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Acetaminophen attenuates pathological pain through a mechanism that requires CB1 cannabinoid receptors and the enzyme diacylglycerol lipase in mice

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Acetaminophen attenuates pathological pain through a mechanism that requires CB1 cannabinoid receptors and the enzyme diacylglycerol lipase in mice

Abstract

Acetaminophen (APAP) is commonly used as a pain and fever reliever, but its mechanisms remain unclear. Conflicting evidence implicates the endocannabinoid system in the effects of APAP. We tested the hypothesis that the analgesic effects of APAP were dependent upon both CB1 cannabinoid receptors and diacylglycerol lipase (DAGL), an enzyme which catalyzes formation of the endocannabinoid 2-arachidonoylglycerol. We examined the impact of APAP, administered in the presence and absence of DAGL inhibitors, on mechanical hypersensitivity in mice using models of inflammatory (induced by intraplantar injection of complete Freund's adjuvant (CFA)) and post-surgical (induced by incisional injury) pain. Pharmacological specificity was assessed using global (Rimonabant, AM251) and peripherally restricted (AM6545) CB1 antagonists. APAP produced a dose-dependent attenuation of inflammation-induced mechanical hypersensitivity, but did not alter peripheral edema in the CFA-injected paw. APAP also attenuated mechanical hypersensitivity in mice with incisional injury. The DAGL inhibitors, RHC-80267 or DO34, attenuated the anti-allodynic effects of APAP in both models of pain. CB1 receptor antagonists (Rimonabant and/or AM251) suppressed the antinociceptive effect of APAP in both pain models. The peripherally-restricted CB1 antagonist AM6545 did not alter the anti-allodynic effects of APAP. We also assessed the impact of APAP on tail-flick antinociception, locomotor behavior, and body temperature. APAP produced hypothermia and hypolocomotion at the highest dose, but these effects were not blocked by RHC-80267 or AM251. APAP did not produce tail flick antinociception. Our studies demonstrate that the analgesic effects of APAP observed in mouse models of pathological pain require both DAGL and CB1 activation. Our findings support a potential mechanism of APAP-induced analgesic action involving the enzyme DAGL and CB1 receptors.

1. Introduction

Acetaminophen (N-acetyl-para-aminophenol, APAP, or paracetamol) has been used as an over-the-counter pain and fever reliever for more than a century. It is usually prescribed when there is contraindication for non-steroidal anti-inflammatory (NSAIDs) drugs (e.g. renal, hepatic, and cardiovascular disease), with consistent efficacy, good tolerability, and lack of multiple risk factors and adverse events typically observed with NSAIDs [1]. *In vitro* and *in vivo* studies suggest that multiple targets may contribute to APAP's analgesic effect, but, to date, its pharmacological mechanism remains unclear.

The endocannabinoid system, which consists of cannabinoid receptors, endocannabinoids, and enzymes implicated in endocannabinoid synthesis and degradation, represents an important target for analgesic drug development. Activation of cannabinoid receptor type 1 (CB1) and type 2 (CB2) receptors produce analgesic effects in animal models of inflammatory and chronic pain (for review see [2]). Enzymes catalyzing endocannabinoid hydrolysis (e.g. fatty acid amide hydrolase, FAAH and monoacylglycerol lipase, MGL) represent potential targets for pain pharmacotherapies in pre-clinical pain models [3]. The endocannabinoid system has been repeatedly implicated in the mechanism of action of APAP (for review see [4]), based predominantly, but not exclusively, upon studies evaluating acute nociceptive pain (e.g. hotplate test).

The antinociceptive effects of APAP in inflammatory pain models have been linked to CB1 receptor activation, as these effects are abrogated in CB1 knock-out mice [5] and in rodents treated with CB1 receptor antagonists [6]. Active metabolism of APAP leads to production of p-aminophenol, that is subsequently conjugated to arachidonic acid, by FAAH, resulting into an *N*-arachidonoylphenolamine (AM404) [7]. FAAH is the primary enzyme responsible for the degradation of anandamide (AEA) in the nervous system. In turn, the effect of APAP was also abrogated in mice lacking the enzyme FAAH [8]. AM404 is known to produce analgesia and central effects through activation of transient receptor potential vanilloid 1 (TRPV1) [9,10]. In addition, AM404 is a known inhibitor of AEA transport, which may lead to CB1 receptor activation by elevating levels of endogenous AEA [11]. However, the analgesic mechanisms of APAP and involvement of CB1 receptors remain incompletely understood, and it is unclear whether physiologically active

concentrations of AM404 would be produced by *in vivo* metabolism of APAP. We previously showed that 2-AG is produced on demand and suppresses pain responsiveness through a mechanism that requires both diacylglycerol lipase and CB1 receptors [13]. Furthermore, injection of exogenous 2-AG also produces antinociceptive effects in preclinical models of acute pain [14]. APAP has also been hypothesized to promote analgesia through modulation of diacylglycerol lipase (DAGL), an enzyme responsible for hydrolysis of diacylglycerols and production of the endocannabinoid 2-arachidonoylglycerol (2-AG) [12] and its downstream metabolite AA [10], although there is no direct evidence to suggest that such a mechanism is responsible for analgesic effects of APAP observed *in vivo*.

Conflicting evidence implicates a role for the endocannabinoid system and its components in the antinociceptive effects of APAP. However, published studies have largely relied on *in vitro* approaches or *in vivo* models of acute nociception (e.g. hotplate test) that lack the translational relevance [15]. We, consequently, tested the hypothesis that the analgesic effects of APAP in pre-clinical pain models were dependent upon both DAGL and activation of CB1 in mice using two mechanistically distinct clinically relevant models of pathological pain - the complete Freund's Adjuvant (CFA) model of inflammatory pain and the incisional injury model of post-operative pain. We used these models to ascertain whether the antinociceptive effects of APAP were dependent upon the activation of DAGL, using enzyme inhibitors RHC-80267 and DO34. We also evaluated whether the analgesic effects of APAP were mediated by CB1 receptors. Pharmacological specificity was evaluated by administration of global (Rimonabant and AM251) and peripherally restricted CB1 receptor antagonists (AM6545).

Methods

1.1. Subjects

All experiments were conducted using either male mice on a C57BL/6 background (Jackson Laboratories), or mice of both sexes on a CD1 (CD1 IGS, also known as Crl:CD1(ICR)) background (Inotiv). Mice were maintained in standard conditions of temperature ($21 \pm 2^\circ \text{C}$), humidity (45%) and under a light/dark cycle (12h/12h). All the experimental procedures conducted in this work were approved by the Bloomington

Institutional Animal Care and Use Committee of Indiana University (BIACUC) and followed the guidelines for the treatment of animals of the International Association for the Study of Pain.

1.2. Drugs and Chemicals

Acetaminophen (APAP, Sigma Aldrich, St. Louis MO, USA), RHC-80267 was purchased from Cayman Chemicals (MI, USA). DO34 was synthesized by the laboratory of Dr. Mario van der Stelt (Leiden University, Leiden, the Netherlands). RHC80267 and DO34 were dissolved in a vehicle consisting of cremophor (Sigma-Aldrich, St. Louis, MO), ethanol (Sigma-Aldrich), and saline (Aquilite System, Hospira Inc, Lake Forest, IL) in a 1:1:18 ratio for intraperitoneal (i.p.) administration. Acetaminophen was administered orally in a vehicle consisting of DMSO, Kolliphore, and Mannitol in a 1:1:8 ratio. AM6545 was synthesized by the laboratory of Alex Makriyannis (Center for Drug Discovery, Northeastern University, Boston, MA, USA). AM6545 was dissolved in a vehicle consisting of 20% dimethyl sulfoxide (DMSO) and a 1:1:8 ratio of ethanol, emulphor and saline. APAP was delivered via intraperitoneal injection, after sonication for 1 hour, to mice in the CFA model in a volume of 10 ml/Kg. Other drugs given via intraperitoneal injection in the CFA model were not sonicated. APAP was administered via oral gavage (metal orogastric gavage tube) to mice in the incisional injury model (volume of 10 ml/kg). All other drugs used in studies employing the incisional injury model were given via intraperitoneal injection.

1.3. CFA-induced inflammatory pain

Complete Freund's adjuvant (CFA) was dissolved in saline in a ratio of 1:1 [16] and injected (20 μ l i.pl.) subcutaneously into the plantar surface of the right hind paw of male (C57BL/6) or female (CD1) mice. Baseline mechanical withdrawal thresholds were measured before CFA injection and two days after CFA injection to confirm that mice exhibited mechanical CFA-induced mechanical hypersensitivity prior to pharmacological manipulations.

1.4. Incision pain model

Male and female mice (CD1) were anesthetized with isoflurane and a 5-mm longitudinal incision was made with a n°11 blade through the skin and fascia of the plantar region of the right paw. The incision started 2 mm from the proximal edge of the heel and was extended toward the toes [17]. The plantaris muscle was then elevated and incised longitudinally. The muscle origin and insertion were kept intact. After hemostasis with gentle pressure, the skin was closed with single suture of 8-0 nylon. Mechanical paw withdrawal thresholds were measured before (baseline) and one day after surgery to confirm that mice exhibited incisional injury-induced mechanical hypersensitivity prior to assessments of drug efficacy.

1.5. Assessment of mechanical paw withdrawal thresholds

Paw withdrawal threshold (i.e. force in grams) and development of mechanical hypersensitivity were evaluated as previously described by our group [16]. Briefly, mice were habituated for at least 30 minutes to individual inverted plexiglass chambers placed on a metal mesh platform positioned on a stable wooden table. Paw withdrawal thresholds were evaluated with a semi-flexible plastic filament with a 0.8 mm diameter that was placed in a 90-g probe connected to an electronic von Frey anesthesiometer (IITC Life Science, Woodland Hills, CA). Mechanical paw withdrawal threshold was defined as the gram force necessary to produce paw withdrawal and was recorded in duplicate for each hind paw with a 2-minute interval between stimulations to avoid sensitization. Mechanical withdrawal thresholds were averaged for each paw separately to provide a single value per paw/per animal/per timepoint. Paw withdrawal thresholds were determined in both ipsilateral and contralateral paws separately given that these pain models produce unilateral hypersensitivity in the injured (ipsilateral) paw [16,17].

1.6. Assessment of peripheral edema

Paw thickness was assessed as previously performed by our group with minor alterations [18]. Briefly, animals were restrained with a cloth and had their paw thickness of both hind paws assessed using an electronic caliper (World Precision Instruments, INC; Sarasota, FL, USA; Digital Caliper 501601) and expressed in millimeters (mm). Paw thickness was measured before (baseline) and at different times after CFA or drug injection by an

experimenter blinded to the experimental conditions. Paw thickness was evaluated in duplicate and averaged for each paw (ipsilateral and contralateral) in CFA-injected mice.

1.7. Locomotor activity

Drug-induced changes in locomotor activity was assessed by placing CD1 mice in an Omnitech Superflex Node activity meter (Dimensions: 42 × 42 x 30 cm). The total distance travelled (cm) during a 30 min testing period was calculated using the Fusion 6.5 software (Omnitech Electronics, Columbus, OH). Mice were habituated to the test room in their home cages for at least 1 hour before placed in the activity meter. Mice were also habituated to the activity meter for 30 min duration 24 h prior to pharmacological manipulations. Then, locomotor activity was assessed for 30 minutes after drug treatments (i.p.). Time spent in the center of the apparatus (this area was defined as the eight-by-eight square center portion of the activity meter), rest time (a resting period was defined as a period of inactivity greater than or equal to 1 second), vertical time (time accumulated by the animal when breaking beams in the vertical layer) and total distance exploring the apparatus were the main behaviors assessed during the activity meter assessment [19].

1.8. Body temperature

Body temperature (°C) was assessed using a thermometer (Physitemp Instruments, Inc., Clifton, NJ) attached to a rectal probe (Braintree Laboratories, Inc., Braintree, MA). Body temperature was measured prior to pharmacological manipulations (i.e. baseline) and was reassessed at different time points after pharmacological treatments [20].

1.9. Tail-flick (tail immersion test)

Hot water tail immersion test was used to evaluate antinociception to noxious heat stimuli after drug treatment (i.p.). The test was performed by submerging the distal portion (2 cm) of the mouse tail in a hot water bath (52°C) and the latency to response (tail flick) was measured as previously described [13,21]. Three baseline values were obtained and averaged prior to drug treatment with a 10-minute interval between them with a cut-off latency of 15 seconds to avoid tissue damage. Tail flick latency was taken as a baseline and at different times after drug treatments.

1.10. Experimental procedures

Therapeutic efficacy of APAP was evaluated acutely in mice 48 h after CFA injection and paw incisional injury based upon the following treatments and experimental paradigms:

Experiment 1: Dose response of APAP in CFA-induced inflammatory pain. We evaluated the dose response of the antinociceptive effect of APAP (30, 100 and 300 mg/kg, i.p.) in male (C57BL/6) and female (CD1) mice 2 days following unilateral intraplantar injection of CFA in one of the hind paws. Mechanical threshold and paw thickness were determined before and after CFA injection and 30, 90 and 240 minutes after APAP treatment.

Experiment 2: Effect of DAGL inhibition on the anti-allodynic effect of APAP in CFA-induced inflammatory pain. First, we assessed the effect of a DAGL inhibitors (RHC-80267, 20 mg/kg; or DO34, 30 mg/kg; i.p. [17]) in the antinociception exerted by APAP (300 mg/kg, i.p.) on inflammatory pain induced by CFA injection. RHC-80267 or DO34 were given 30 minutes before APAP injection. Mechanical paw withdrawal thresholds were measured before and after CFA injection as well as 30 minutes after RHC-80267 or DO34 injection, as well as 30, 90, and 240 minutes after APAP treatment in male mice (C57BL/6). Female mice (CD1) also had their mechanical threshold evaluated before and after CFA injection, and at different time points after RHC-80267/DO34 and APAP treatment.

Experiment 3: Effect of CB1 receptor antagonists on the anti-allodynic effect of APAP in CFA-induced inflammatory pain. We examined the effect of global CB1 receptor antagonists (AM251 or Rimonabant, 5 mg/kg; i.p. [18]) on the antinociception produced by APAP (300 mg/kg, i.p.) treatment in male and/or female mice. AM251 or Rimonabant were given 30 minutes before APAP injection (i.p.). Mechanical threshold was determined before and after CFA injection, as well as 30 minutes after CB1 receptor antagonist injection, and 30, 90, and 240 minutes after APAP treatment in male mice. Female mice with CFA-injected paws were given AM251 30 minutes before APAP and their mechanical threshold was reevaluated 30, 90, and 240 minutes after APAP treatment.

Experiment 4: Effect of peripherally restricted CB1 receptor antagonist on the anti-allodynic effect of APAP in CFA-induced inflammatory pain. We further assessed the

effect of a peripherally restricted CB1 receptor antagonist AM6545 (10 mg/kg; i.p. [3]) on the antinociceptive efficacy of APAP (300 mg/kg, i.p.) in male and female mice with CFA-injected paws. AM6545 was given 30 minutes before APAP injection. Mechanical threshold was determined before and after CFA injection, as well as 30 minutes after AM6545 injection, and 30, 90, and 240 minutes after APAP treatment in male mice. AM6545 or vehicle was administered 30 min prior to and APAP in female mice with CFA-injected paws and mechanical thresholds were assessed before and 30, 90 and 240 minutes after APAP treatment.

Experiment 5: Dose response of APAP in an incisional injury model. We evaluated the dose response of the antinociceptive effect of APAP (100 and 300 mg/kg, i.p.), compared to vehicle, in suppressing mechanical hypersensitivity induced by plantar paw incision using male (CD1) mice. Mechanical paw withdrawal thresholds and paw thickness were determined before and after CFA injection and 30, 90 and 240 minutes after pharmacological treatment.

Experiment 6: Effect of DAGL inhibition on the anti-allodynic effect of APAP in a mouse model of incisional injury. Mechanical paw withdrawal thresholds were evaluated before and 24 h after hindpaw incisional injury in separate cohorts of male and female (CD1) mice. RHC-80267 (20 mg/kg), DO34 (30 mg/kg) or vehicle were administered i.p. 30 minutes prior to APAP (300 mg/kg, p.o.) or vehicle treatment. Mechanical paw withdrawal thresholds were reevaluated 30, 90 and 240 minutes after APAP treatment, as previously described.

Experiment 7: Effect of CB1 receptor antagonists on the anti-allodynic effect of APAP in CFA-induced inflammatory pain and incisional injury pain. We examined the effect of CB1 receptor antagonists (AM251, 5 mg/kg; i.p. [18]) on the antinociception produced by APAP (300 mg/kg, i.p.) treatment in male and/or female mice with incisional injury. Male and female CD1 mice with incisional injury were received AM251 (i.p.) or vehicle 30 minutes before APAP (300 mg/kg, p.o.). Mechanical paw withdrawal thresholds were assessed at the same time points described above.

Experiment 8: Effect of APAP on body temperature, tail-flick latency and locomotor activity in naïve mice. Tail flick antinociception in the tail immersion test (52 °C) and rectal

temperature was measured at baseline and at 30-,90- and 240-minutes post APAP or vehicle treatment, using the same male CD1 mice. We also examined effects of pretreatment with either the CB1 antagonist AM251 (5 mg/kg), DAGL inhibitor RHC-80267 (20 mg/kg), or their respective vehicles on responses induced by APAP in each assay. After a 7-day washout period, baseline activity meter assessments were performed. Then, 24 hours later, locomotor activity was reassessed in the activity meter 30 minutes treatment with drug combinations consisting of CB1 receptor antagonist AM251 (5 mg/kg) and APAP (300 mg/kg), DAGL inhibitor RHC-80267 (20 mg/kg) and APAP, vehicle, and APAP, or the combination of antagonist and APAP's respective vehicles. Antagonist/inhibitor injection was administered i.p. 30 minutes before APAP injection.

