect mean ± SEM, n=12 mice per group.

Figure 2. Assessment of behavior reveals DO34 treatment does not produce common cannabimimetic effects in naïve mice. DO34 does not produce (A), catalepsy. (B), antinociception. (C), body temperature or (D), change in locomotion. Data reflect mean ± SEM, n=6 mice per group.

Figure 3. Pharmacological inhibition of DAGL with DO34 reverses LPS-induced mechanical and cold allodynia. (A) DO34 reverses LPS-induced mechanical allodynia in a dose and time dependent manner. (B) DO34 reverses LPS-induced cold allodynia in a dose-dependent manner 2h after i.p. administration. (C) DO34 significantly reversed allodynia in a time and dose dependent manner, within reversal onset at 30 minutes, lasting beyond 8 hours, after i.p. administration. Data reflect mean ± SEM, n=6 mice per group. *** $p <0.0001$, ** $p<0.001$, * $p < 0.05$ vs. LPS + vehicle. Filled circles = $p < 0.05$ vs. LPS + vehicle.

Figure 4. Repeated administration of DO34 prevents LPS-induced mechanical and cold allodynia. (A) Acute or repeated administration of DO34 (30 mg/kg) prevents the expression of LPS-induced mechanical allodynia. (B) Acute or repeated administration of DO34 (30 mg/kg) prevents the expression of LPS-induced cold allodynia. Data reflect mean ± SEM, n=6 mice per group. ** $p<0.001$ vs. LPS + vehicle.

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Figure 5. Genetic inhibition of DAGL-β in the mediation of LPS-induced mechanical and cold allodynia. (A) LPS-treated DAGL-β (-/-) mice do not develop mechanical allodynia. The DAGL-α and DAGL-β inhibitor DO34 (30 mg/kg), reverses LPS-induced mechanical allodynia in DAGL-β (+/+) mice, and does not further alter the antinociceptive effects in DAGL-β (-/-) mice. The ABHD6 inhibitor DO53 did not produce reversal of mechanical allodynia in DAGL-β (+/+) mice. (B) LPS-treated DAGL-β (-/-) mice do not develop cold allodynia. DO34 (30 mg/kg), reverses LPS-induced cold allodynia in DAGL-β (+/+) mice, and does not further alter the antinociceptive effects in DAGL-β (-/-) mice. The ABHD6 inhibitor DO53 did not produce reversal of cold allodynia in DAGL-β (+/+) mice. N = 6 mice/group. Data reflect mean ± SEM, **** $p < 0.0001$ vs. respective genotype control paw.

Figure 6. The role of DAGL-α inhibition in the mediation of DO34 reversal of LPS-induced mechanical and cold allodynia. (A) LPS-treated DAGL-α (-/-) normally develop mechanical allodynia. DO34 (30 mg/kg), reverses LPS-induced mechanical allodynia in DAGL-α (+/+) and (-/-) mice. There is no significant shift in the dose response curve due to genotype. (B) LPS-treated DAGL-α (-/-) mice develop cold allodynia. DO34 (30 mg/kg), reverses LPS-induced cold allodynia in DAGL-α (+/+) and (-/-) mice. There is no significant shift in the dose response curve due to genotype. N = 6 mice/group. Data reflect mean ± SEM. Filled circles = $p < 0.05$ vs. LPS + vehicle in the respective genotype paw.

