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Acetaminophen effects upon formalin-evoked flinching, postformalin, and postincisional allodynia and conditioned place preference

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Abstract

Introduction: We explored in mice, the analgesic, tolerance, dependency, and rewarding effects of systemic acetaminophen (APAP).
Methods: Studies employed adult mice (C57Bl6). (1) *Intraplantar formalin flinching + post formalin allodynia*. Mice were given intraperitoneal APAP in a DMSO (5%)/Tween 80 (5%) or a water-based formulation before formalin flinching on day 1 and tactile thresholds assessed before and after APAP at day 12. (2) *Paw incision.* At 24 hours and 8 days after hind paw incision in male mice, effects of intraperitoneal APAP on tactile allodynia were assessed. (3) *Repeated delivery*. Mice received daily (4 days) analgesic doses of APAP or vehicle and tested upon formalin flinching on day 5. (4) *Conditioned place preference*. For 3 consecutive days, vehicle was given in the morning in either of 2 chambers and in each afternoon, an analgesic dose of morphine or APAP in the other chamber. On days 5 and 10, animals were allowed to select a "preferred" chamber.
Results: Formalin in male mice resulted in biphasic flinching and an enduring postformalin tactile allodynia. Acetaminophen dose dependently decreased phase 2 flinching, and reversed allodynia was observed postflinching. At a comparable APAP dose, female mice showed similarly reduced phase 2 flinching. Incision allodynia was transiently reversed by APAP. Repeated APAP delivery showed no loss of effect after sequential injections or signs of withdrawal. Morphine, but not APAP or vehicle, resulted in robust place preference.
Conclusions: APAP decreased flinching and allodynia observed following formalin and paw incision and an absence of tolerance, dependence, or rewarding properties.

Keywords: Acetaminophen, Paracetamol, Tactile allodynia, Formalin, Flinching, Mouse, Tolerance, Dependence, Reward, Conditioned place preference

1. Introduction

Acetaminophen (APAP) is a modestly efficacious pain therapeutic reducing mild to moderate pain.[15,22,39,53,74] It is the most widely used analgesic, with over-the-counter use exceeding 25×10^9 doses/year, worldwide with a market of almost USD 1.6 billion in 2022.[82]

Preclinical work has demonstrated APAP efficacy in inflammatory and neuropathic pain paradigms.[26,33,86] Work has demonstrated the robust effect of APAP upon the facilitated state underlying the biphasic hind paw flinching evoked by intraplantar formalin.[8,12,27,46,55] The early phase has been argued to reflect the acute afferent drive initiated by the actions of formalin mediated through the TRPA1 channel.[49] Late-phase flinching is considered to reveal the facilitated state initiated by the afferent barrage generated during phase 1.[3,64] Although most work has focused on flinching, there is a postflinching, persistent, allodynia (phase 3) that has a neuropathic phenotype.[24,25,84]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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We have undertaken studies focusing on 4 issues related to effects of APAP. (1) Would APAP reverse postintraplantar formalin evoked late-phase allodynia said to have a neuropathic phenotype and in parallel have comparable effects upon allodynia observed early and late after paw incision? (2) Does APAP at analgesic doses display intrinsic rewarding properties? Clinical experience indicates that APAP is without an intrinsic rewarding property, as confirmed by its FDA designation for over-the-counter use worldwide and consistent with preclinical self-administration work.[36,56] Accordingly, we sought to determine using the conditioned place preference (CPP) paradigm if repeated delivery of APAP is associated with the development of a rewarding state. (3) As the CPP model required repeated dosing to develop place preference, we queried whether repeated delivery would result in tolerance with repeated exposure, and subsequently, if such repeated dosing led to any sign of dependence/withdrawal upon termination of dosing, a property which, while accepted, is poorly documented. (4) Finally, APAP is poorly soluble in water and is frequently studied with a variety of vehicles (dimethylsulfoxide [DMSO], Tween 80) with varying degree of intrinsic activity. Recent work led to a water-based formulation created by taking advantage of APAP's temperature sensitive solubility and storage in sealed ampoules.