1.11. Statistical analysis

Mechanical paw withdrawal thresholds, latency to tail-flick and body temperature were analyzed by Two-way repeated measures ANOVA. One-way ANOVA was used to analyze locomotor activity data. One-way ANOVA was also used to assess effect of antagonists and enzyme inhibitors 30 minutes after injection in male mice with CFA-inflammatory pain. Bonferroni's post hoc test using GraphPad Prism 10 (GraphPad Software, La Jolla, CA) was used for all data sets with $p < 0.05$ was considered significant.

2. Results

2.1. General experimental results

Intraplantar injection of CFA lowered paw withdrawal threshold in the ipsilateral (CFA-injected) paw of male and female mice, relative to baseline in all studies. Overall, no differences were detected in the paw withdrawal threshold of the paws contralateral to CFA injection.

2.2. APAP attenuated mechanical allodynia, but not paw edema in CFA-injected male and female mice.

APAP produced a dose- and time-dependent increase in ipsilateral paw withdrawal thresholds and paw withdrawal thresholds also changed across time, irrespective of drug treatment (**Figure 1A**: Treatment: $F_{3,22}=16.43$, $p < 0.0001$; Time: $F_{2,154,47.38}=18.18$,

$p < 0.0001$; Interaction: $F_{9,66} = 3.474$, $p = 0.0015$). APAP at 100 ($p = 0.0037$) and 300 mg/kg (i.p.) ($p < 0.0001$) increased paw withdrawal thresholds in the CFA-injected (ipsilateral) paw in male mice, in comparison to the vehicle-treated group (**Figure 1A**). APAP 300 mg/kg also increased mechanical paw withdrawal thresholds across the observation interval to a greater extent than the low 30 mg/kg dose ($p = 0.0208$).

All doses of APAP (30 mg/kg: $p = 0.0028$; 100 mg/kg: $p = 0.0210$; 300 mg/kg: $p = 0.0003$, respectively) elevated paw withdrawal thresholds at 30 min post-injection relative to the vehicle treated group. The highest dose of APAP, 300 mg/kg (i.p.) ($p = 0.0344$), also elevated paw withdrawal thresholds at 90 min post-injection. No alterations in paw withdrawal threshold were detected in the contralateral paw of male mice after pharmacological treatments (**Figure 1B**; Treatment: $F_{3,22} = 0.7138$, $p = 0.5542$; Time: $F_{2,843,65.56} = 0.9434$, $p = 0.4213$; Interaction: $F_{9,66} = 0.4997$, $p = 0.8695$).

CFA injection also increased paw thickness, consistent with paw edema, in the ipsilateral paw. APAP did not affect paw edema in male mice at any dose (**Figure 1C**; Treatment: $F_{3,22} = 0.3116$, $p = 0.8168$; Time: $F_{2,386,52.49} = 2.840$, $p = 0.0583$; Interaction: $F_{9,66} = 1.338$, $p = 0.2347$). Furthermore, no alterations in paw thickness were detected in the contralateral paw of male mice injected with CFA and pharmacologically treated with APAP (**Figure 1D**; Treatment: $F_{3,22} = 0.3116$, $p = 0.8168$; Time: $F_{2,386,52.49} = 2.840$, $p = 0.0583$; Interaction: $F_{9,66} = 1.338$, $p = 0.2347$).

In female mice, APAP produced dose- and time-dependent increases in ipsilateral paw withdrawal thresholds, which also changed across time irrespective of drug treatment (**Figure 1E**; Treatment: $F_{3,16} = 3.384$, $p = 0.0442$; Time: $F_{2,856,45.69} = 15.10$, $p < 0.0001$; Interaction: $F_{9,48} = 2.522$, $p = 0.0187$). APAP, at doses of 30 ($p = 0.0028$) and 300 mg/kg ($p < 0.0001$), increased mechanical thresholds of the CFA-injected (ipsilateral) paw of female mice relative to the vehicle-treated group. APAP (300 mg/kg) increased ipsilateral paw withdrawal thresholds at 30 ($p = 0.0017$) and 90 minutes post-injection ($p = 0.0042$). Pharmacological treatments did not alter paw withdrawal thresholds contralateral to CFA injection (**Figure 1F**; Treatment: $F_{3,16} = 0.5173$, $p = 0.6763$; Time: $F_{2,576,41.22} = 2.455$, $p = 0.0850$; Interaction: $F_{9,48} = 1.025$, $p = 0.4344$).

2.3. *DAGL inhibitors, RHC-80267 and DO34, attenuate the anti-allodynic effect of APAP in CFA-induced inflammatory pain.*

Pre-treatment with RHC-80367 (20 mg/kg) did not alter paw withdrawal thresholds in the CFA-injected (ipsilateral) paw of male mice 30 minutes after treatment (**Figure 2A**, Data not shown; $F_{3,24}=2.317$, $p=0.1011$). In CFA-injected mice receiving RHC80267/vehicle pretreatment prior to APAP/vehicle, paw withdrawal threshold changed as a function of drug treatment and in a time dependent manner and withdrawal thresholds also changed across time irrespective of pharmacological treatment (**Figure 2A**; Treatment: $F_{3,24}=12.27$, $p<0.0001$; Time: $F_{2,416,57.99}=9.10$, $p=0.0002$; Interaction: $F_{9,72}=4.183$, $p=0.0002$). In male mice, APAP, administered at a dose of 300 mg/kg (i.p.) following vehicle pre-treatment (Veh-APAP), increased mechanical paw withdrawal thresholds in the CFA-injected paw overall in comparison to groups that received either vehicle (vs. Veh-Veh, $p<0.0001$) or RHC-80267 (vs. RHC-Veh, $p<0.0001$) prior to vehicle treatment. RHC-80267 pretreatment before APAP blocked the anti-allodynic effect of APAP in male mice (vs. RHC-APAP; $p=0.0021$). APAP (300 mg/kg, i.p.) induced reliable anti-allodynic efficacy at 30 minutes post injection in comparison to groups pretreated with either vehicle or RHC-80267 prior to vehicle treatment (vs. Veh-Veh, $p=0.0005$; vs RHC+Veh, $p=0.0029$).

APAP (300 mg/kg, i.p.) administration following vehicle pre-treatment (Veh-APAP) also increased paw withdrawal thresholds in the CFA-injected paw overall in female mice (**Figure 2B**; Treatment: $F_{3,16}=19.49$, $p<0.0001$; Time: $F_{2,319,37.10}=3.315$, $p=0.0408$; Interaction: $F_{9,48}=3.813$, $p=0.0011$) relative to either vehicle-vehicle or RHC-Veh treatment ($p<0.0001$ for each comparison). Pretreatment with RHC80267 blocked the anti-allodynic effect of APAP (VEH-APAP vs. RHC-APAP, $p=0.0003$). APAP (Veh-APAP) elevated paw withdrawal thresholds in the CFA-injected paw at 30- and 90-minutes post treatment, in comparison to control groups that were given only vehicle (vs. Veh-Veh, 30 min: $p=0.0017$, 90 min: $p=0.0042$) and/or RHC (vs. RHC-Veh, 30 min: $p=0.0054$). RHC-80267 blocked the anti-allodynic effect of APAP at 30- and 90-minutes post treatment (vs. RHC-APAP, 30 min: $p=0.0165$, 90 min: $p=0.0128$) in female mice with CFA-induced inflammatory pain (**Figure 2B**).

APAP (300 mg/kg, i.p.), administered after vehicle pretreatment (Veh-APAP) increased paw withdrawal thresholds in the CFA-injected paw of male mice compared to either vehicle (vs. Veh-Veh, $p < 0.0001$) or DO34 (30 mg/kg) alone (DO34-Veh, $p \leq 0.0001$) (**Figure 2C**; Treatment: $F_{3,20}=9.825$, $p=0.0003$; Time: $F_{2.857,57.13}=1.940$, $p=0.1360$; Interaction: $F_{9,60}=1.887$, $p=0.0713$). One-way ANOVA revealed DO34 30 mg/kg (i.p.) did not alter paw withdrawal thresholds in the CFA-injected (ipsilateral) paw (Data not shown; $F_{3,20}=2.106$, $p=0.1315$). Pre-treatment with DO34 30 minutes before APAP injection attenuated the anti-allodynic effect of APAP in the CFA injected paw (vs. DO34-APAP, $p=0.0003$). No interaction of time and treatment was detected in this subset of experiments with male mice

Similarly, DO34 30 mg/kg did not alter mechanical paw withdrawal thresholds in female mice receiving intraplantar CFA (**Figure 2D**; Treatment: $F_{3,19}=11.39$, $p=0.0002$; Time: $F_{2.523,47.94}=2.308$, $p=0.0982$; Interaction: $F_{9,57}=1.564$, $p=0.1485$). APAP (300 mg/kg, i.p.), administered after pretreatment with vehicle (Veh-APAP) elevated mechanical paw withdrawal thresholds in the CFA-injected paw relative to groups pretreated with vehicle that subsequently received either vehicle or DO34 (30 mg/kg) (vs. Veh- Veh, $p < 0.0001$; vs. DO34-Veh, $p < 0.0001$). DO34 pretreatment prior to APAP blocked the anti-allodynic effect of APAP in the CFA-injected paw in female mice (vs. DO34-APAP, $p=0.0071$). No interaction between treatment and time was detected.

RHC-80267 did not alter paw withdrawal thresholds in the contralateral paw of male mice (**Supplemental Figure 1A**, Data not shown; $F_{3,24}=1.108$, $p=0.3654$). Moreover, no changes in paw withdrawal thresholds were detected in the contralateral paw of CFA-injected male or female mice during the course of the study (**Supplementary Figure 1A**; Treatment: $F_{3,24}=1.260$, $p=0.3105$; Time: $F_{2.707,64.96}=0.5988$, $p=0.6014$; Interaction: $F_{9,72}=0.3182$, $p=0.9665$; **Supplementary Figure 1B**; Treatment: $F_{3,16}=1.351$, $p=0.2933$; Time: $F_{2.645,42.32}=0.290$, $p=0.8528$; Interaction: $F_{9,48}=1.053$, $p=0.4137$). Similarly, DO34 (30 mg/kg, i.p.) alone did not alter paw withdrawal threshold of the contralateral paw of CFA-injected male mice (**Supplemental Figure 1C**, Data not shown; $F_{3,20}=1.513$, $p=0.2417$). No alterations in the paw withdrawal thresholds were observed in the contralateral paws of CFA-injected male and female mice (**Supplementary Figure 1C**;

Treatment: $F_{3,20}=1.148$, $p=0.3540$; Time: $F_{2,594,51.89}=0.1358$, $p=0.9179$; Interaction: $F_{9,60}=0.9860$, $p=0.4610$; **Supplementary Figure 1D**; Treatment: $F_{3,19}=1.677$, $p=0.2057$; Time: $F_{2,415,45.88}=0.4300$, $p=0.6904$; Interaction: $F_{9,57}=1.121$, $p=0.3633$).

2.4. *Global, but not peripherally restricted CB1 receptor antagonists attenuate the effect of APAP on CFA-induced inflammatory pain mice.*

The CB1 antagonist AM251 (5 mg/kg i.p.) blocked the anti-allodynic effect of APAP in the CFA-injected paw (**Figure 3A**; Treatment: $F_{3,27}=12.40$, $p<0.0001$; Time: $F_{2,642,71.33}=9.900$, $p<0.0001$; Interaction: $F_{9,81}=6.107$, $p<0.0001$). Pre-treatment with AM251, by itself, did not alter the mechanical threshold of the CFA-injected (ipsilateral) paw compared to pre-injection levels ($F_{3,27}=1.774$, $p=0.1759$). Paw withdrawal thresholds were lower in the AM251-Veh group compared to Veh-Veh group ($p=0.0222$) but did not differ from the post-CFA threshold determined prior to pharmacological manipulations (vs. 30 minutes $p>0.99$; vs 90 minutes $p=0.61$; vs 240 minutes $p>0.99$). APAP elevated paw withdrawal thresholds in the CFA-injected (ipsilateral) paw (Veh-APAP), in comparison to control groups receiving either vehicle or AM251 alone (vs. Veh-Veh, $p=0.0222$; vs. AM251-Veh, $p<0.0001$). AM251 attenuated the anti-allodynic effect of APAP'S antiallodynic effect in CFA-injected paw of male mice (Veh-APAP vs. AM251-APAP, $p<0.0001$). APAP elevated paw withdrawal thresholds in the CFA-injected paw at 30- and 90-minutes post treatment relative to vehicle (vs. Veh-Veh, 30 min: $p=0.0043$, 90 min: $p=0.0254$) or AM251 alone (vs. AM251-Veh, 30 min: $p=0.0002$; 90 min: $P=0.0004$). AM251 attenuated the anti-allodynic effect of APAP at 30 and 90 minutes after treatment (30 min: $p=0.0093$, 90 min: $p=0.0056$)

In females, APAP treatment (Veh-APAP) increased paw withdrawal threshold in the CFA-injected paw across the observation interval and in a time-dependent manner (**Figure 3B**; Treatment: $F_{3,27}=14.98$, $p<0.0001$; Time: $F_{2,466,66.59}=14.66$, $p<0.0001$; Interaction: $F_{9,81}=4.850$, $p<0.0001$) in comparison to control groups treated with Vehicle (vs. Veh-Veh, $p<0.0001$) or AM251 (vs. AM251-Veh, $p=0.0005$). The CB1 antagonist AM251, given before APAP, blocked the anti-allodynic effect of APAP in female mice (vs. AM251-APAP, $p<0.0001$). APAP increased mechanical paw withdrawal thresholds in the CFA-injected paw at 30 and 90 minutes after administration (vs. Veh-Veh, 30 min: $p<0.0001$, 90 min:

p=0.0007; vs. AM251-Veh, 30 min: p=0.0164, 90 min p=0.0199). AM251 blocked the anti-allodynic effect of APAP at both 30- and 90-minutes post treatment (vs. Veh-APAP, 30 min: p=0.0020, 90 min: p=0.0480).

The peripherally restricted CB1 receptor antagonist AM6545 (10 mg/kg) did not alter paw withdrawal thresholds in the CFA-injected paw in male mice (**Figure 3C**, ipsilateral paw, data not shown, $F_{3,20}=0.0125$, p=0.9980). Subsequent APAP treatment increased paw withdrawal thresholds in the CFA-injected paw overall and in a time-dependent manner (**Figure 3C**; Treatment: $F_{3,20}=5.833$, p=0.0049; Time: $F_{2.582,51.64}=9.367$, p=0.0001; Interaction: $F_{9,60}=3.641$, p=0.0011). compared to control groups receiving vehicle or AM6545 (vs. Veh-Veh, p=0.0150; AM6545-Veh, p=0.0064). AM6545 did not attenuate the anti-allodynic effect of APAP in male mice (vs. AM6545-APAP, p>0.9999) under conditions in which APAP elevated paw withdrawal thresholds compared to control groups (vs. Veh-Veh, p=0.0023; vs. AM6545-Veh, p=0.0009). APAP elevated mechanical paw withdrawal thresholds in the CFA-injected paw in comparison to the control group receiving vehicle (vs. Veh-Veh, p=0.0494). APAP elevated paw withdrawal relative to groups treated with vehicle or AM6545 at 30 minutes (p=0.0165 for each comparison) post-injection, whereas paw withdrawal thresholds in the group receiving the combination of AM6545 and APAP were higher than those observed in the Veh-Veh group (vs. Veh-Veh, p=0.0006)

In female mice, APAP increased paw withdrawal thresholds in the CFA-injected paw (**Figure 3D**; Treatment: $F_{3,26}=12.39$, p<0.0001; Time: $F_{2.314,60.16}=14.64$, p<0.0001; Interaction: $F_{9,78}=3.538$, p=0.0010) irrespective of AM6545 pre-treatment. APAP elevated paw withdrawal thresholds (vs. Veh-Veh, p<0.0001), regardless of pre-treatment with AM6545 (vs. Veh-Veh, p<0.0001). APAP treatment elevated paw withdrawal thresholds at 30- and 90-minutes post treatment, relative to the vehicle-treated group (vs. Veh-Veh, 30 min: p=0.0026, 90 min: p=0.0028). Treatment with AM6545 followed by APAP also increased paw withdrawal thresholds in the CFA-injected paw in female mice at 30 minutes post injection (vs. Veh-Veh, p=0.0107). Although AM6545 alone was associated with higher paw withdrawal thresholds than the control group (vs. Veh-Veh, p=0.0380), withdrawal thresholds did not differ from post-CFA pre-injection thresholds at any post-

injection timepoint (vs 30 minutes $p=0.1546$; vs 90 minutes $p>0.99$; vs 240 minutes $p=0.5057$) (**Figure 3D**).

Treatment with AM251 or AM6545 in the presence or absence of APAP did not alter paw withdrawal thresholds in the paw contralateral to CFA injection in male mice (**Supplemental Figure 2A**, data not shown, $F_{3,28}=0.3904$, $p=0.7608$; **Supplemental Figure 2C**, data not shown, $F_{3,20}=1.069$, $p=0.3844$). Furthermore, no group differences in paw withdrawal thresholds were detected in the contralateral paw in either male and female mice throughout the course of the study or following administration of different pharmacological treatments (**Supplemental Figure 2A**; Treatment: $F_{3,28}=0.7344$, $p=0.5403$; Time: $F_{2,947,82.51}=1.279$, $p=0.2871$; Interaction: $F_{9,84}=1.203$, $p=0.3045$; **Supplemental Figure 2B**; Treatment: $F_{3,25}=2.164$, $p=0.1175$; Time: $F_{2,915,72.88}=0.1801$, $p=0.9050$; Interaction: $F_{9,75}=0.3695$, $p=0.9461$; **Supplemental Figure 2C**; Treatment: $F_{3,20}=1.939$, $p=0.1557$; Time: $F_{2,636,52.72}=0.3209$, $p=0.7846$; Interaction: $F_{9,60}=0.9353$, $p=0.5018$; **Supplemental Figure 2D**; Treatment: $F_{3,26}=1.290$, $p=0.2987$; Time: $F_{2,782,72.32}=0.7108$, $p=0.5386$; Interaction: $F_{9,78}=0.7765$, $p=0.6384$).