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Figure 1

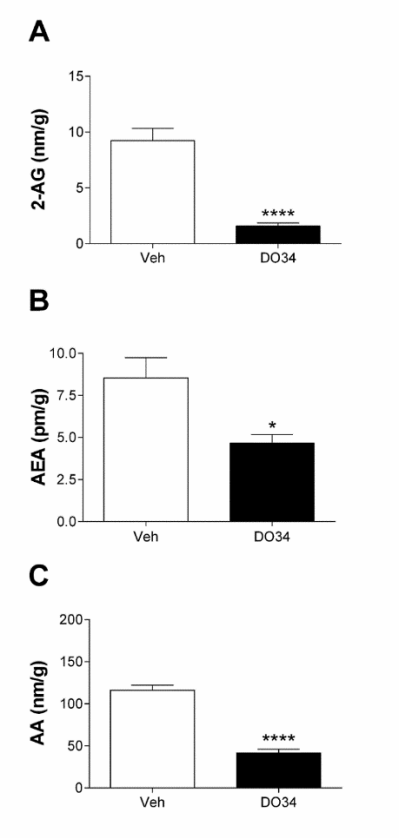

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Figure 2

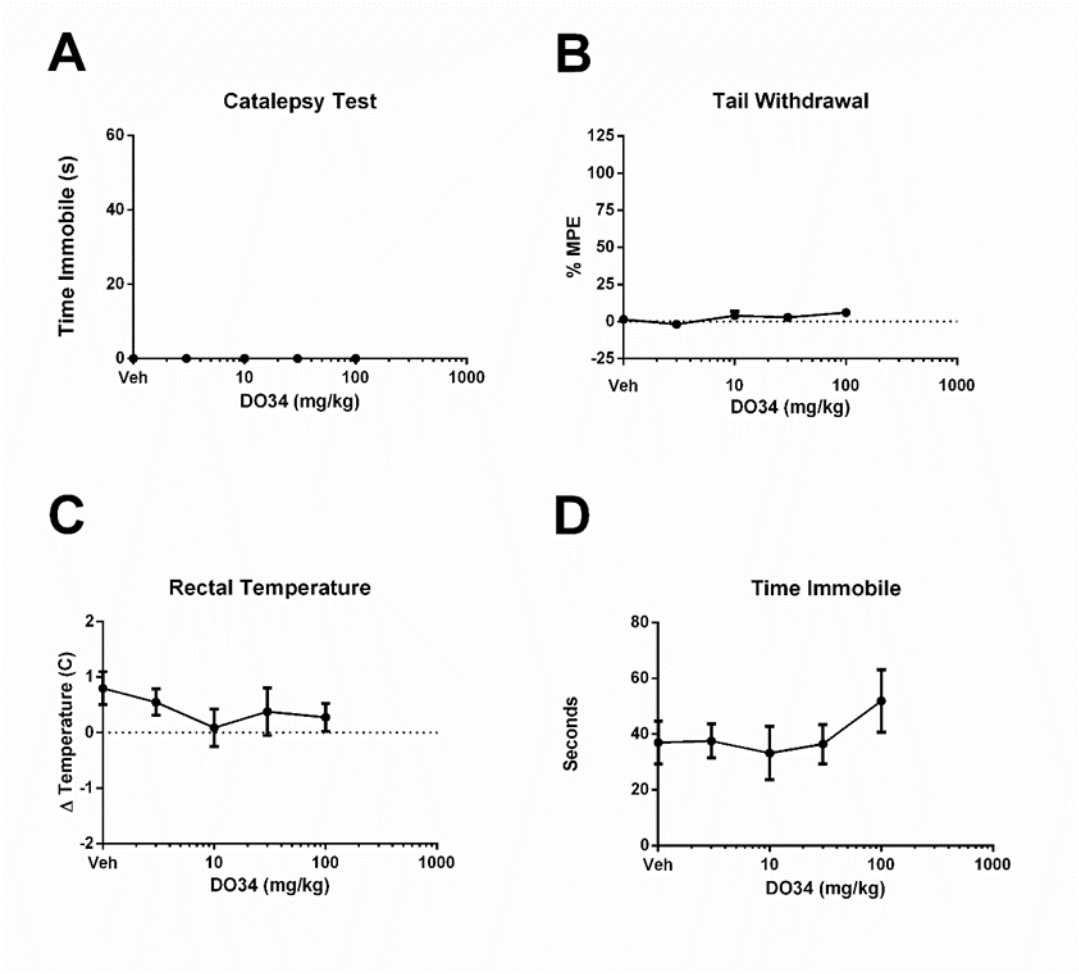

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Figure 3

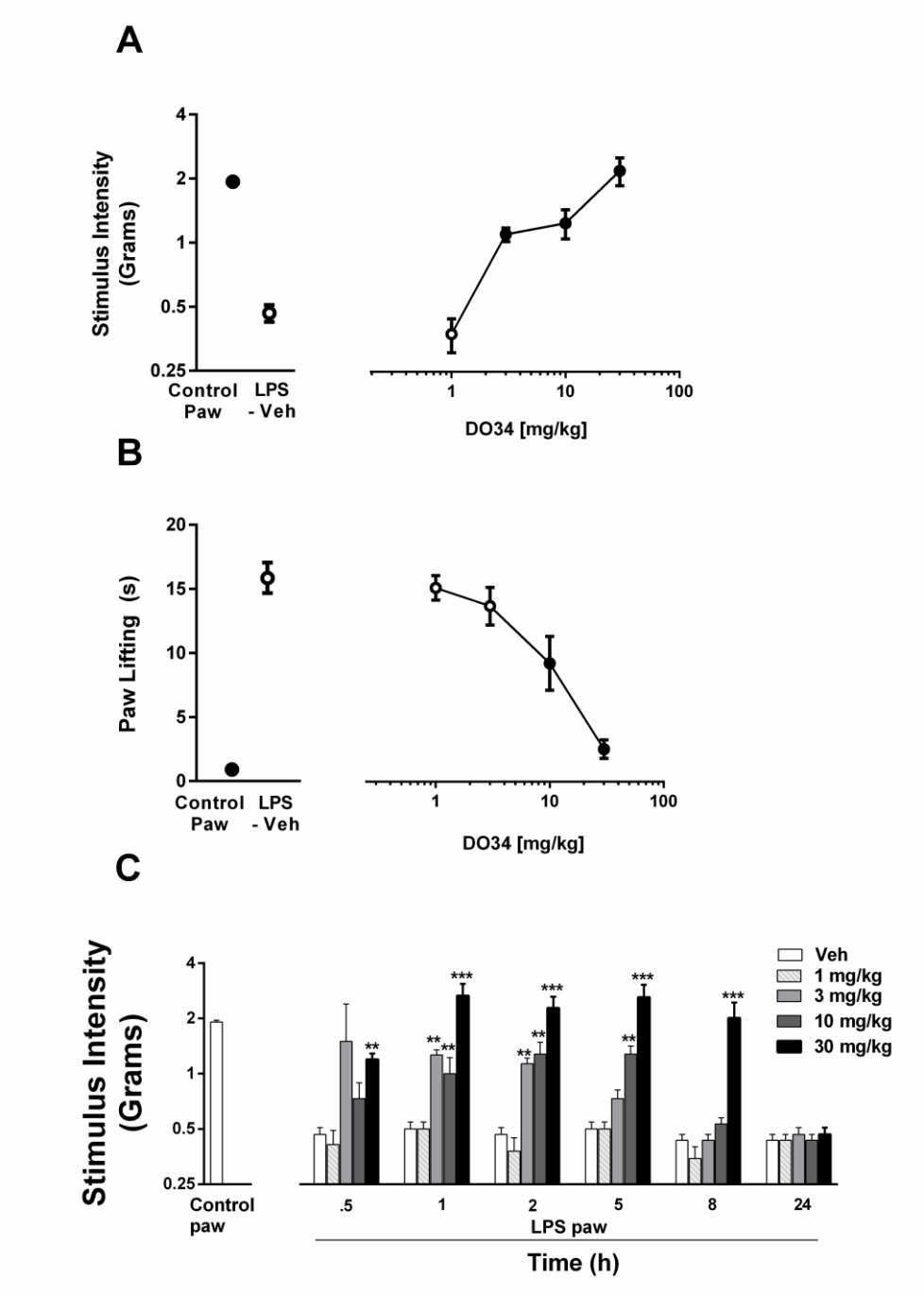