2. Methods

All studies were performed according to protocols that have been approved by the University of California, San Diego (UCSD) Animal Research Committee to ensure compliance with all tenets of the Animal Welfare Act and Public Health service policy.

2.1. Animals

Adult wild type C57Bl/6 male and females 20 to 25 g. were obtained from Envigo. All mice were held in the vivarium for a minimum of 5 days before use.

2.2. Behavioral testing

Behavioral tests were conducted between 9:00 AM and 5:00 PM Considering reports of a possible contribution of sex of the experimenter,[77] we note that each of these studies were performed by one female investigator, without knowledge as to treatment assignment.

2.3. Drugs

Acetaminophen as a powder (APAPp) (Sigma) was formulated by dissolving in DMSO (5%)/Tween 80 (5%) and brought to a concentration of 10 mg/mL by the addition of sterile water. Acetaminophen was also delivered in a water-based solution provided in sealed ampoules (APAPa) (courtesy of Sintetica Pharma, Switzerland) in a concentration of 30 mg/mL (https://patents.google.com/patent/DK2874602T3/en). Ampoules were opened just before use, and solutions were diluted in sterile water to the desired concentration for injection. In the conditioned place preference studies, morphine sulfate was prepared in saline. Drugs were prepared for delivery by diluting to a final volume of 0.1 mL/10 grams of body weight.

2.4. Drug delivery

2.4.1. Mouse intraplantar injection

Mice were lightly restrained. A 30-G needle was inserted subcutaneously into the plantar surface of the left paw and 20 μL of 2.5%. Formalin was injected over 10 seconds.

2.4.2. Mouse intraperitoneal injection

The mouse was restrained, and its head tilted facing downward with the abdomen exposed. A 30-G needle was inserted through the abdominal skin and musculature on the right side of the animal, and fluid was injected. Aspiration ensured the needle had not punctured a blood vessel, intestines, or bladder.

2.5. Study paradigm

2.5.1. Study 1. Acetaminophen and formalin-evoked phase 1/2 flinching and postformalin allodynia

To assess formalin-evoked flinching, a metal band was placed around the left hind paw of the mouse. Following a 1-hour acclimation, the mouse received an injection of intraplantar (IPLT) formalin (20 μL/2.5%) to induce flinching. The movement of the metal band (mouse flinching) was detected by an automated device.[85] Data were collected continuously as flinch counts/minute for a period of 45 minutes following formalin injection. In formalin studies, groups of male mice were randomly assigned to receive intraperitoneal (IP) injections of APAPp (100 or 300 mg/kg) vs vehicle or APAPa (100 or 300 mg/kg) vs saline 30 minutes before the injection of intraplantar formalin and phase 1 and phase 2 formalin was assessed. In female mice, effects of IP APAPp and APAPa at 300 mg/kg vs vehicle on formalin-induced flinching was also examined.

Mice were assessed for tactile thresholds before the injections of APAPa/APAPp or the respective vehicles and the intraplantar formalin. At 1 hour after cessation of flinching, tactile thresholds were assessed. On day 12 after formalin, allodynia was again assessed, and mice assigned to receive APAPa/APAPp or the respective vehicles. Allodynia thresholds were assessed over the next 4 hours. Following the completion of these studies, animals were euthanized by CO_2 inhalation, according to Institutional Animal Care and Use Committee (IACUC)-approved protocols. To assess mechanical thresholds, animals were placed in clear plexiglass chambers (base: 6 cm × 6 cm base 20 cm height), placed on a wire mesh-bottomed cages for 45 minutes before the initiation of testing. Tactile thresholds were measured with a series of von Frey filaments (Seemes Weinstein von Frey Anesthesiometer; Stoelting Co., Wood. Dale, IL) ranging from 2.44 to 4.31 (0.02–2.00 g), using the Dixon up–down method[18] to calculate the 50% probability of withdrawal threshold in grams.[10]

2.5.2. Study 2. Paw incision

Under isoflurane anesthesia, a cutaneous incision was made in male mice on the plantar surface of the left hind paw, and the underlying muscle elevated, but not severed. The cutaneous wound was closed with 3 sutures, and the animal allowed to recover.[47] In these studies, APAP or vehicle was delivered after 24 hours, and the effects upon the tactile allodynia assessed, as described in study 1 above. On day 8, given persistent allodynia, a second injection of APAP was given, and effects on tactile threshold are assessed.