We also examined whether a structurally distinct CB1 receptor antagonist, Rimonabant (5 mg/kg, i.p.), would block the anti-allodynic effect of APAP in male mice with CFA-induced inflammatory pain. In this study, intraplantar CFA decreased paw withdrawal thresholds in the ipsilateral (**Supplemental Figure 3A**), but not in the contralateral paw (**Supplemental Figure 3B**). Rimonabant alone did not alter paw withdrawal thresholds of either the ipsilateral (data not shown, $F_{3,28}=1.300$, $p=0.2939$) or contralateral paw (data not shown, $F_{3,28}=1.327$, $p=0.2856$) compared to vehicle treatment. APAP elevated paw withdrawal thresholds in the CFA-injected paw (**Supplemental Figure 3A**; Treatment: $F_{3,28}=14.42$, $p<0.0001$; Time: $F_{2,811,78.81}=5.464$, $p=0.0023$; Interaction: $F_{9,84}=7.180$, $p<0.0001$) in comparison to control groups receiving either vehicle or Rimonabant alone (vs. Veh-Veh, $p=0.0001$, vs. Rimonab-Veh, $p<0.0001$). Rimonabant, given 30 minutes before APAP, attenuated the anti-allodynic effect of APAP (vs. Rimonab-APAP, $p<0.0001$). APAP effectively attenuated allodynia in CFA-injected male mice at 30- and 90-minutes post treatment (vs. Veh-Veh, 30 min: $p=0.0052$, 90 min: $p=0.0082$; vs. Rimonab-Veh, 30 min: $p=0.0025$, 90 min: $p=0.0004$). Combination treatment with Rimonabant and APAP

only transiently elevated paw withdrawal thresholds relative to rimonabant alone at 90 minutes post-injection (vs. Rimonab-Veh, $p=0.0031$), but thresholds did not differ in comparison to the vehicle group (vs Veh-Veh, $p>0.99$). Rimonabant attenuated the antiallodynic effect of APAP at 30- and 90-minutes post final injection (vs. Rimonab-APAP, 30' $p=0.0128$, 90' $p=0.0221$). None of the pharmacological treatments altered withdrawal thresholds in the contralateral paw (**Supplemental Figure 3B**; Treatment: $F_{3,28}=0.1319$, $p=0.9403$; Time: $F_{2,685,75.19}=1.310$, $p=0.2779$; Interaction: $F_{9,84}=1.349$, $p=0.2247$).

2.5. APAP attenuated mechanical allodynia in a model of incisional injury-induced post-surgical pain.

Incisional injury of the hind paw lowered paw withdrawal threshold of the ipsilateral paw of male and female mice, relative to baseline in all studies. No differences were detected in the paw withdrawal threshold of the contralateral paw to incisional injury.

Oral (p.o.) administration of APAP increased ipsilateral paw withdrawal thresholds overall and in a time-dependent manner and paw withdrawal thresholds also changed across time irrespective of drug treatment (**Figure 4A**: Treatment: $F_{2,60}=15.92$, $p<0.0001$; Time: $F_{3,60}=7.313$, $p=0.0003$; Interaction: $F_{6,60}=4.125$, $p=0.0016$). APAP at 100 and 300 mg/kg (p.o.) increased paw withdrawal thresholds in incisional-injury (ipsilateral) paw in male mice, in comparison to vehicle-treated group (Figure 4A). APAP elevated paw withdrawal thresholds at 30 (APAP 100 $p=0.0453$; APAP 300 $p=0.0259$) and 90 min post-injection (APAP 100 $p=0.0024$; APAP 300 $p=0.0003$), relative to the vehicle-treated group. No alterations in paw withdrawal thresholds were detected in the contralateral paw of male mice after pharmacological treatments (**Figure 4B**; Treatment: $F_{2,15}=0.0449$, $p=0.9561$; Time: $F_{2,193,32.90}=0.7018$, $p=0.5155$; Interaction: $F_{6,45}=0.8998$, $p=0.5034$).

2.6. RHC-80267 and DO34 attenuate the antiallodynic effect of APAP in a model of incisional injury-induced post-surgical pain.

In general, prior to pharmacological treatments, incisional injury lowered mechanical paw withdrawal thresholds of the ipsilateral hind paw, relative to baseline in male and female mice in all studies. APAP (300 mg/kg p.o.; Veh-APAP) increased paw withdrawal thresholds in male mice in the ipsilateral (injured) paw across the observation interval and in a time-dependent manner (**Figure 5A**; Treatment: $F_{3,20}=5.202$, $p=0.0081$; Time:

$F_{2,537,50.75}=6.549$, $p=0.0014$; Interaction: $F_{9,60}=3.210$, $p=0.0031$) in comparison to control groups given only vehicle (vs. Veh-Veh, $p<0.0001$) or RHC-80267 (vs. RHC-Veh, $p=0.0051$), consistent with anti-allodynic efficacy of APAP. Pre-treatment with RHC blocked the anti-allodynic effect of APAP in male mice with incisional injury (vs. RHC-APAP, $p=0.0082$). APAP (300 mg/kg, p.o.) preferentially increased paw withdrawal thresholds in the injured paw in comparison to control groups, at 90 minutes post injection (vs. Veh-Veh, $p=0.006$; vs. RHC-Veh, $p=0.0078$).

APAP (300 mg/kg, p.o.) also increased paw withdrawal thresholds in the injured paw of female mice (**Figure 5B**; Treatment: $F_{3,20}=10.07$, $p=0.0003$; Time: $F_{2,189,43.77}=2.145$, $p=0.1250$; Interaction: $F_{9,60}=1.421$, $p=0.1995$). APAP, administered after pretreatment with vehicle, increased paw withdrawal thresholds in the injured paw compared to female mice receiving only vehicle (vs. Veh-Veh, $p<0.0001$) or RHC-80267 (vs. RHC-Veh, $p<0.0001$). Pre-treatment with RHC-80267 blocked the anti-allodynic effect of APAP in female mice with incisional injury (vs. RHC-APAP, $p=0.0011$).

DO34 (30 mg/kg) pre-treatment attenuated the anti-allodynic effect of APAP in the incisional injury model (**Figure 5C**; Treatment: $F_{3,20}=8.708$, $p=0.0007$; Time: $F_{2,218,44.35}=6.206$, $p=0.0032$; Interaction: $F_{9,60}=3.387$, $p=0.0020$). Paw withdrawal thresholds were higher overall in the APAP (300 mg/kg, p.o.) group (Veh-APAP), in comparison to groups receiving either vehicle (vs. Veh-Veh, $p<0.0001$), DO34 alone (vs. DO34-Veh, $p<0.0001$) or DO34 prior to APAP (vs. DO34-APAP, $p<0.0001$). APAP elevated paw withdrawal thresholds at 30 and 90 minutes after injection in male mice with incisional pain, when compared to all groups that did not receive APAP (vs. Veh-Veh 30 min: $p=0.0122$, 90 min: $p=0.0214$; vs. DO34-Veh, 30 min: $p=0.0229$, 90 min: $p=0.0293$). DO34 maximally attenuated the therapeutic effect of APAP 30 minutes post treatment (vs. DO34-APAP, $p=0.0394$).

APAP (Veh-APAP) increased paw withdrawal thresholds in female mice with incisional injury across the observation interval and in a time-dependent manner (**Figure 5D**; Treatment: $F_{3,20}=8.442$, $p=0.0008$; Time: $F_{2,066,41.33}=6.883$, $p=0.0024$; Interaction: $F_{9,60}=4.388$, $p=0.0002$) compared to treatment with either vehicle or DO34 alone (Veh-Veh, $p<0.0001$; vs. DO34-Veh, $p<0.0001$). Pre-treatment with DO34 attenuated the anti-

allodynic effect of APAP in female mice with incisional pain (vs. DO34-APAP, $p=0.0003$). APAP treatment (Veh-APAP) increased paw thresholds at 30 and/or 90 minutes after injection in compared to groups that were not given APAP (vs. Veh-Veh 30 min: $p=0.0465$, 90 min: $p=0.0011$; vs. DO34-Veh 90 min: $p=0.0019$). DAGL inhibitor, DO34 (30 mg/kg, i.p.) effectively attenuated the anti-allodynic effect of APAP in female mice with incisional pain at 90 minutes post treatment with APAP (vs. DO34– APAP, $p=0.0018$)).

Neither the surgical procedure or any of the pharmacological treatments altered withdrawal thresholds in the paw contralateral to incisional injury in either male or female mice (**Supplementary Figure 4A**; Treatment: $F_{3,20}=0.1950$, $p=0.8985$; Time: $F_{2,851,57.02}=0.9214$, $p=0.4324$; Interaction: $F_{9,60}=1.189$, $p=0.3189$; **Supplementary Figure 4B**; Treatment: $F_{3,20}=1.272$, $p=0.3108$; Time: $F_{2,430,48.60}=2.609$, $p=0.0735$; Interaction: $F_{9,60}=0.2853$, $p=0.9763$; **Supplementary Figure 4C**; Treatment: $F_{3,20}=0.1931$, $p=0.8998$; Time: $F_{2,446,48.93}=1.354$, $p=0.2686$; Interaction: $F_{9,60}=1.633$, $p=0.1263$; **Supplementary Figure 4D**; Treatment: $F_{3,20}=0.7376$, $p=0.5419$; Time: $F_{2,936,58.73}=0.8237$, $p=0.4839$; Interaction: $F_{9,60}=1.710$, $p=0.1064$).

2.7. *Global CB1 receptor antagonist attenuates the anti-allodynic effect of APAP in a model of incisional injury-induced post-surgical pain in mice*

The CB1 receptor antagonist, AM251, attenuated the anti-allodynic effect of APAP in incisional injury-induced post-surgical pain across the observation interval and in a time-dependent manner (**Figure 6A**; Treatment: $F_{3,20}=9.131$, $p=0.0005$; Time: $F_{2,594,51.88}=1.678$, $p=0.1889$; Interaction: $F_{9,60}=4.293$, $p=0.0002$). APAP elevated paw withdrawal thresholds in the paw ipsilateral to incisional injury in male mice (**Figure 6A**), relative to control groups not treated with APAP (vs. Veh-Veh, $p<0.0001$; vs. AM251-Veh, $p<0.0001$). The CB1 antagonist, AM251, attenuated the anti-allodynic effect of APAP in male mice (vs. AM251-APAP, $p=0.0020$). APAP effectively attenuated incisional injury-induced mechanical hypersensitivity at 90 minutes following oral administration (p.o.) (vs. Veh-Veh, $p=0.0006$; vs. AM251-Veh, $p=0.0007$), and AM251 blocked this therapeutic effect (vs. AM251-ACAP, $p=0.0009$)

In female mice with incisional injury, APAP (Veh-APAP) increased paw withdrawal thresholds (**Figure 6B**; Treatment: $F_{3,20}=35.91$, $p<0.0001$; Time: $F_{2,614,52.28}=2.447$,

$p=0.0819$; Interaction: $F_{9,60}=1.208$, $p=0.3074$) overall relative to groups receiving either vehicle or AM251 alone (**Figure 6B**; Veh-Veh, $p<0.0001$; vs. AM251-Veh, $p<0.0001$). AM251 (5 mg/kg, i.p.) given 30 minutes before APAP treatment, attenuated the anti-allodynic of APAP in the injured paw (vs. AM251-APAP, $p=0.0005$). Paw withdrawal thresholds were lower in the group receiving combination treatment with AM251 (i.p.) and APAP (p.o.) than the group receiving AM251 alone through the course of study (vs. AM251-Veh, $p=0.0187$), but did not differ from the group receiving vehicle alone ($p=0.1583$). Neither surgery nor pharmacological treatments altered paw withdrawal thresholds in the paw contralateral to incisional injury in either male or female mice (**Supplemental Figure 5A**; Treatment: $F_{3,20}=1.342$, $p=0.2890$; Time: $F_{2,626,52.52}=0.8230$, $p=0.4733$; Interaction: $F_{9,60}=1.288$, $p=0.2626$; **Supplemental Figure 5B**; Treatment: $F_{3,20}=20.79$, $p=0.3736$; Time: $F_{2,524,50.49}=2.417$, $p=0.0869$; Interaction: $F_{9,60}=0.3178$, $p=0.9661$).

2.8. DAGL inhibitor RHC-80267 does not attenuate cannabimimetic effects of a high dose of APAP.

APAP (300 mg/kg, i.p.) (Veh-APAP) reduced body temperature in otherwise naïve male mice across the observation interval and in a time-dependent manner (**Figure 7A**, Treatment: $F_{3,25}=9.418$, $p=0.0002$; Time: $F_{2,112,52.81}=31.79$, $p<0.0001$; Interaction: $F_{9,75}=12.92$, $p<0.0001$); APAP reduced body temperature overall compared to groups receiving only vehicle or RHC-20267 (20 mg/kg, i.p.) (vs. Veh-Veh, $p<0.0001$; vs. RHC-Veh, $p<0.0001$). Pre-treatment with DAGL inhibitor, RHC-80267 (RHC- APAP), did not attenuate hypothermia in male mice (vs. Veh-Veh, $p<0.0001$; vs. RHC-Veh, $p<0.0001$; vs. Veh-APAP, $p<0.0001$). APAP decreased body temperature at 30- and 90-minutes post treatment, when compared to control groups that were not given APAP (vs. Veh-Veh, 30 min: $p<0.0001$, 90 min: $p<0.0001$; vs. RHC-Veh, 30 min: $p<0.0001$, 90 min: $p=0.0023$). Similarly, mice that received APAP following pre-treatment with the DAGL inhibitor, RHC-80267, showed reductions in body temperature at 30- and 90-minutes post treatment with APAP (vs. Veh-Veh, 30 min: $p<0.0001$, 90 min: $p<0.0001$; vs RHC-Veh, 30 min: $p<0.0001$, 90 min: $p=0.0113$), which did not differ from treatment with APAP alone (vs. RHC- APAP, $p>0.9999$). Treatment with APAP did not alter tail flick latencies in otherwise naïve male

mice (**Figure 7B**, Treatment: $F_{3,26}=1.268$, $p=0.3060$; Time: $F_{2,367,61.55}=1.553$, $p=0.2166$; Interaction: $F_{9,78}=0.9100$, $p=0.5211$).

Activity meter parameters (i.e., parameters at baseline) did not differ between experimental groups prior to pharmacological manipulations in any of the parameters ($p>0.05$ for each parameter). With respect to total distance traveled, a main effect of time and significant interaction between time and treatment were detected (**Figure 7C**, Treatment: $F_{3,25}=1.277$, $p=0.3038$; Time: $F_{1,25}=25.02$, $p<0.0001$; Interaction: $F_{3,25}=3.858$, $p=0.0214$). APAP decreased total distance traveled after drug treatment, when compared to baseline levels (Veh-APAP, $p=0.0001$; RHC-APAP, $p=0.0183$).

APAP treatment altered rest time in the activity meter overall in a time-dependent manner and rest time also differed across time irrespective of pharmacological manipulations (**Figure 7D**, Treatment: $F_{3,25}=5.032$, $p=0.0073$; Time: $F_{1,25}=152.7$, $p<0.0001$; Interaction: $F_{3,25}=19.87$, $p<0.0001$). APAP (Veh-APAP) increased rest time overall in comparison to Veh-Veh group ($p=0.0301$) and mice given RHC-APAP showed higher rest time compared to both control groups (vs. Veh-Veh, $p=0.0002$; vs. RHC-Veh, $p=0.0017$). Mice receiving Veh-APAP showed greater post-injection rest time compared to control conditions (vs. Veh-Veh, $p=0.0013$; vs. RHC-Veh, $p=0.0062$), irrespective of pre-treatment with RHC-80267 (RHC/APAP vs. Veh-Veh, $p<0.0001$; vs. RHC -Veh, $p=0.0002$). Comparison to baseline also indicated greater rest time in the APAP group irrespective of pre-treatment with RHC-80267 (Veh-APAP $p<0.0001$; RHC-APAP $p<0.0001$).

APAP also decreased vertical time in the activity meter overall and in a time-dependent manner (**Figure 7E**, Treatment: $F_{3,25}=5.571$, $p=0.0042$; Time: $F_{1,25}=81.61$, $p<0.0001$; Interaction: $F_{3,25}=8.639$, $p=0.0004$). Overall, treatment with Veh-APAP decreased vertical time compared to vehicle (Veh-APAP, $p=0.0017$). RHC-APAP treatment reduced vertical time in comparison to all control groups (vs Veh-Veh, $p=0.0002$; vs RHC-Veh $p=0.0258$) (**Figure 7E**). Specifically at post-drug time point, Veh-APAP lowered vertical time when compared to control groups that did not receive APAP (vs. Veh-Veh, $p=0.0002$; vs RHC-Veh, $p=0.0245$), irrespective of pre-treatment with RHC (RHC-APAP vs. Veh-Veh, $p<0.0001$, vs. RHC-Veh, $p=0.0098$). Treatment with Veh-APAP, RHC-APAP and RHC-

Veh reduced vertical time in comparison to respective baseline numbers (Veh-APAP $p < 0.0001$; RHC-APAP $p < 0.0001$; RHC-Veh $p < 0.0410$).