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Figure 4

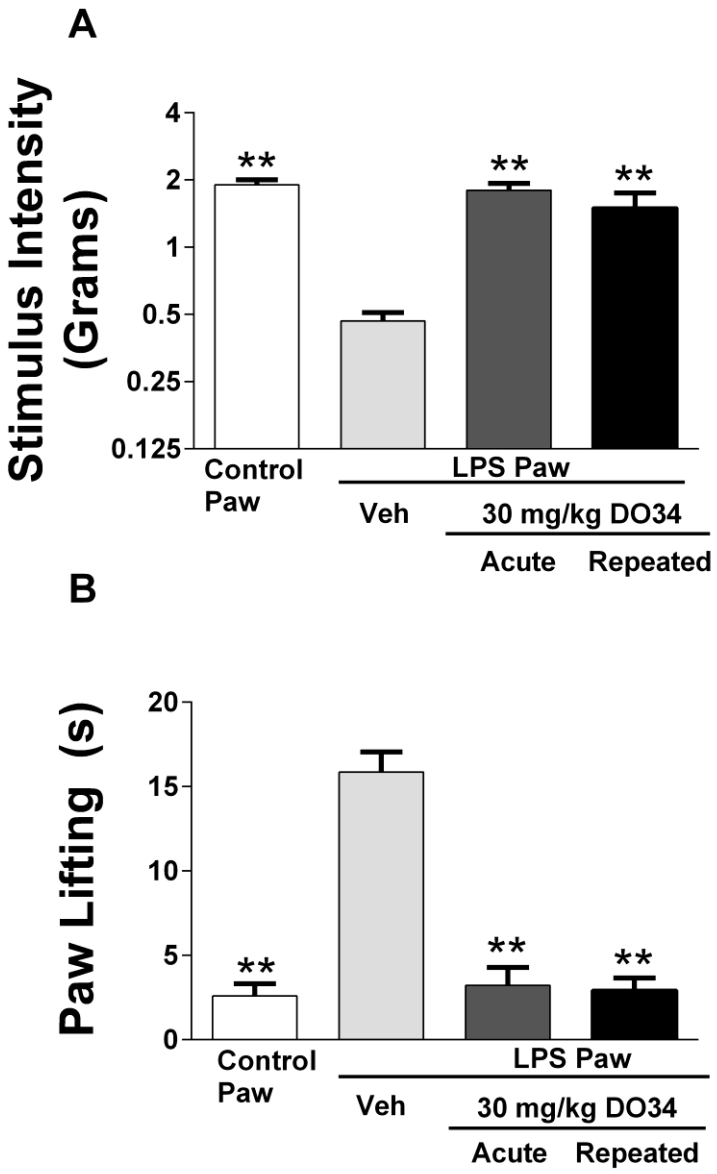

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Figure 5