2.5.3. Study 3. Conditioned place preference

Using a modification of the previously reported method,[57,58] we tested for place preference induced by vehicle, APAP, and morphine in male mice. Each unit consisted of 3 adjoining clear Plexiglas compartments measuring 90 × 90 × 165 mm, with the middle compartment separated from the other 2 by walls with removable entries. The middle compartment was a neutral

chamber, whereas the other 2 compartments were distinctly different in terms of wall pattern (diagonal black stripes vs black squares with total light admitted into each chamber being identical) and removable flooring with 1 of 2 textures (granular vs grid). Time the animal spent in each chamber was monitored by the disruption of LED light paths crossing the space of each of the 2 nonneutral chambers. The testing paradigm occurred over 10 days. During the first 2 "adaptation" days (days 1 and 2), mice were placed in the middle chamber and allowed to freely move between the 3 chambers for 30 minutes. The time spent in each chamber was recorded. On the mornings of the following 2 "conditioning" days (days 3 and 4), 10 minutes after vehicle injection, mice were placed in one of 2 outer chambers for 30 minutes. In the afternoons, mice received drug treatments: vehicle (10 mL/kg), morphine (3 mg/kg), or APAP (200 mg/kg), and 10 minutes later, they were restricted to the other outer chamber for 30 minutes. Exposure doses were chosen based on their ability to produce a significant analgesia. On days 5 and 10, mice were placed in the middle chamber to have free access to all 3 compartments for 30 minutes to determine whether they had developed a preference for the drug-paired chamber and whether this drug-chamber pairing was persistent. Time spent in each chamber was recorded. To define drug effect, average time spent in the drug-paired chamber during the 2 adaptation days was subtracted from the time spent in the same chamber on the test days. Assignment of the chambers to drug-paired or vehicle-paired compartments was counterbalanced.

2.5.4. Study 4. Tolerance and withdrawal

Male mice were assigned to receive 4 daily injections of either acetaminophen (IP APAPp, 300 mg/kg) or vehicle (IP, 10 mL/kg) (days 1–4). On day 5, all animals received IP APAPp (300 mg/kg), 30 minutes before intraplantar formalin. Animals from the 2 groups were then systematically noted at 6, 24, and 48 hours for indices of withdrawal (agitation, hyperactivity, and weight loss).[35]

2.6. General behavioral assessment

During the study, assessment of behavioral function was undertaken by the categorical assessment of the following indices. (1) righting (regaining quadruped posture after inversion), (2) symmetrical ambulation, (3) placing and stepping (dragging of the dorsum of the hind paw over an edge leading to an elevation of the paw and placement), (4) pinnae (twitch of the ear with application of a flexible probe into the auditory canal), or (5) blink (twitch of the eyelid with a light touch of the eye).