APAP also reduce center time in the activity meter post-injection, and center time also changed across time (**Figure 7F**, Treatment: $F_{3,25}=2.559$, $p=0.0777$; Time: $F_{1,25}=44.09$, $p < 0.0001$; Interaction: $F_{3,25}=5.062$, $p=0.0071$). Veh-APAP ($p=0.0190$) and RHC-APAP ($p=0.0023$) groups showed reduced center time compared to control Veh-Veh. Accordingly, these groups also showed less time in the center in comparison to their respective baseline numbers (Veh-APAP, $p < 0.0001$; RHC-APAP, $p < 0.0001$).

2.9. CB1 receptor antagonist, AM251, does not attenuate side effects of high dose APAP treatment in naïve mice.

The APAP-induced reduction of body temperature observed in otherwise naïve mice was not mediated by CB1 receptors (**Figure 8A**, Treatment: $F_{3,28}=18.37$, $p < 0.0001$; Time: $F_{2.577,72.15}=1.879$, $p=0.1488$; Interaction: $F_{9,84}=11.23$, $p < 0.0001$). APAP (300 mg/kg) decreased body temperature compared with groups treated with vehicle (vs. Veh-Veh, $p < 0.0001$) or AM251 alone (AM251-Veh, $p < 0.0001$), regardless of pre-treatment with CB1 receptor antagonist, AM251 (AM251-APAP vs Veh-Veh, $p < 0.0001$, vs. AM251-Veh, $p < 0.0001$) (**Figure 8A**). APAP lowered body temperature at 30- and 90- minutes post-injection, relative to control groups receiving vehicle or AM251 alone (vs. Veh-Veh, 30 min: $p=0.0018$, 90 min: $p < 0.0001$; vs. AM251-Veh, 30 min: $p=0.0113$, 90 min: $p < 0.0001$) or APAP following AM251 pre-treatment (vs. Veh-Veh, 30 min: $p < 0.0001$, 90 min: $p=0.0004$; vs. AM251-Veh, 30 min: $p=0.0003$, 90 min: $p=0.0022$). None of the pharmacological treatments altered tail-flick latencies in otherwise naïve mice (**Figure 8B**, Treatment: $F_{3,28}=0.6814$, $p=0.5708$; Time: $F_{2.522,70.62}=1.048$, $p=0.3686$; Interaction: $F_{9,84}=1.158$, $p=0.3322$).

APAP, with or without AM251 pretreatment, did not reduce total distance travelled in the activity meter overall and the interaction did not reach statistical significance (**Figure 8C**, Treatment: $F_{3,27}=2.687$, $p=0.0664$; Time: $F_{1,27}=24.34$, $p < 0.0001$; Interaction: $F_{3,27}=0.7625$, $p=0.5250$).

A hypolocomotion phenotype was evidenced by an APAP-induced increase in rest time in the activity meter (**Figure 8D**, Treatment: $F_{3,27}=4.366$, $p=0.0125$; Time: $F_{1,27}=82.63$, $p<0.0001$; Interaction: $F_{3,27}=7.727$, $p=0.0007$). APAP (Veh-APAP) increased rest time overall compared to control groups receiving Veh-Veh or Veh-AM251 (vs. Veh-Veh, $p=0.0142$, vs. AM251-Veh, $p=0.0045$), and rest time was elevated in groups receiving the combination of AM251-APAP, when compared to the AM251-Veh group ($p=0.0249$). APAP increased post-injection levels of rest time (vs. Veh-Veh, $p=0.0019$; vs AM251-Veh, $p=0.0013$), irrespective of AM251 pre-treatment (vs. Veh-Veh, $p=0.0023$, vs. AM251-Veh, $p=0.0016$). Accordingly, rest time was reduced compared to baseline numbers in all mice given APAP (Veh-APAP $p<0.0001$; AM251-APAP $p<0.0001$).

APAP also reduced vertical time in the activity meter post-injection (**Figure 8E**, Treatment: $F_{3,27}=2.550$, $p=0.0767$; Time: $F_{1,27}=75.76$, $p<0.0001$; Interaction: $F_{3,27}=6.037$, $p=0.0028$). Treatment with either Veh-APAP or AM251-APAP lowered vertical time compared to Veh-Veh or AM251-Veh (Veh-APAP vs. Veh-Veh $p=0.0229$, vs. AM251-Veh $p=0.0409$; AM251-APAP vs Veh-Veh $p=0.0283$, vs AM251-Veh $p=0.0499$). In comparison to pre-injection levels (baseline) of vertical time, APAP also reduced vertical time irrespective of pre-treatment with AM251 (Veh-APAP $p<0.0001$; AM251-APAP $p<0.0001$, **Figure 8E**).

APAP, administered alone or following AM251 pre-treatment, did not alter time spent in the center of the activity meter overall in comparison to vehicle or AM251 alone but the interaction between treatment and time was significant (**Figure 8F**, Treatment: $F_{3,27}=0.0356$, $p=0.9908$; Time: $F_{1,27}=40.01$, $p<0.0001$; Interaction: $F_{3,27}=3.045$, $p=0.0459$). APAP decreased time in the center of the apparatus when compared to their respective pre-injection (baseline) levels (Veh-APAP $p=0.0016$; AM251-APAP $p<0.0001$), but center time was similar between groups assessed after pharmacological manipulations. Baseline responding in the activity meter were not different between groups in this subset of experiments for any of the parameters mentioned above.

Lastly, APAP (100 mg/kg i.p.) did not alter body temperature (**Supplemental Figure 7A**, Treatment: $F_{1,56}=2.295$, $p=0.1354$; Time: $F_{3,56}=2.006$, $p=0.1236$; Interaction: $F_{3,56}=0.5643$, $p=0.6408$), tail-flick latency (**Supplemental Figure 7B**, Treatment: $F_{1,14}=0.1524$, $p=0.7022$; Time: $F_{1,977,2768}=0.1558$, $p=0.8542$; Interaction: $F_{3,42}=0.4211$, $p=0.7388$),

distance traveled, rest time, vertical time or center time in the activity meter apparatus (**Supplementary Figure 7C-F**; **7C**, Treatment: $F_{1,14}=0.5245$, $p=0.4808$; Time: $F_{1,14}=0.0570$, $p=0.8146$; Interaction: $F_{1,14}=0.0063$, $p=0.9376$); **7D**, Treatment: $F_{1,14}=0.0009$, $p=0.9761$; Time: $F_{1,14}=5.463$, $p=0.0348$; Interaction: $F_{1,14}=1.987$, $p=0.1804$; **7E**, Treatment: $F_{1,14}=0.6794$, $p=0.4236$; Time: $F_{1,14}=5.591$, $p=0.0330$; Interaction: $F_{1,14}=3.928$, $p=0.0675$; **7F**, Treatment: $F_{1,14}=0.0002$, $p=0.9877$; Time: $F_{1,14}=5.493$, $p=0.0344$; Interaction: $F_{1,14}=0.0550$, $p=0.8179$).

3. Discussion

Treatment with APAP has previously been shown to suppress pathological pain in different rodent models [5,22,23]. Here we show that APAP attenuates inflammatory and post-surgical pain in mice through a mechanism that requires both the enzyme DAGL and CB1 cannabinoid receptors. In our studies, the antinociceptive effect of APAP in these models was blocked by pre-treatment with DAGL inhibitors or global CB1 receptor antagonists. Importantly, these results were validated in two mechanistically distinct preclinical pain models, in both sexes, in two different strains of mice and using two different routes of administration (i.p. and p.o.).

APAP exerted a dose-dependent antinociceptive effect in male and female mice with CFA-induced inflammatory pain. Not surprisingly, APAP did not exert any effect on CFA-induced paw edema, since it does not possess a strong anti-inflammatory property [24]. In addition, throughout all experiments, we did not detect significant alterations in the mechanical threshold of the contralateral paw in either inflammatory or incisional injury models in mice, which suggests no alteration in the normal mechanical threshold in the absence of injury. Furthermore, APAP did not produce spinally-mediated antinociception in the tail-immersion test. By contrast, the analgesic efficacy of the high dose of APAP (300 mg/kg i.p.) was reliable throughout the course of testing in both the CFA model and the incisional injury and was also shown to be efficacious in both male and female mice in each model.

APAP (300 mg/kg i.p.) reduced body temperature and produced hypolocomotion, but these effects of APAP were not mediated by CB1 or DAGL in our studies. Previous work has reported important discrepancies in thermal (i.e. heat) responses after APAP

treatment. Rodent models of acute nociception show that APAP treatment can lead to increases in latency to paw or tail withdrawal in the hot plate and tail-flick tests, respectively [25,26], or no effect in thermal responses [27], which agrees with our findings. In fact, initial reports of the antinociceptive effects of APAP have mainly focused on acute *in vivo* models of nociception (e.g. hot plate test, paw pressure test) [6,28]. To reliably translate the therapeutic potential of APAP, testing in robust preclinical models of pathological pain is needed.

In our studies, systemic administration of APAP at 300 mg/kg (i.p. or p.o.) consistently attenuated CFA and incisional injury-induced mechanical allodynia in male and female mice of different strains. The antinociceptive effect of APAP and its pharmacological mechanisms seem to be classically linked to multiple targets within the endocannabinoid system. Our groups showed that the biosynthesis of 2-AG in the PAG is mediated by a DAGL α -dependent mechanism and suppresses stress-induced analgesia through a CB1 dependent mechanism [13]. Production and mobilization of endogenous ligands such as 2-AG can produce antinociception in models of pathological pain [13]. Importantly, inhibition of DAGL, the primary enzyme responsible for the synthesis of endocannabinoid ligand 2-AG, in the ventrolateral periaqueductal grey (vlPAG) abolished the analgesic action of APAP in rats submitted to the paw immersion test (acute thermal test) [12]. In the present study, pharmacological inhibition of DAGL using structurally distinct enzyme inhibitors (RHC-80267 or DO34), reliably attenuated the anti-allodynic effect of APAP in two different models of pathological pain and in both sexes in mice. This is the first report indicating the involvement of DAGL in the antinociceptive mechanism of APAP specifically in models of chronic pain (i.e., inflammatory (CFA) and post-surgical pain (incisional injury)).

Systemic administration of DAGL inhibitors RHC-80267 or DO34, in the absence of APAP, did not exert any antinociceptive effect in the inflammatory or post-surgical pain models at the doses used in the present studies. However, DAGL inhibitors, DO34 or KT109, administered systemically, attenuated mechanical allodynia in models of LPS-induced inflammatory pain [29,30], chronic constriction injury-induced neuropathic pain and chemotherapy-induced neuropathic pain [31]. These effects were abrogated in mice

lacking the enzyme DAGL β [29,31], but not DAGL α [29]. Further studies are necessary to determine whether the differences in the effect of DAGL inhibitors found in our study are due to the onset or severity of the pain models, polypharmacology of DAGL inhibitor or the dose of enzyme inhibitor used.

Administration of APAP leads to production of its active metabolite, AM404, which is known to modulate activity within the central nervous system. Moreover, pharmacokinetic analysis indicates that concentrations of AM404 in rat brain is similar to APAP and other metabolites when APAP is administered in human therapeutic doses (20 mg/kg) [32], whereas exposure to APAP at 300 mg/kg in rats leads to significant levels of AM404 in the brain, but not spinal cord, liver or blood. Strikingly, APAP was detected in significant concentrations in liver and blood, but not in the brain or spinal cord [7]. The PAG has previously been implicated in APAP-induced analgesia [33]. Administration of APAP into vPAG is not sufficient to modify thermal or mechanical thresholds in acute tests of nociception, but systemic injection of APAP or a vPAG injection of its metabolites can lead to antinociception, supporting the hypothesis that APAP needs to be metabolized to start engaging targets within the central nervous system [12]. AM404 is a known TRPV1 agonist and conversion of APAP into AM404 leads to activation of TRPV1 receptors within the brain, which is believed to be an important step to achieve the analgesic effect of APAP [8]. In addition, desensitization of TRPV1 receptors in the brain by intracerebroventricular injection of a potent TRPV1 agonist, resiniferatoxin, leads to failure in APAP-induced analgesia in resiniferatoxin-induced licking and biting behavior, formalin test and acute mechanical nociception [34]. However, TRPV1 blockade is not the only mechanism through which in vivo effects of APAP can be suppressed. The TRPV1 antagonist capsazepine did not block the antinociceptive effect of AM404, APAP active metabolite, in the formalin-induced nociception model [35]. Therefore, downstream targets of TRPV1 such as supraspinal calcium channels [36], metabotropic glutamate receptors and ultimately DAGL activation, mobilization of 2-AG and CB1 receptor activation, are likely to represent critical steps in the cascade that contributes to APAP's analgesic effects [13,25].

The endocannabinoid 2-AG is one of the main sources of arachidonic acid (AA) in the brain [37]. As previously mentioned, AM404 formation requires conjugation of p-aminophenol to AA [7]. Thus, manipulation of 2-AG metabolism (e.g. DAGL inhibition) could impact the formation of AM404. In addition, studies have indicated that AM404 can inhibit the uptake of 2-AG [38], similarly to what is observed with AEA. Increased availability of 2-AG can lead to TRPV1 and CB1 receptor activation, and in turn, analgesia [39]. Altogether, these observations suggest that APAP and its metabolite AM404 may exert their antinociceptive effect by interfering with the metabolism of 2-AG. Parallely, inhibition of DAGL, by RHC-80267 and DO34, indeed interfere with 2-AG metabolism and the formation of AM404, which may reduce the efficacy of APAP.

Here we also report that the antinociceptive effect of APAP in two distinct pain models was suppressed by CB1 receptor antagonists (AM251 and rimonabant) in male and female mice. Previous studies indicate that APAP-induced antinociception is dependent on CB1 receptor activation, as demonstrated by suppression of this effect after administration of receptor antagonists such as rimonabant, AM251 or AM281, but these initial reports have mainly relied on *in vivo* models of acute nociception [6]. Later studies have demonstrated the antinociceptive effect of APAP in models of pathological pain [5,25,40], which our findings validate further, using CFA-induced inflammatory pain and incisional injury in mice. However, the participation of CB1 receptors in the effect of APAP remains controversial. Even though global antagonists of the CB1 receptor effectively block the therapeutic effect of APAP, APAP's mechanism may not solely rely on CB1 receptors because the active metabolite of APAP, AM404, interacts with multiple targets [41]. The contribution of other targets engaged by AM404 to the antinociceptive effects of APAP should be further assessed in robust models of pathological pain.

APAP and its active metabolite AM404, exert antinociception that is linked to targets such as TRPV1, DAGL and CB1 receptors within brain regions such as the rostral ventromedial medulla (RVM). For instance, administration of rimonabant into the RVM suppresses the systemic antinociceptive effect of APAP, whereas intra-RVM administration of AM404 mimics the systemic antinociceptive effect of APAP. However, ablation of CB1 receptors into the RVM is not enough to abrogate the systemic antinociceptive effect of APAP [5].

Although APAP does not affect spinally mediated antinociception in the tail-flick test, in vitro, AM404 suppresses excitatory post-synaptic currents in the dorsal horn of the spinal cord [41]. Local injection of AM404 in the paw also reduced pain behavior in the formalin model [35]. These latter observations are consistent with our data which demonstrates that a peripherally restricted CB1 receptor antagonist, AM6545, did not attenuate the antinociceptive effect of APAP in inflammatory pain induced by CFA injection. Therefore, these observations reinforce a central mechanism for APAP and AM404.

In our study, APAP (300 mg/kg .p.) produced hypothermia in otherwise naïve male mice. This a phenomenon that occurs in a dose dependent manner, through different routes of administration, as previously demonstrated [8,25,26]. Although APAP's antipyretic mechanism remains unclear, APAP is reported to exert thermoregulatory properties through inhibition of cyclooxygenase, leading to reduced production of pyrogenic prostaglandins in the hypothalamus and reduction of fever (for review see [42]). This is a mechanism that can be dissociated from direct CB1 receptor activation, which also leads to hypothermia [26]. In fact, other studies have reported that neither CB1, nor opioid receptors mediate the thermoregulatory properties of APAP [43]. In the present study, systemic treatment with CB1 receptor antagonist AM251 did not attenuate APAP-induced hypothermia in naïve male mice. Additionally, in otherwise naïve mice, systemic inhibition of the enzyme DAGL, by injection of RHC-80267, did not attenuate APAP-induced hypothermia in our studies and treatment with DAGL inhibitor alone did not exert an effect in body temperature. Conversely, treatment with DO34, another DAGL inhibitor, has been shown to significantly attenuate lipopolysaccharide (LPS)-induced anapyrexia in mice, an effect that was mitigated in mice lacking the enzyme DAGL α [10]. Therefore, more studies are necessary to investigate the effects of APAP associated with DAGL inhibitors in animal models of anapyrexia.

Our data show that mice treated with a high dose of APAP (300 mg/kg), regardless of pre-treatment with DAGL inhibitor or CB1 receptor antagonist, exhibited a reduction of locomotor activity that was evidenced by increased rest time (period of inactivity), decreased vertical time (less time in rearing position) and decreased center time (time spent in the center of the apparatus), as well as total distance travelled in the apparatus.