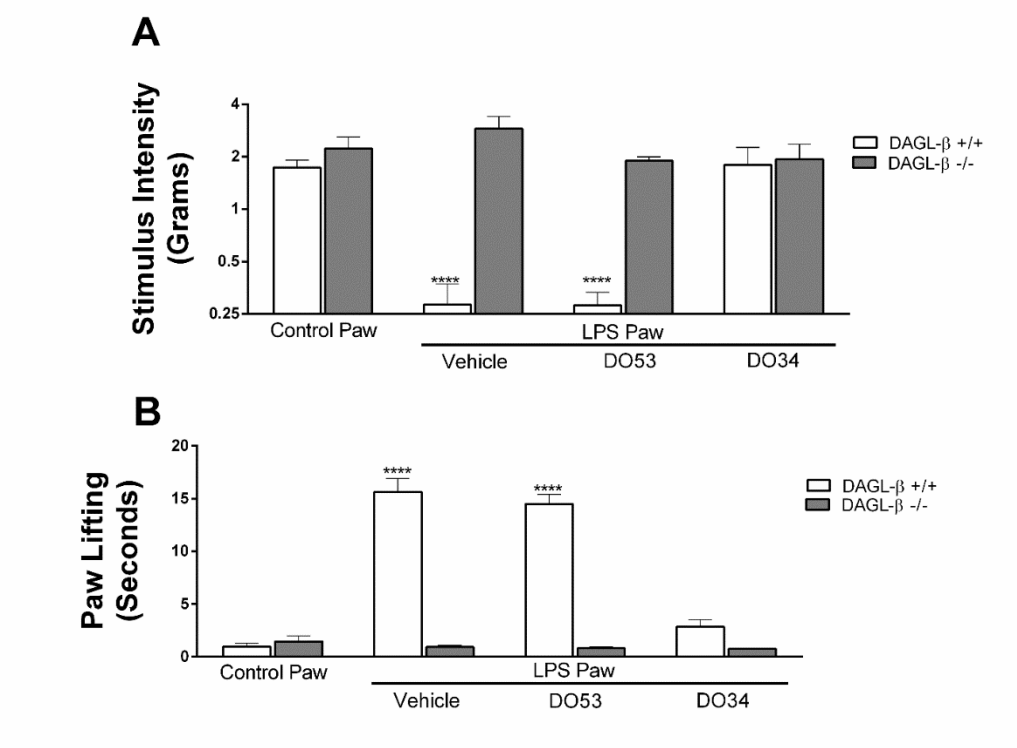

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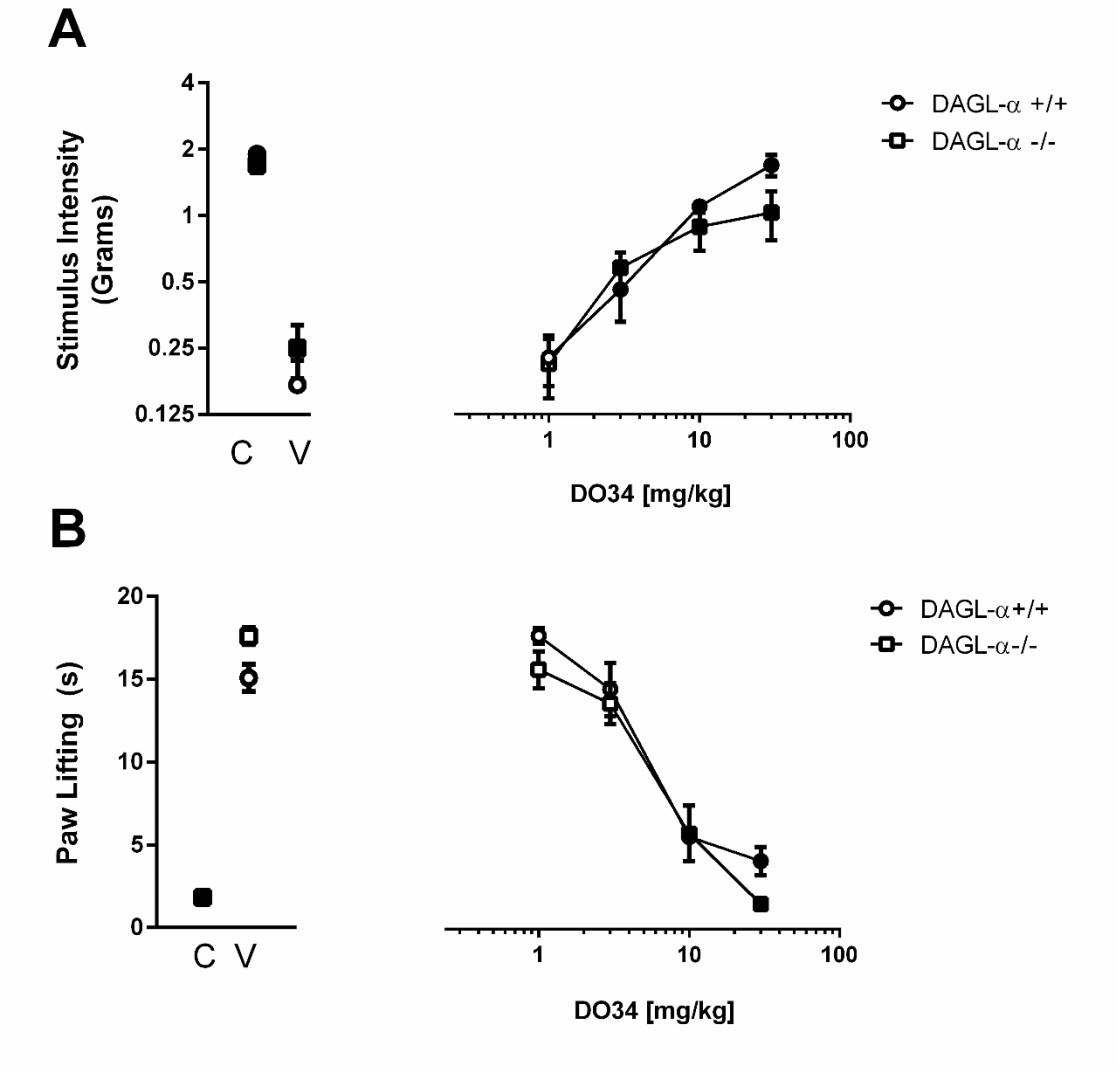
Figure 6