However, these effects were not observed with a lower dose of APAP (100 mg/kg), that nonetheless was efficacious in the CFA and incisional injury models. The mechanisms by which APAP produces sedation are not well elucidated. Classical cannabinoid ligands exert tetrad effect in rodents, which includes hypothermia, reduced locomotor activity, analgesia and catalepsy, but not all these effects are observed after APAP treatment (lack of catalepsy), as previously demonstrated [25]. Conversely, the active metabolite AM404, injected systemically or intracerebroventricularly, did not alter locomotor activity, emotional behavior or catalepsy in rodents that were otherwise naïve or used in a neuropathic pain model [44,45], and ameliorated motor and sensorimotor deficits in parkinsonian rats [46]. These therapeutic effects are conflicting, given that other studies found that AM404 can dose dependently induce hypolocomotion [47], as well as promote conditioned place preference (CPP) and reverse drug-induced CPP in rats (e.g. nicotine) [48]. Furthermore, CB1 receptors do not seem to be involved in the motor impairment induced by APAP [29–31], a conclusion supported by our studies. Thus, it cannot be concluded that APAP's side effects are cannabimimetic, and our overall results suggest a differential mechanism between analgesic and side effect profiles of APAP.

In conclusion, our results demonstrate that APAP shows efficacy in reducing mechanical hypersensitivity in two different pathological models of pain and in both sexes in mice. Our data contributes to the elucidation of the pharmacological mechanism of APAP, suggesting that the analgesic effects of APAP are dependent not only on activation of CB1 receptors, but also the enzyme DAGL. The present findings also indicate that APAP exerts its therapeutic effects through the endogenous cannabinoid system, whereas adverse side effects (hypothermia, hypolocomotion) of APAP are mediated through a different mechanism.

Declaration of interest.

None

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REFERENCES

1. Freo U, Ruocco C, Valerio A, Scagnol I, Nisoli E, Murphy L. Clinical Medicine Paracetamol: A Review of Guideline Recommendations. *J Clin Med*. Published online 2021. doi:10.3390/jcm10153420
2. Donvito G, Nass SR, Wilkerson JL, et al. The Endogenous Cannabinoid System: A Budding Source of Targets for Treating Inflammatory and Neuropathic Pain. *Neuropsychopharmacology* 2018 43:1. 2017;43(1):52-79. doi:10.1038/npp.2017.204
3. Slivicki RA, Saberi SA, Iyer V, Vemuri VK, Makriyannis A, Hohmann AG. Brain-Permeant and -Impermeant Inhibitors of Fatty Acid Amide Hydrolase Synergize with the Opioid Analgesic Morphine to Suppress Chemotherapy-Induced Neuropathic Nociception Without Enhancing Effects of Morphine on Gastrointestinal Transit. *J Pharmacol Exp Ther*. 2018;367(3):551. doi:10.1124/JPET.118.252288
4. Przybyła GW, Szychowski KA, Gmiński J. Paracetamol – An old drug with new mechanisms of action. *Clin Exp Pharmacol Physiol*. 2021;48(1):3-19. doi:10.1111/1440-1681.13392
5. Klinger-Gratz PP, Ralvenius WT, Neumann E, et al. Acetaminophen Relieves Inflammatory Pain through CB1 Cannabinoid Receptors in the Rostral Ventromedial Medulla. *J Neurosci*. 2018;38(2):322-334. doi:10.1523/JNEUROSCI.1945-17.2017
6. Ottani A, Leone S, Sandrini M, Ferrari A, Bertolini A. The analgesic activity of paracetamol is prevented by the blockade of cannabinoid CB1 receptors. *Eur J Pharmacol*. 2006;531(1-3):280-281. doi:10.1016/J.EJPHAR.2005.12.015
7. Högestätt ED, Jönsson BAG, Ermund A, et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. *J Biol Chem*. 2005;280(36):31405-31412. doi:10.1074/JBC.M501489200
8. Mallet C, Barrière DA, Ermund A, et al. TRPV1 in Brain Is Involved in Acetaminophen-Induced Antinociception. *PLoS One*. 2010;5(9):e12748. doi:10.1371/JOURNAL.PONE.0012748
9. Roberts LA, Christie MJ, Connor M. Anandamide is a partial agonist at native vanilloid receptors in acutely isolated mouse trigeminal sensory neurons. *Br J Pharmacol*. 2002;137(4):421. doi:10.1038/SJ.BJP.0704904
10. Ogasawara D, Deng H, Viader A, et al. Rapid and profound rewiring of brain lipid signaling networks by acute diacylglycerol lipase inhibition. *Proc Natl Acad Sci U*

- S A. 2016;113(1):26-33. doi:10.1073/PNAS.1522364112/-/DCSUPPLEMENTAL/PNAS.1522364112.SD02.XLSX
11. Bühner C, Endesfelder S, Scheuer T, Schmitz T. Paracetamol (Acetaminophen) and the Developing Brain. *International Journal of Molecular Sciences* 2021, Vol 22, Page 11156. 2021;22(20):11156. doi:10.3390/IJMS22011156
12. Barrière DA, Boumezbeur F, Dalmann R, et al. Paracetamol is a centrally acting analgesic using mechanisms located in the periaqueductal grey. *Br J Pharmacol.* 2020;177(8):1773. doi:10.1111/BPH.14934
13. Gregg LC, Jung KM, Spradley JM, et al. Activation of type 5 metabotropic glutamate receptors and diacylglycerol lipase- α initiates 2-arachidonoylglycerol formation and endocannabinoid-mediated analgesia. *J Neurosci.* 2012;32(28):9457-9468. doi:10.1523/JNEUROSCI.0013-12.2012
14. Khasabova IA, Chandiramani A, Harding-Rose C, Simone DA, Seybold VS. Increasing 2-arachidonoyl glycerol signaling in the periphery attenuates mechanical hyperalgesia in a model of bone cancer pain. *Pharmacological research : the official journal of the Italian Pharmacological Society.* 2011;64(1):60. doi:10.1016/J.PHRS.2011.03.007
15. Kandasamy R, Morgan MM. "Reinventing the wheel" to advance the development of pain therapeutics. *Behavioural pharmacology.* 2021;32(2-#x000263):142. doi:10.1097/FBP.0000000000000596
16. Li AL, Lin X, Dhopeswarkar AS, et al. Cannabinoid CB2 Agonist AM1710 Differentially Suppresses Distinct Pathological Pain States and Attenuates Morphine Tolerance and Withdrawal. *Mol Pharmacol.* 2019;95(2):155-168. doi:10.1124/MOL.118.113233
17. Pogatzki EM, Raja SN. A Mouse Model of Incisional Pain. *Anesthesiology.* 2003;99(4):1023-1027. doi:10.1097/00000542-200310000-00041
18. Griffioen MA, Dernetz VH, Yang GS, Griffith KA, Dorsey SG, Renn CL. Evaluation of Dynamic Weight Bearing for Measuring Nonevoked Inflammatory Hyperalgesia in Mice. *Nurs Res.* 2015;64(2):81. doi:10.1097/NNR.0000000000000082
19. Iyer V, Saberi SA, Pacheco R, et al. Negative allosteric modulation of CB1 cannabinoid receptor signaling suppresses opioid-mediated tolerance and withdrawal without blocking opioid antinociception. *Neuropharmacology.* 2024;257:110052. doi:10.1016/J.NEUROPHARM.2024.110052
20. Deng L, Guindon J, Cornett BL, Makriyannis A, Mackie K, Hohmann AG. Chronic cannabinoid CB2 activation reverses paclitaxel neuropathy without tolerance or CB1-dependent withdrawal. *Biol Psychiatry.* 2014;77(5):475. doi:10.1016/J.BIOPSYCH.2014.04.009

21. Lee WH, Xu Z, Ashpole NM, et al. Small molecule inhibitors of PSD95-nNOS protein-protein interactions as novel analgesics. *Neuropharmacology*. 2015;97:464-475. doi:10.1016/J.NEUROPHARM.2015.05.038
22. Cabañero D, Maldonado R. Synergism between oral paracetamol and nefopam in a murine model of postoperative pain. *Eur J Pain*. 2021;25(8):1770-1787. doi:10.1002/EJP.1787
23. Yun M, Regen ND, Anchondo Y, et al. Acetaminophen effects upon formalin-evoked flinching, postformalin, and postincisional allodynia and conditioned place preference. *Pain Rep*. 2024;9(5):e1168. doi:10.1097/PR9.0000000000001168
24. Hanel AM, Lands WEM. Modification of anti-inflammatory drug effectiveness by ambient lipid peroxides. *Biochem Pharmacol*. 1982;31(20):3307-3311. doi:10.1016/0006-2952(82)90565-2
25. Mallet C, Daulhac L, Bonnefont J, et al. Endocannabinoid and serotonergic systems are needed for acetaminophen-induced analgesia. *Pain*. 2008;139(1):190-200. doi:10.1016/J.PAIN.2008.03.030
26. Gentry C, Andersson DA, Bevan S. TRPA1 mediates the hypothermic action of acetaminophen. *Scientific Reports* 2015 5:1. 2015;5(1):1-8. doi:10.1038/srep12771
27. Li Q, Zhuang Q, Gu Y, et al. Enhanced analgesic effects of nefopam in combination with acetaminophen in rodents. *Biomed Rep*. 2017;8(2):176. doi:10.3892/BR.2017.1032
28. Courade JP, Chassaing C, Bardin L, Alloui A, Eschaliere A. 5-HT receptor subtypes involved in the spinal antinociceptive effect of acetaminophen in rats. *Eur J Pharmacol*. 2001;432(1):1-7. doi:10.1016/S0014-2999(01)01464-9
29. Wilkerson JL, Donvito G, Grim TW, et al. Investigation of Diacylglycerol Lipase Alpha Inhibition in the Mouse Lipopolysaccharide Inflammatory Pain Model. *J Pharmacol Exp Ther*. 2017;363(3):394-401. doi:10.1124/JPET.117.243808
30. Shin M, Snyder HW, Donvito G, et al. Liposomal Delivery of Diacylglycerol Lipase-Beta Inhibitors to Macrophages Dramatically Enhances Selectivity and Efficacy in Vivo. *Mol Pharm*. 2018;15(3):721-728. doi:10.1021/ACS.MOLPHARMACEUT.7B00657
31. Wilkerson JL, Ghosh S, Bagdas D, et al. Diacylglycerol lipase β inhibition reverses nociceptive behaviour in mouse models of inflammatory and neuropathic pain. *Br J Pharmacol*. 2016;173(10):1678. doi:10.1111/BPH.13469
32. Muramatsu S, Shiraishi S, Miyano K, et al. Metabolism of AM404 From Acetaminophen at Human Therapeutic Dosages in the Rat Brain. *Anesth Pain Med*. 2016;6(1):32873. doi:10.5812/AAPM.32873

33. Bazan H, Bhattacharjee S, Reid M, et al. Transcriptomic signature, bioactivity and safety of a non-hepatotoxic analgesic generating AM404 in the mid-brain PAG region. *Res Sq*. Published online May 4, 2023. doi:10.21203/RS.3.RS-2883310/V1
34. Fukushima A, Mamada K, Iimura A, Ono H. Supraspinal-selective TRPV1 desensitization induced by intracerebroventricular treatment with resiniferatoxin. *Scientific Reports* 2017 7:1. 2017;7(1):1-9. doi:10.1038/s41598-017-12717-5
35. Schreiber AK, Neufeld M, Jesus CHA, Cunha JM. Peripheral antinociceptive effect of anandamide and drugs that affect the endocannabinoid system on the formalin test in normal and streptozotocin-diabetic rats. *Neuropharmacology*. 2012;63(8):1286-1297. doi:10.1016/J.NEUROPHARM.2012.08.009
36. Kerckhove N, Mallet C, François A, et al. Ca(v)3.2 calcium channels: the key protagonist in the supraspinal effect of paracetamol. *Pain*. 2014;155(4):764-772. doi:10.1016/J.PAIN.2014.01.015
37. Chen C. Inhibiting degradation of 2-arachidonoylglycerol as a therapeutic strategy for neurodegenerative diseases. *Pharmacol Ther*. 2023;244:108394. doi:10.1016/J.PHARMTHERA.2023.108394
38. Bisogno T, Maccarrone M, De Petrocellis L, et al. The uptake by cells of 2-arachidonoylglycerol, an endogenous agonist of cannabinoid receptors. *Eur J Biochem*. 2001;268(7):1982-1989. doi:10.1046/J.1432-1327.2001.02072.X
39. Jiang M, Huizenga MCW, Wirt JL, et al. A monoacylglycerol lipase inhibitor showing therapeutic efficacy in mice without central side effects or dependence. *Nature Communications* 2023 14:1. 2023;14(1):1-19. doi:10.1038/s41467-023-43606-3
40. Hama AT, Sagen J. Cannabinoid receptor-mediated antinociception with acetaminophen drug combinations in rats with neuropathic spinal cord injury pain. *Neuropharmacology*. 2010;58(4-5):758-766. doi:10.1016/J.NEUROPHARM.2009.12.010
41. Ohashi N, Uta D, Sasaki M, Ohashi M, Kamiya Y, Kohno T. Acetaminophen metabolite n-acylphenolamine induces analgesia via transient receptor potential vanilloid 1 receptors expressed on the primary afferent terminals of c-fibers in the spinal dorsal horn. *Anesthesiology*. 2017;127(2):355-371. doi:10.1097/ALN.0000000000001700
42. Ayoub SS. Paracetamol (acetaminophen): A familiar drug with an unexplained mechanism of action. *Temperature*. 2021;8(4):351-371. doi:10.1080/23328940.2021.1886392/ASSET/5A2FB576-4CA5-4103-92A9-67BC67E4CB3C/ASSETS/IMAGES/KTMP_A_1886392_F0009_OC.JPG

43. Corley G, Rawls SM. Opioid, cannabinoid CB1 and NOP receptors do not mediate APAP-induced hypothermia in rats. *Pharmacol Biochem Behav.* 2009;92(3):503-507. doi:10.1016/J.PBB.2009.01.020
44. Campolongo P, Ratano P, Manduca A, et al. The endocannabinoid transport inhibitor AM404 differentially modulates recognition memory in rats depending on environmental aversiveness. *Front Behav Neurosci.* 2012;6(FEBRUARY 2012):11. doi:10.3389/FNBEH.2012.00011
45. Beltramo M, Rodríguez De Fonseca F, Navarro M, et al. Reversal of Dopamine D2 Receptor Responses by an Anandamide Transport Inhibitor. *Journal of Neuroscience.* 2000;20(9):3401-3407. doi:10.1523/JNEUROSCI.20-09-03401.2000
46. Fernandez-Espejo E, Caraballo I, Rodriguez De Fonseca F, et al. Experimental Parkinsonism Alters Anandamide Precursor Synthesis, and Functional Deficits are Improved by AM404: A Modulator of Endocannabinoid Function. *Neuropsychopharmacology* 2004 29:6. 2004;29(6):1134-1142. doi:10.1038/sj.npp.1300407
47. Cippitelli A, Bilbao A, Gorriti MA, et al. The anandamide transport inhibitor AM404 reduces ethanol self-administration. *Eur J Neurosci.* 2007;26(2):476-486. doi:10.1111/J.1460-9568.2007.05665.X
48. Scherma M, Justinová Z, Zanettini C, et al. The anandamide transport inhibitor AM404 reduces the rewarding effects of nicotine and nicotine-induced dopamine elevations in the nucleus accumbens shell in rats. *Br J Pharmacol.* 2012;165(8):2539-2548. doi:10.1111/J.1476-5381.2011.01467.X

Figure Legends

Figure 1. APAP dose-dependently suppresses CFA-induced mechanical hypersensitivity without altering paw edema in mice. APAP elevated mechanical paw withdrawal threshold in the (A) CFA-injected (ipsilateral) paw at all doses (30, 100 and 300 mg/kg, i.p.) without altering responsiveness in the (B) contralateral paw in male mice. APAP did not alter thickness of the paw (C) ipsilateral or (D) contralateral to CFA injection in male mice. In female mice, APAP increased mechanical paw withdrawal thresholds in the (E) CFA-injected (ipsilateral) paw at the doses of 30 and 100 mg/kg, i.p. without altering responsiveness in the (F) contralateral paw (n = 5-7 per group). Data show mean ($\pm$ SEM) for ipsilateral and contralateral paws. Two-way ANOVA followed by Bonferroni's *post hoc* test. Interaction between time points and treatment effect: ** $P < 0.01$, *** $P < 0.001$ Vehicle vs. APAP 300; # $P < 0.05$ Vehicle vs. APAP 100; ++ $P < 0.01$ Vehicle vs. APAP 30; ^ $P < 0.05$ APAP 30 VS. APAP 300. Brackets (main treatment effect only): **** $P < 0.0001$ Vehicle vs. APAP 300; ## $P < 0.01$ Vehicle vs. APAP 100; ++ $P < 0.01$ Vehicle vs. APAP 30; ^ $P < 0.05$ APAP 30 VS. APAP 300. & $P < 0.05$ APAP 100 vs. APAP 300.

Figure 2. Anti-allodynic effects of APAP are prevented by pharmacological inhibitors of DAGL in a mouse model of inflammatory pain. DAGL inhibitor RHC-80267 (20 mg/kg, i.p.) attenuates the anti-allodynic effect of APAP (300 mg/kg, i.p.) in the CFA-injected (ipsilateral) paw of (A) male and (B) female mice. DAGL inhibitor DO34 (30 mg/kg, i.p.) attenuates the anti-allodynic effect of APAP (300 mg/kg, i.p.) in the CFA-injected (ipsilateral) paw of (C) male and (D) female mice. (n = 5-7 per group). Data show mean ($\pm$ SEM). Two-way ANOVA followed by Bonferroni's *post hoc* test. Interaction between time points and treatment effect: ** $P < 0.01$, *** $P < 0.001$ Veh-Veh vs. Veh-APAP; # $P < 0.05$ Veh-APAP vs. RHC-APA or Veh-APAP vs. DO34-APAP; ++ $P < 0.01$ RHC-Veh vs. Veh-APAP or DO34-Veh vs. Veh-APAP. Brackets (main treatment effect only): **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; ## $P < 0.01$, ### $P < 0.001$ Veh-APAP vs. RHC-APAP or Veh-APAP vs. DO34-APAP; +++++ $P < 0.0001$ RHC-Veh vs. Veh-APAP or DO34-Veh vs. Veh-APAP.

Figure 3. CB1 receptor antagonist AM251 (5 mg/kg, i.p.) (A, B), but not AM6545 (10 mg/kg, i.p.) (C, D) suppress the anti-allodynic effect of APAP (300 mg/kg, i.p.) evaluated

in the ipsilateral paw of male (**A**, **C**) and female (**B**, **D**) mice with CFA-induced inflammatory pain (n=6-9 per group). Data show mean ($\pm$ SEM). Two-way ANOVA followed by Bonferroni's post hoc test. Interaction between time points and treatment effect: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; # $P < 0.05$, ## $P < 0.01$, Veh-APAP vs. AM251-APAP or Veh-APAP vs. AM6545-APAP; + $P < 0.05$, +++ $P < 0.001$ AM251-Veh vs. Veh-APAP or AM6545-Veh vs. Veh-APAP; & $P < 0.05$, && $P < 0.001$ Veh-Veh vs. AM6545-APAP. Brackets (main treatment effect only): ** $P < 0.01$, **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ Veh-APAP vs. AM251-APAP; +++++ $P < 0.0001$ AM251-Veh vs. Veh-APAP; ^ $P < 0.05$ Veh-Veh vs. AM251-Veh; && $P < 0.01$, &&& $P < 0.0001$ Veh-Veh vs. AM6545-APAP; \$\$\$ $P < 0.001$ AM6545-Veh vs. AM6545-APAP. ^ $P < 0.05$ Veh-Veh vs. AM6545-Veh.

Figure 4. APAP suppresses mechanical hypersensitivity induced by incisional injury in the paw (A) ipsilateral but not (B) contralateral to injury. APAP elevated mechanical paw withdrawal threshold in the (**A**) ipsilateral paw (injured paw) at all doses (100 and 300 mg/kg, p.o.) without altering responsiveness in the (**B**) contralateral paw in male mice (n=6 per group). Data show mean ($\pm$ SEM) for ipsilateral and contralateral paws. Two-way ANOVA followed by Bonferroni's *post hoc* test. Interaction between time points and treatment effect: * $P < 0.05$, *** $P < 0.001$ Vehicle vs. APAP 300; # $P < 0.05$, ## $P < 0.01$ Vehicle vs. APAP 100. Brackets (main treatment effect only): *** $P < 0.001$ Vehicle vs. APAP 300; ##### $P < 0.0001$ Vehicle vs. APAP 100.

Figure 5. Anti-allodynic effects of APAP are prevented by pharmacological inhibitors of DAGL in a mouse model of post-surgical pain. DAGL inhibitor RHC-80267 (20 mg/kg, i.p.) suppresses the anti-allodynic effect of APAP (300 mg/kg, p.o) in the ipsilateral paw of (**A**) male and (**B**) female mice with incisional injury (n=6 per group). DAGL inhibitor DO34 (30 mg/kg, i.p.) suppresses the anti-allodynic effect of APAP (300 mg/kg, p.o) in the ipsilateral paw of (**C**) male and (**D**) female mice with incisional injury (n=6 per group). Data show mean ($\pm$ SEM). Two-way ANOVA followed by Bonferroni's post hoc test. Interaction between time points and treatment effect: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ Veh-Veh vs. Veh-APAP; # $P < 0.05$, ## $P < 0.01$ Veh-APAP vs. RHC-APAP or Veh-APAP vs. DO34-APAP; + $P < 0.05$, ++ $P < 0.01$ RHC-Veh vs. Veh-APAP or DO34-Veh vs. Veh-APAP.

*Brackets (main treatment effect only): **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ Veh-APAP vs. RHC-APAP or Veh-APAP vs. DO34-APAP; ++ $P < 0.01$, +++ $P < 0.0001$ RHC-Veh vs. Veh-APAP or DO34-Veh vs. Veh-APAP.*

Figure 6. CB1 receptor antagonist AM251 (5 mg/kg, i.p.) suppresses the anti-allodynic effect of APAP (300 mg/kg, p.o.) evaluated in the ipsilateral paw of male (A) and female mice (B) with incisional injury (n=6 per group). Data show mean ($\pm$ SEM). Two-way ANOVA followed by Bonferroni's post hoc test. Interaction between time points and treatment effect: *** $P < 0.001$ Veh-Veh vs. Veh-APAP; ### $P < 0.001$, Veh-APAP vs. AM251-APAP; +++ $P < 0.001$ AM251-Veh vs. Veh-APAP. *Brackets (main treatment effect only): **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; ## $P < 0.01$, ### $P < 0.001$ Veh-APAP vs. AM251-APAP; +++ $P < 0.0001$ AM251-Veh vs. Veh-APAP; & $p < 0.05$ AM251-Veh vs. AM251-APAP.*

Figure 7. APAP (300 mg/kg, i.p.) produces hypothermia and hypolocomotion, but not tail flick antinociception in naïve male mice. The DAGL inhibitor RHC-80267 (20 mg/kg) does not block the side effects induced by APAP. (A) Body temperature of naïve male mice after treatment with Veh-Veh, Veh-APAP, RHC80267-Veh or RHC80267-APAP. (B) (C-F) Activity meter data reflecting locomotion (center time, rest time, vertical time, and total distance) 30 minutes after pharmacological manipulations (n=7-8 per group). Data show mean ($\pm$ SEM). Two-way ANOVA followed by Bonferroni's post hoc test. Interaction between time points and treatment effect (Panels A and B): **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; ++ $P < 0.01$, +++ $P < 0.0001$ RHC-Veh vs. Veh-APAP; && $P < 0.01$, &&&& $P < 0.0001$ Veh-Veh vs. RHC-APAP; \$ $P < 0.05$, \$\$\$ $P < 0.0001$ RHC-Veh vs. RHC-APAP. *Brackets (main treatment effect only, Panel A-F): * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; +++ $P < 0.0001$ RHC-Veh vs. Veh-APAP; & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$, &&&& $P < 0.0001$ Veh-Veh vs. RHC-APAP; \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.0001$ RHC-Veh vs. RHC-APAP. Lines between bars (interaction treatment and time, Panel C-F): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ group difference. Symbols on top of group bars at baseline (Panel C-F): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ difference from same group at drug administration time point.*

Figure 8. The CB1 receptor antagonist (5 mg/kg, i.p.) does not block the side effects induced by APAP (300 mg/kg, i.p.). **(A)** Body temperature of naïve male mice after treatment with APAP. **(B)** Tail flick latency after treatment with APAP. **(C-F)** Activity meter data reflecting locomotion (center time, rest time, vertical time, and total distance) 30 minutes after treatment with APAP (n=7-8 per group). Data show mean ($\pm$ SEM). Two-way ANOVA followed by Bonferroni's post hoc test. Interaction between time points and treatment effect (Panels A and B): ** $P < 0.01$, **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; + $P < 0.05$, ++++ $P < 0.0001$ AM251-Veh vs Veh-APAP; &&& $P < 0.001$, &&&& $P < 0.0001$ Veh-Veh vs. AM251-APAP; \$\$ $P < 0.01$, \$\$\$ $P < 0.001$ AM251-Veh vs. AM251-APAP. Brackets (main treatment effect only, Panel A-F): * $P < 0.05$, **** $P < 0.0001$ Veh-Veh vs. Veh-APAP; ++ $P < 0.01$, ++++ $P < 0.0001$ AM251-Veh vs. Veh-APAP; & $P < 0.05$, &&&& $P < 0.0001$ Veh-Veh vs. AM251-APAP; \$ $P < 0.05$, \$\$\$\$ $P < 0.0001$ AM251-Veh vs. AM251-APAP. Lines between bars (interaction treatment and time, Panel C-F): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ group difference. Symbols on top of group bars at baseline (Panel C-F): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ difference from same group at drug administration time point.

Supplemental Figure 1. DAGL inhibitor RHC-80267 (20 mg/kg, i.p.) (**A, B**) or DO34 (30 mg/kg, i.p.) (**C, D**) and APAP (300 mg/kg, i.p.) do not alter mechanical paw withdrawal thresholds evaluated in the contralateral paw of male and female mice with CFA-induced inflammatory pain (n= 5-7 per group). Data show mean ($\pm$ SEM) for contralateral paws. Two-way ANOVA followed by Bonferroni's post hoc test.

Supplemental Figure 2. AM251 (5 mg/kg, i.p.) (**A, B**) or AM6545 (10 mg/kg, i.p.) (**C, D**), and APAP (300 mg/kg, i.p.) do not alter mechanical threshold evaluated in the contralateral paw of male and female mice with CFA-induced inflammatory pain (n= 6-9 per group). Data show mean ($\pm$ SEM) for contralateral paws. Two-way ANOVA followed by Bonferroni's post hoc test.

Supplemental Figure 3. CB1 receptor antagonist Rimonabant (5 mg/kg, i.p.) suppresses the anti-allodynic effect of APAP (300 mg/kg, i.p.) evaluated in the ipsilateral paw of male mice with CFA-induced inflammatory pain (**A**). No alterations were detected in the mechanical threshold of the contralateral paw in the same mice (**B**) (n=7-9 per group). Data show mean ($\pm$ SEM). Two-way ANOVA followed by Bonferroni's post hoc test. Interaction between time points and treatment effect: ** $P < 0.01$, Veh-Veh vs. Veh-APAP; # $P < 0.05$, Veh-APAP vs. Rimonab-APAP; ++ $P < 0.01$, +++ $P < 0.001$ Rimonab-Veh vs. Veh-APAP; && $p < 0.01$ Rimonab-Veh vs. Rimonab-APAP. Brackets (main treatment effect only): *** $P < 0.001$ Veh-Veh vs. Veh-APAP; ##### $P < 0.0001$ Veh-APAP vs. Rimonab-APAP; +++++ $P < 0.0001$ Rimonab-Veh vs. Veh-APAP.

Supplemental Figure 4. DAGL inhibitors RHC-80267 (20 mg/kg, i.p.) (**A, B**) or DO34 (30 mg/kg, i.p.) (**C, D**), and APAP (300 mg/kg, p.o.) do not alter mechanical paw withdrawal thresholds evaluated in the contralateral paw of male and female mice with incisional injury (n=6 per group). Data show mean ($\pm$ SEM) for contralateral paws. Two-way ANOVA followed by Bonferroni's post hoc test.

Supplemental Figure 5. AM251 (5 mg/kg, i.p.) and APAP (300 mg/kg, p.o.) do not alter mechanical paw withdrawal thresholds evaluated in the contralateral paw of male (**A**) and female mice (**B**) with incisional injury (n= 6 per group). Data show mean ($\pm$ SEM) for contralateral paws. Two-way ANOVA followed by Bonferroni's post hoc test.

Supplemental Figure 6. APAP (100 mg/kg, i.p.) does not produce hypothermia, hypolocomotion or tail flick antinociception in naïve male mice. **(A)** Body temperature of naïve male mice after treatment with APAP 100 mg/kg. **(B)** Tail flick latency after treatment with APAP 100 mg/kg. **(C-F)** Activity meter data reflecting locomotion (center time, rest time, vertical time, and total distance) 30 minutes after treatment with APAP 100 mg/kg (n=8 per group). Data show mean ($\pm$ SEM). Two-way ANOVA followed by Bonferroni's post hoc test.

Figure 1

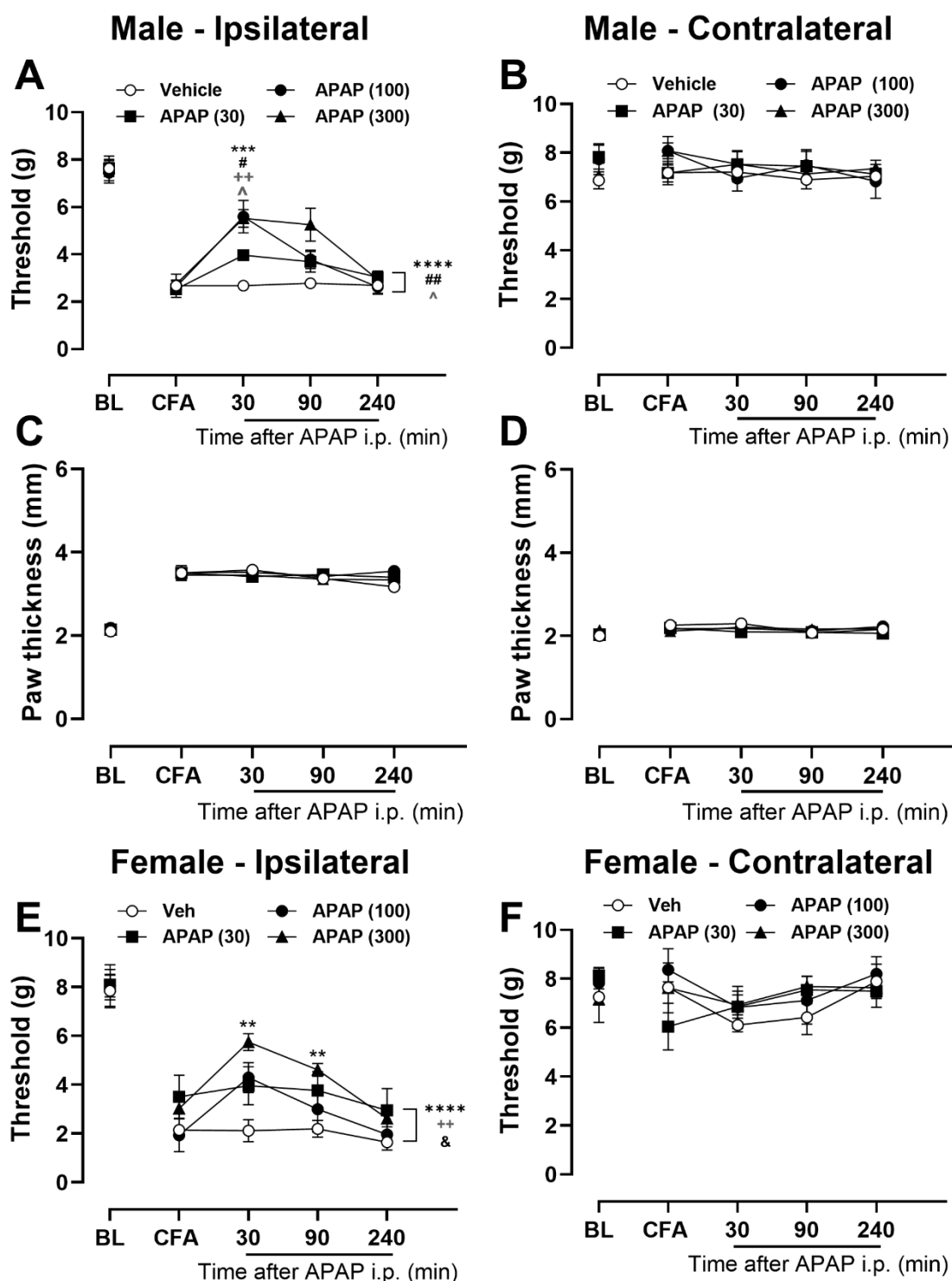


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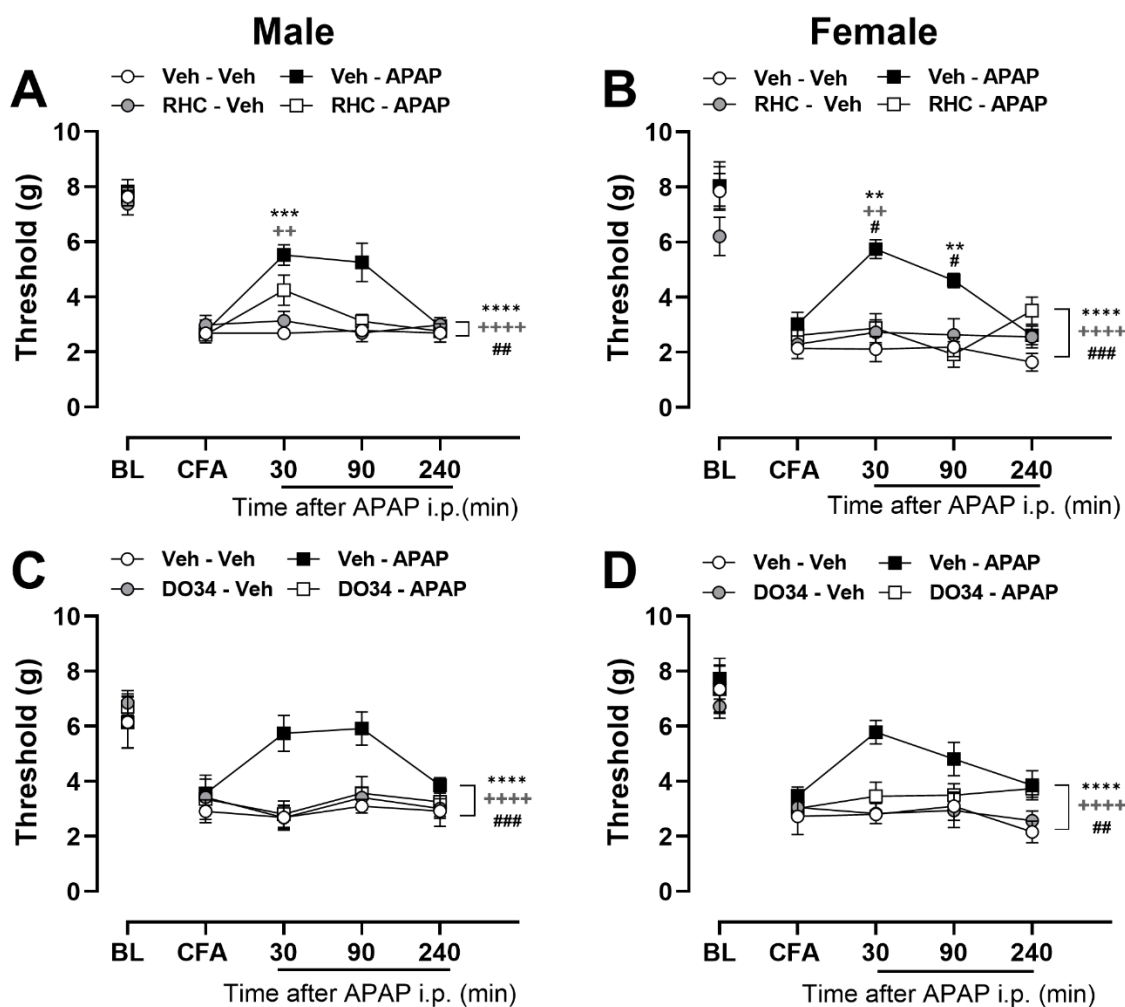