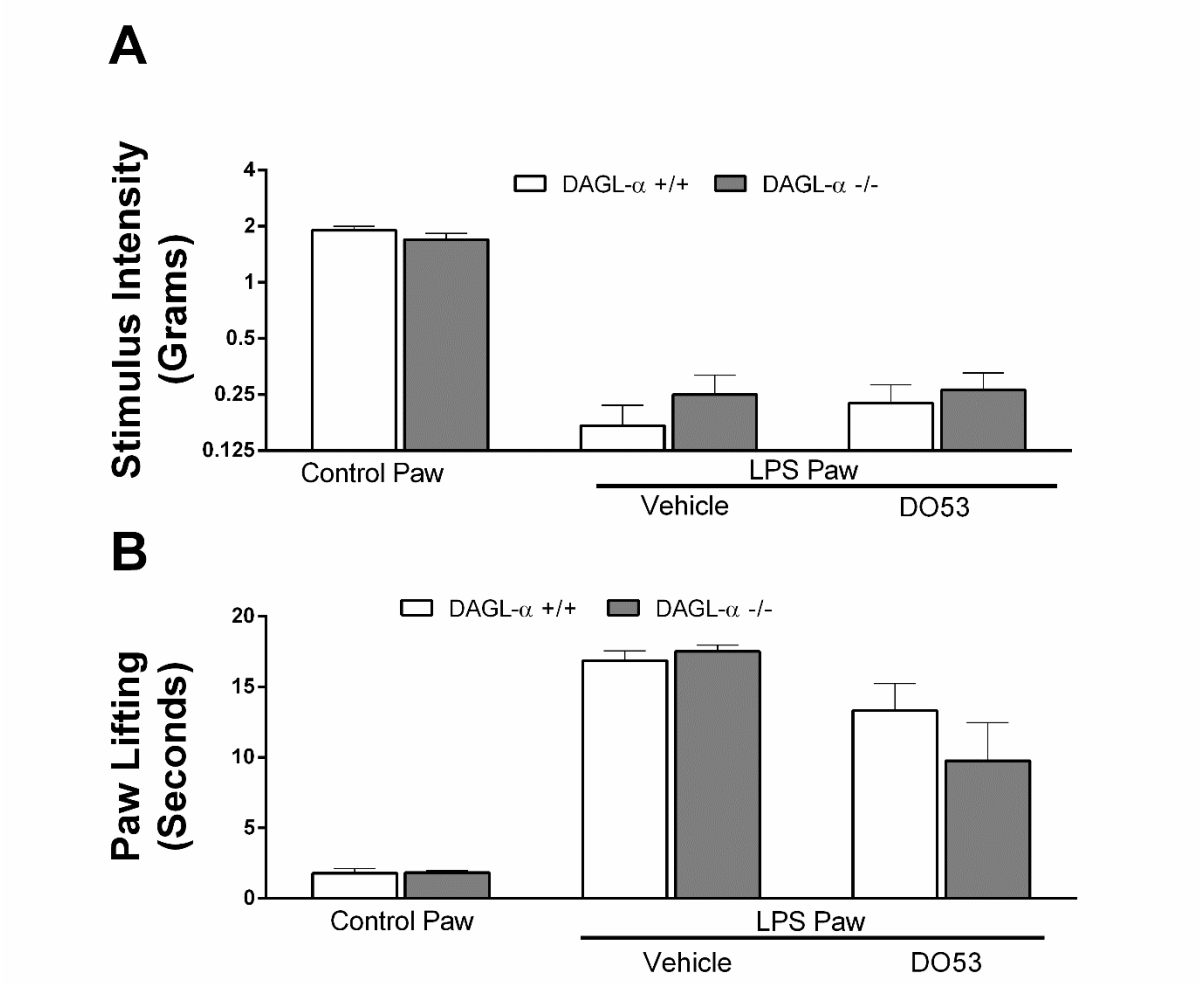
Supplementary methods

Measurement of brain endocannabinoid levels

On the day of processing, tissues were weighed and homogenized with 1.4 ml of chloroform/methanol (2:1 v/v containing 0.0348 g of phenylmethylsulfonyl fluoride/ml) after the addition of internal standards to each sample (8 pmol of AEA-d8, 1 nmol of 2-AG-d8, 3.3 nmol and 1 nmol AA-d8, Cayman Chemical). Homogenates were then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and then centrifuged for 10 min at 3220 × g (4 ^{0}C). The aqueous phase plus debris were collected and extracted two more times with 0.8 ml of chloroform. The organic phases from the three extractions were pooled and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml of chloroform and mixed with 1 ml of ice-cold acetone. The mixtures were then centrifuged for 5 min at 1811 × g and 4 ^{0}C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 ml of methanol and placed in autosample vials for analysis.

Liquid chromatography-tandem mass spectrometry with an electrospray ionization source was used to identify and quantify the endocannabinoids and AA. The chromatographic separation was performed