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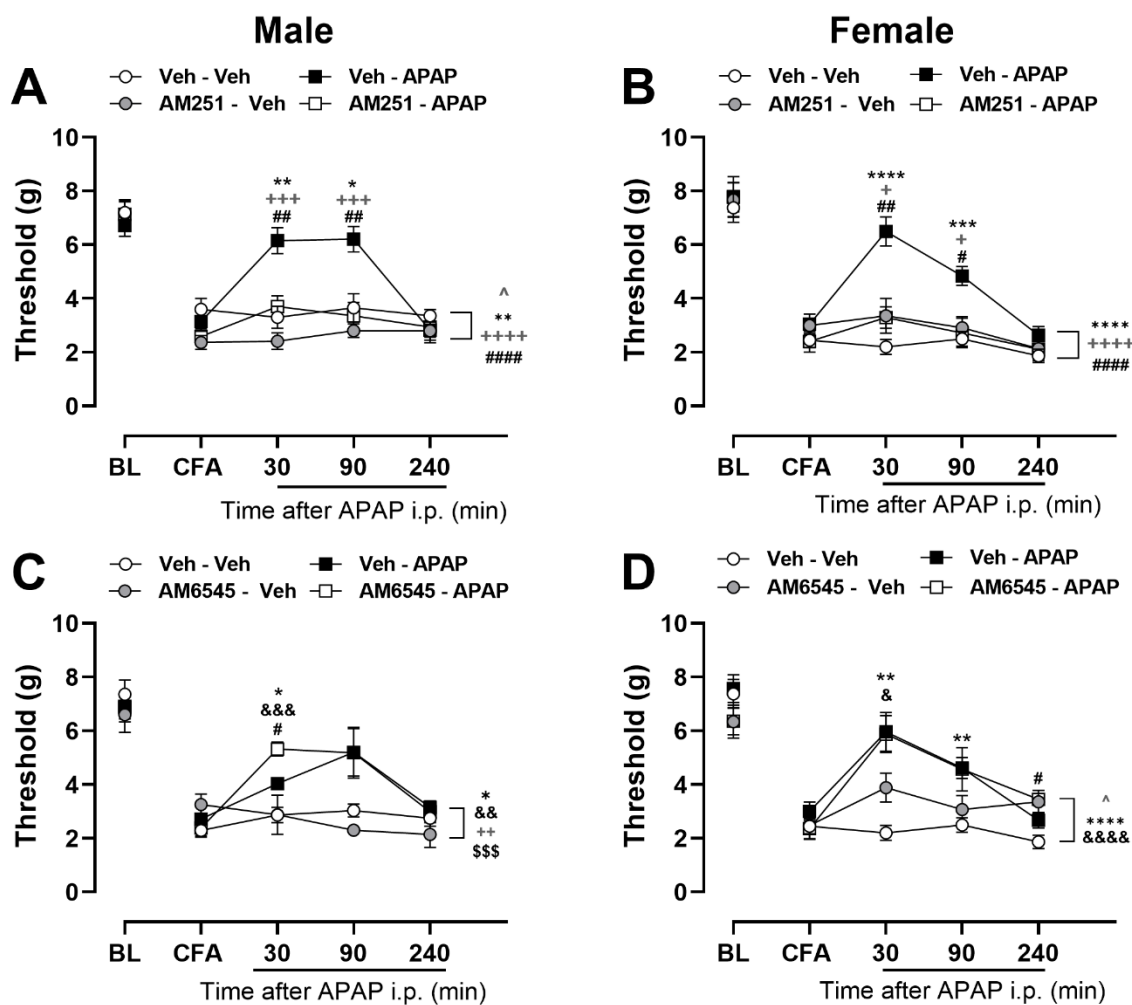


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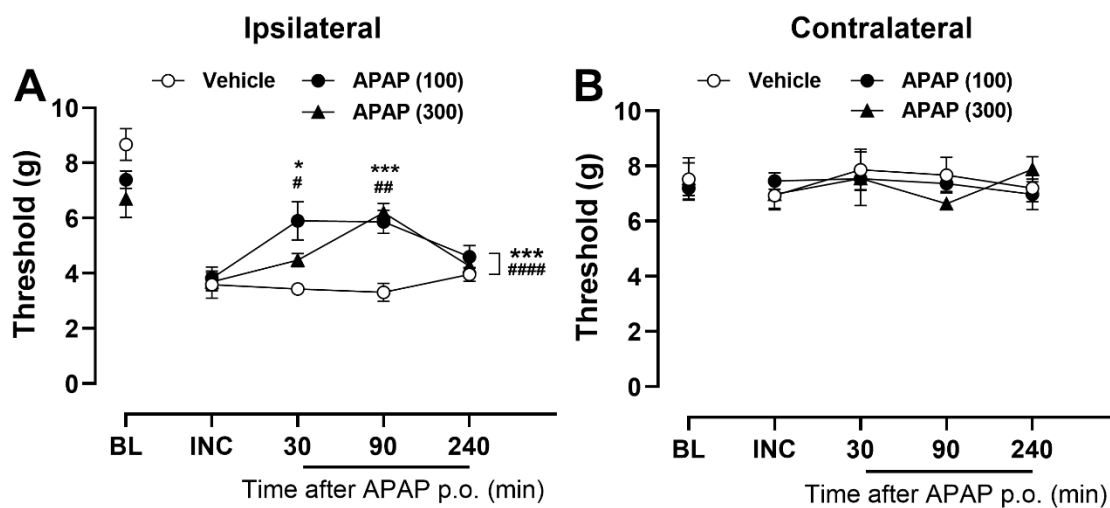


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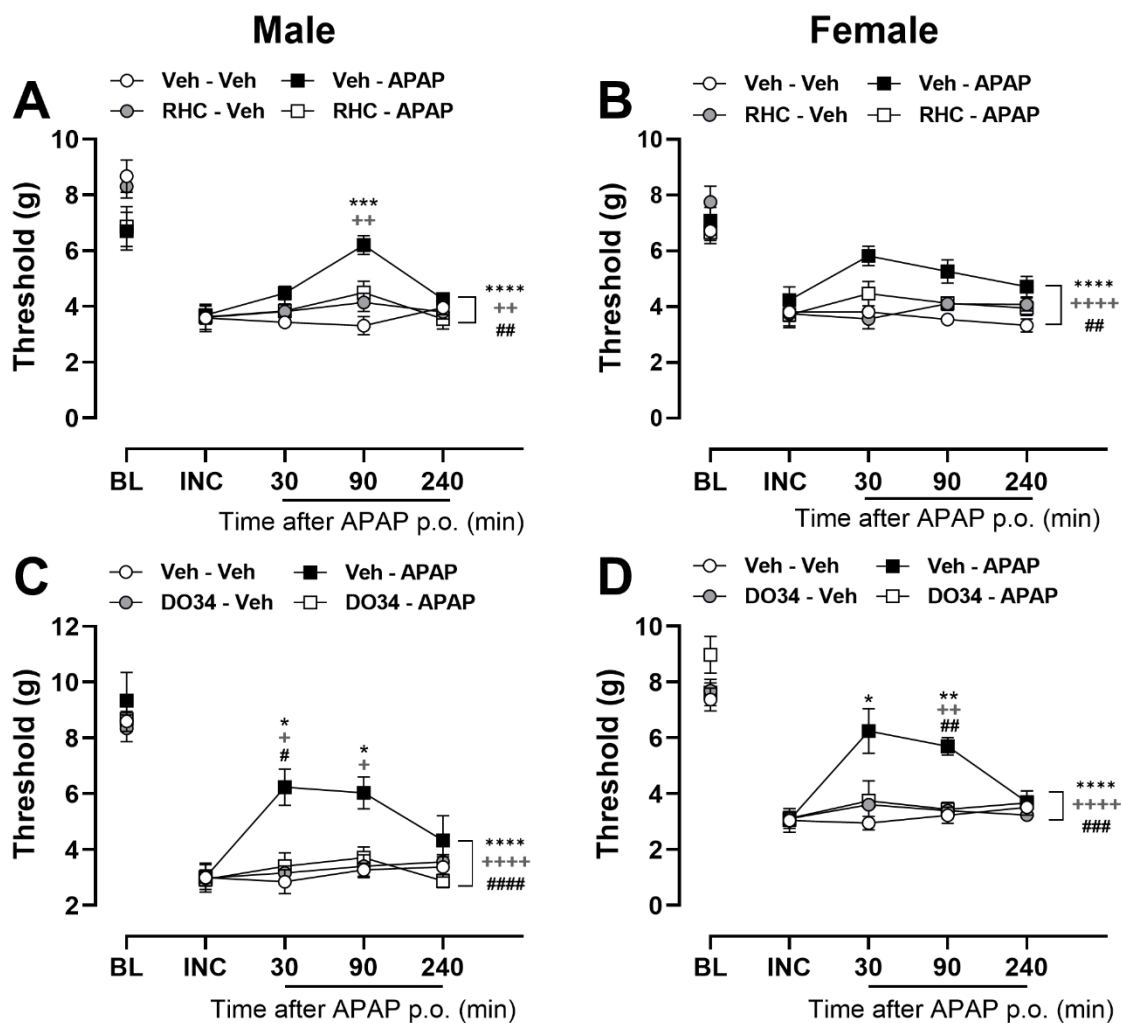


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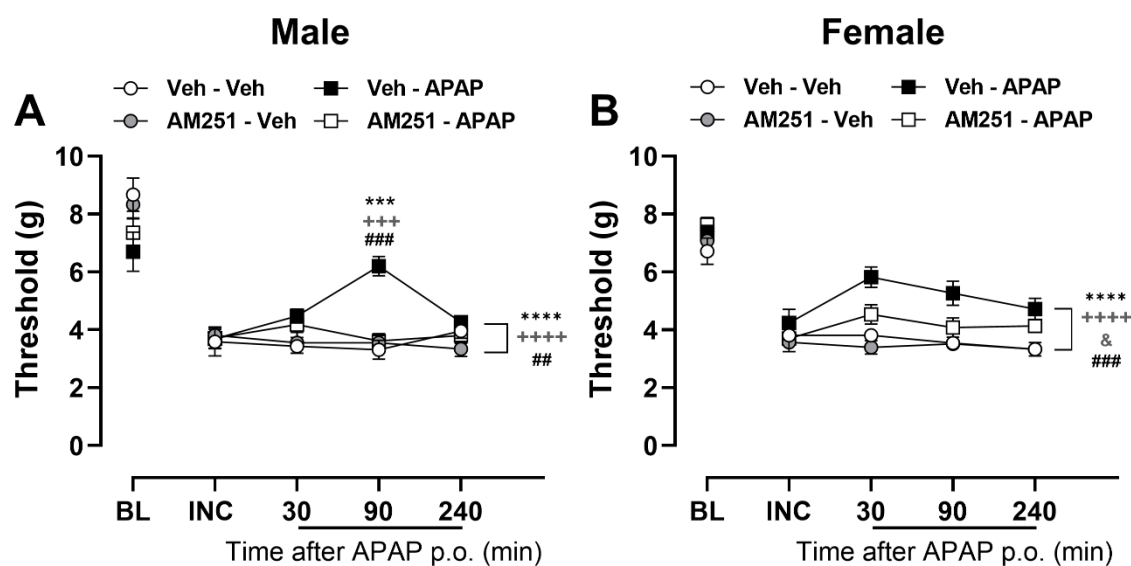


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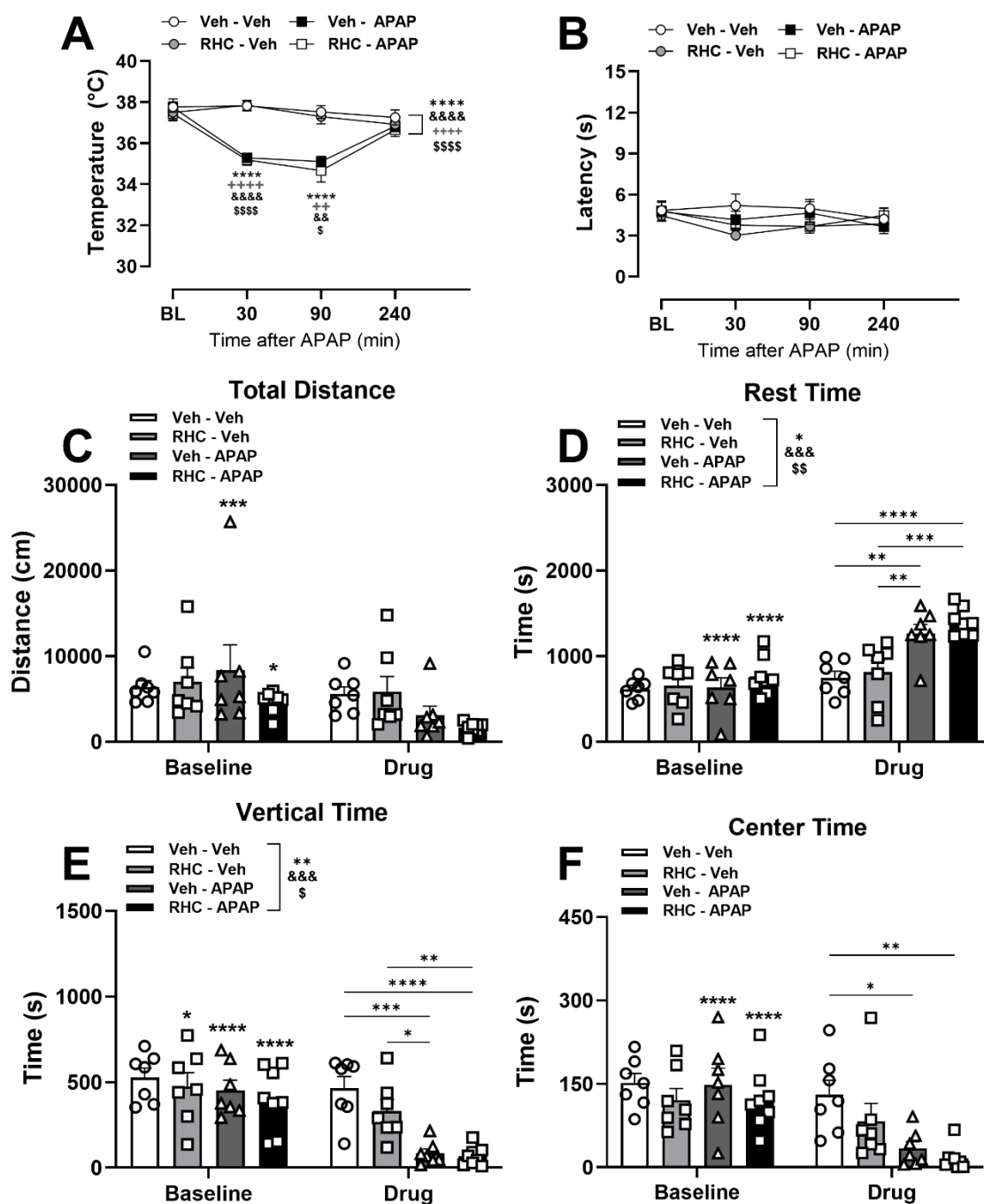
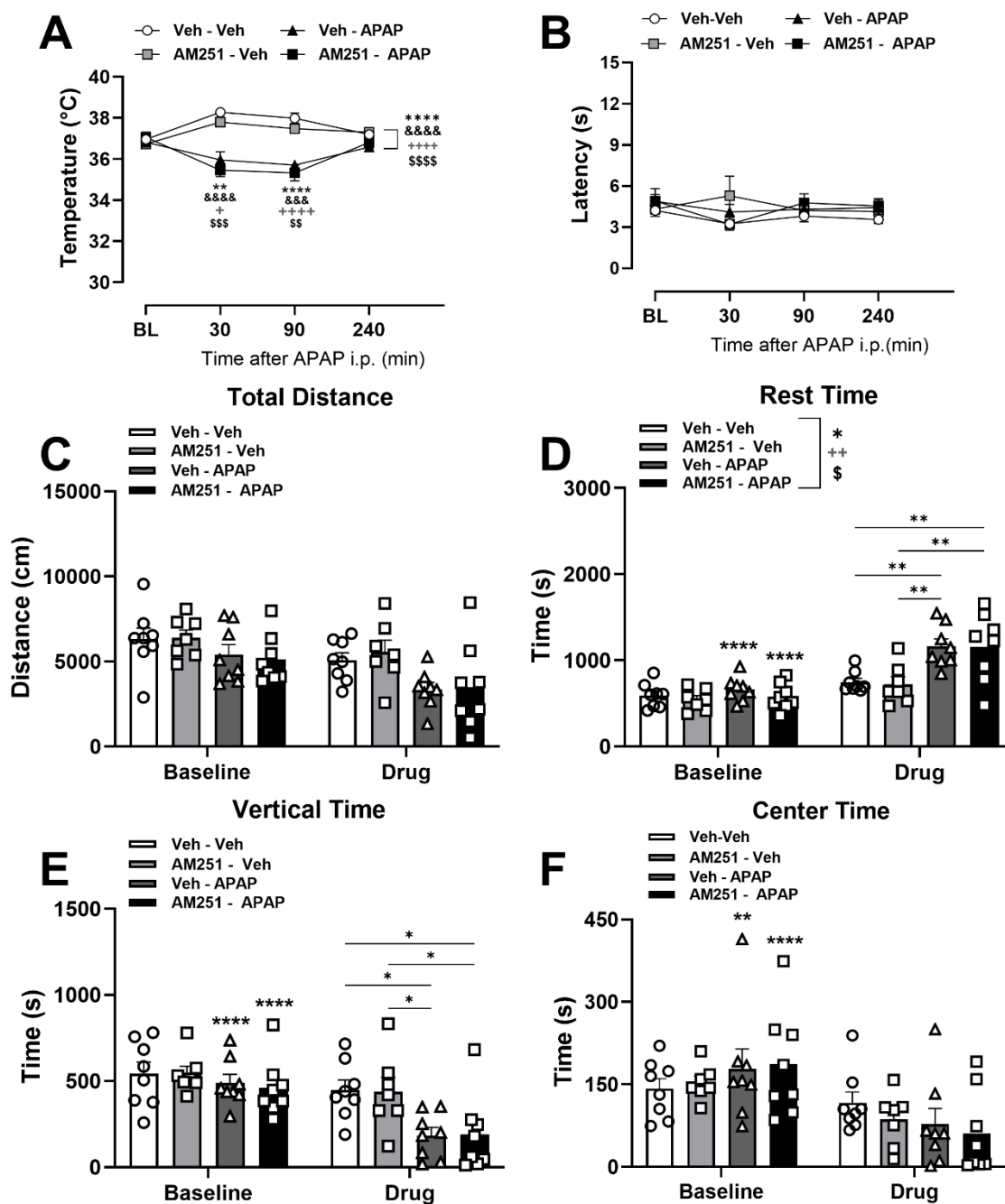
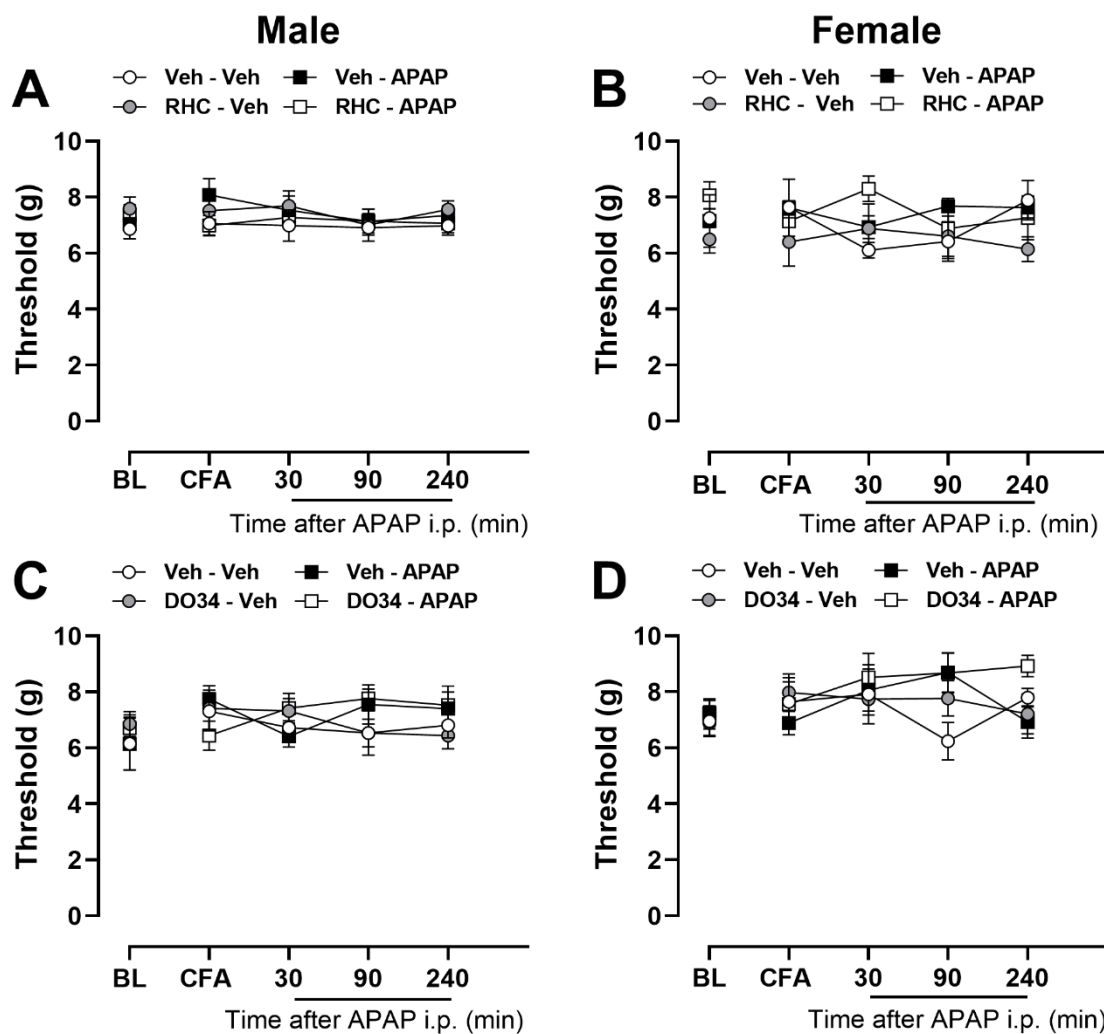


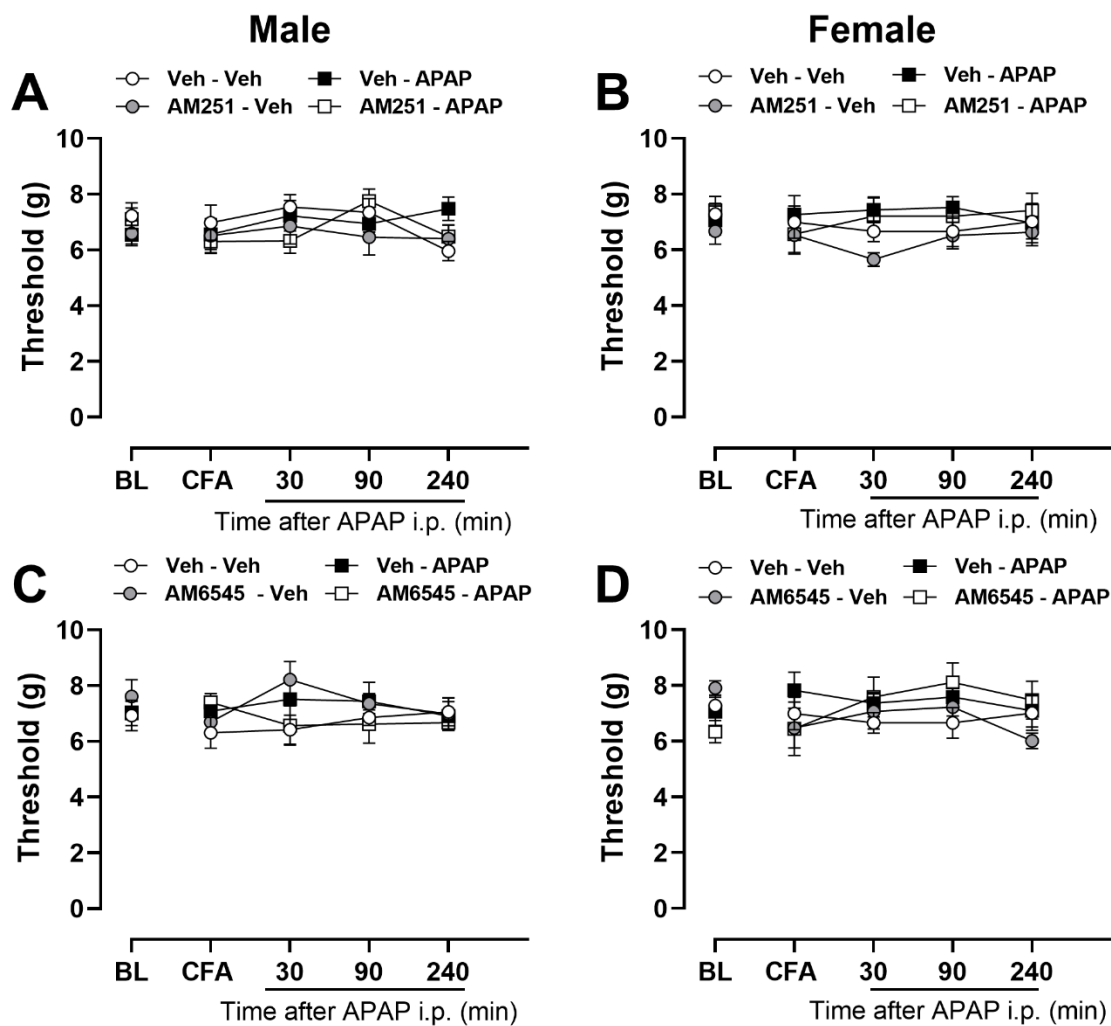
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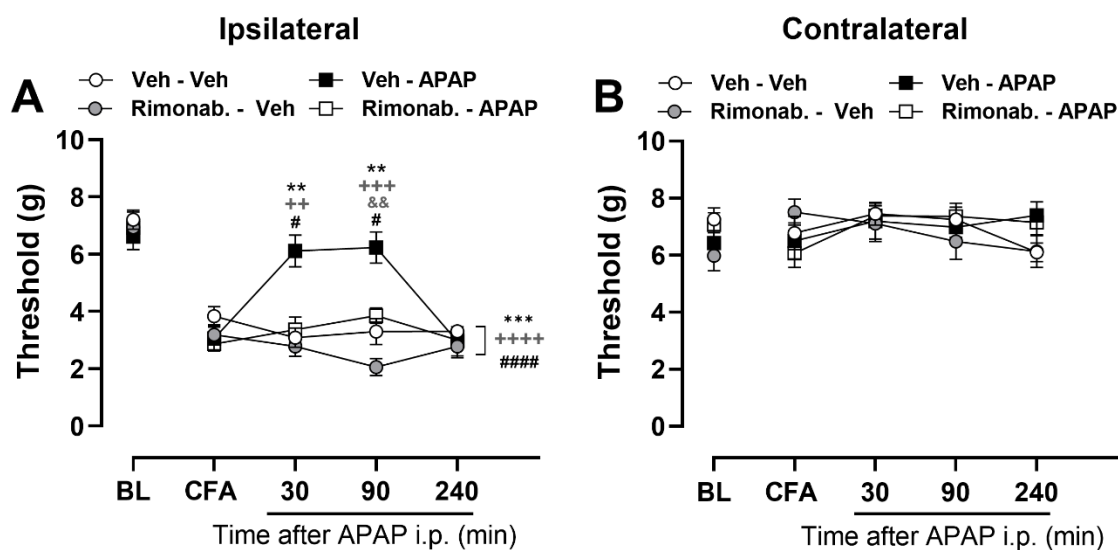
Supplemental Figure 1



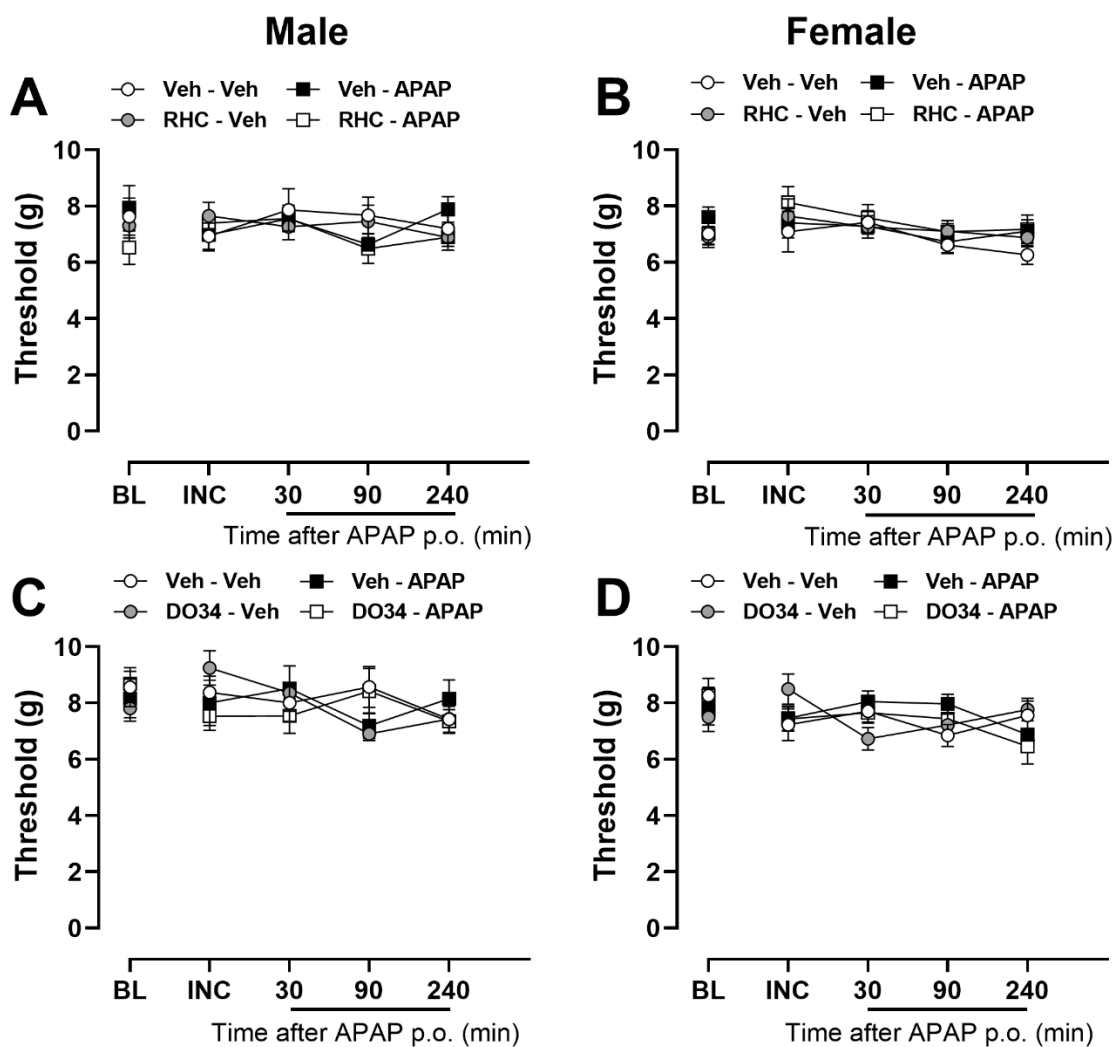
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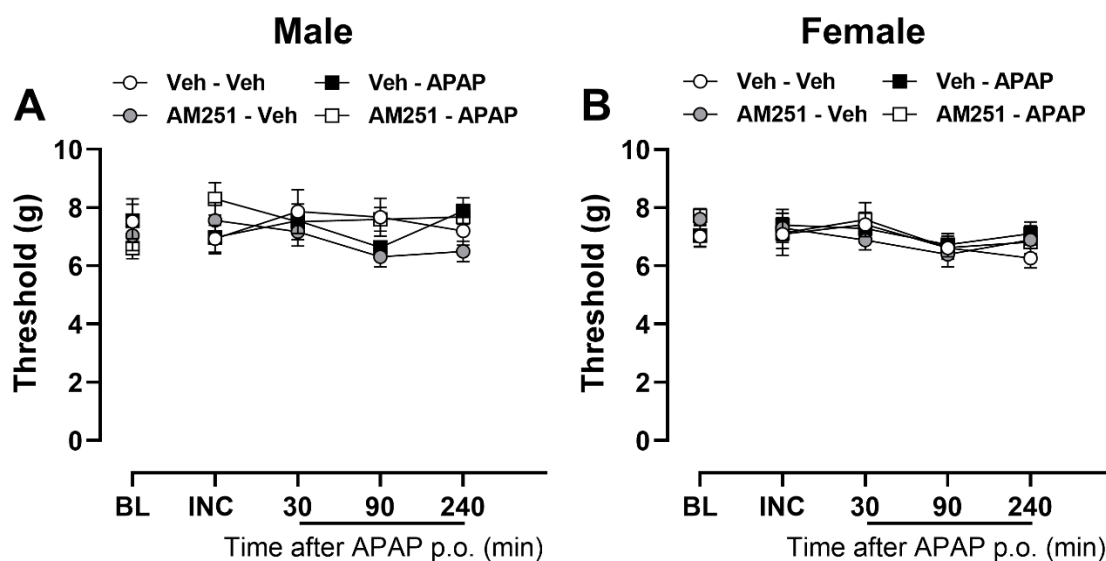
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6