using a Discovery HS C18, 2.1 × 15 cm, 3 μm (Supelco, Bellefonte, PA). The mobile phase consisted of water/methanol (10:90) with 0.1% ammonium acetate and 0.1% formic acid. The following ions were monitored in a multiple-reaction-monitoring positive mode: (348 > 62) and (348 > 91) for AEA; (356 > 62) for AEA-d8; (379 > 287) and (379 > 269) for 2-AG; (387 > 96) for 2AG-d8; (300 > 62) and in negative mode: (303 > 259) and (303 > 59) for AA and (311 > 267) for AA-d8. A calibration curve was constructed for each assay based on linear regression with use of the peak area ratios of the calibrators. The extracted standard curves ranged from 0.039 to 40 pmol for AEA and from 0.0625 to 64 nmol for 2-AG, and from 1 nmol to 32 nmol for AA.

Supplementary Results



Supplemental Figure 1. Pharmacological inhibition of ABHD6 in DAGL-α (-/-) mice with LPS-induced mechanical and cold allodynia. (A) The ABHD6 inhibitor DO53 did not produce reversal of mechanical allodynia in either DAGL-α (+/+) or (-/-) mice. (B) DO53 did not produce reversal of cold allodynia in either DAGL-α (+/+) or (-/-) mice. N = 6 mice/group. Data reflect mean ± SEM, **** $p < 0.0001$ vs. respective genotype control paw.