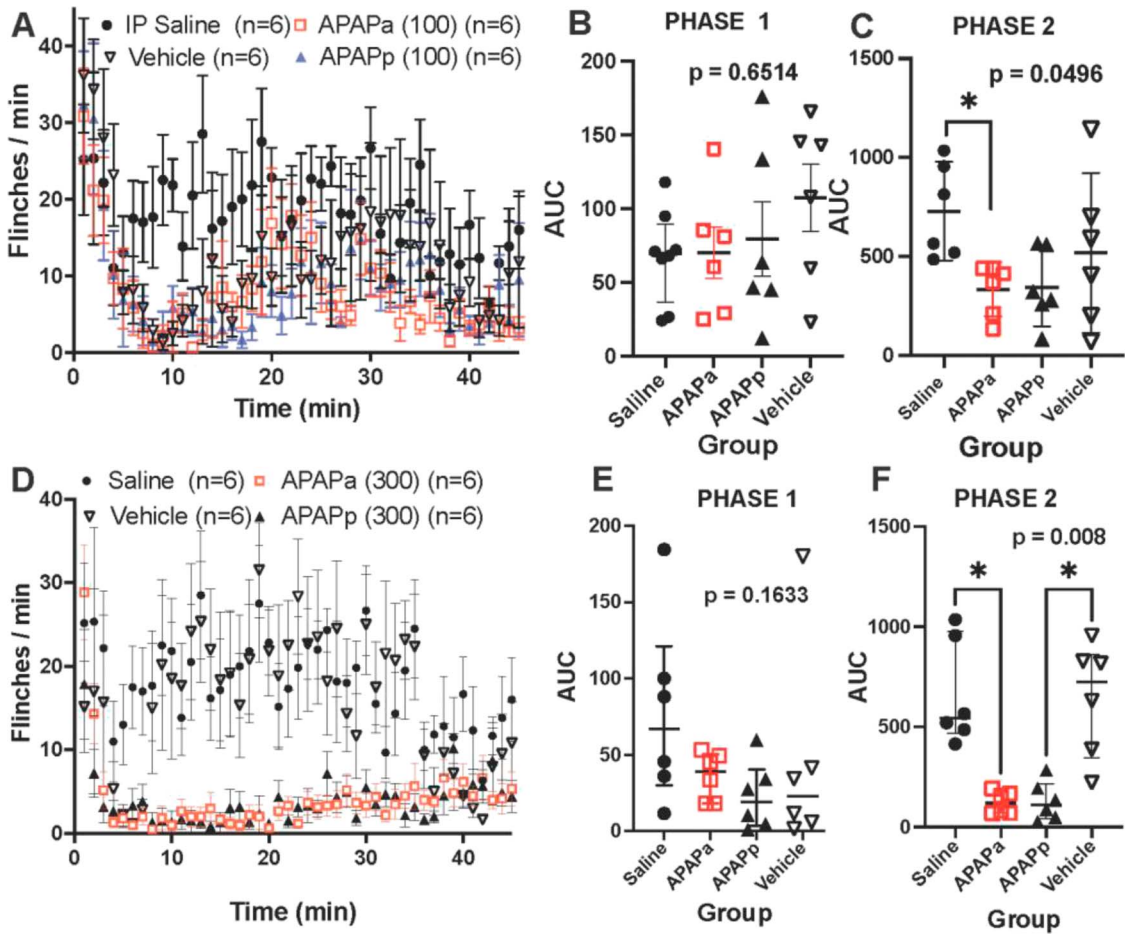

Figure 1. (A, C) Figure presents flinch count (mean ± SEM) for male mice following intraplantar formalin delivered at time 0. Animals received treatment: IP saline, IP vehicle, IP APAPa (ampoule), or IP APAPp (powder) 30 minutes before formalin. (B and E) Scattergram for cumulative flinching count (AUC: median with quartiles) for early phase (phase 1: 0–5 minutes) and (C and F) late phase (phase 2: 6–45 minutes), respectively. (A–C) Data for 100 mg/kg APAP and (D–F) for 300 mg/kg. AUC analysis was performed nonparametrically with Kruskal–Wallis test. Respective *P* values are presented in the respective scattergrams. *Post hoc* analysis for significant effects is shown (*$P < 0.05$). APAP, acetaminophen; AUC, area under the curve; IP, intraperitoneal.

2.7. Power analysis–group size calculation

Group sizes for formalin-evoked flinching were estimated based on group mean and SD for phase 2 flinching in groups receiving formalin would nominally be 1100 ± 240 flinches. In this screening work, we wished to show a behaviorally significant reversal (30% = ≈ 330 count change from 1050) with power (1-β) = 80% and α = 0.05. Analysis indicated a minimum group size of 6 to 7 for flinching.

Group sizes for the tactile allodynic comparisons (post formalin, incision model) were based on the estimates of mean ± SD in groups of arthritic allodynic mice as nominally 0.49 ± 0.45 grams (n = 6). Given that normal mouse thresholds are 1.5 to 2 grams and that a 30% reversal of allodynia is judged to be behaviorally significant and assuming an $\alpha = P < 0.05$ and a power (1-β) = 80%, we calculated sample sizes in the allodynia studies also in the range of 6 to 8/group.

2.8. Data analysis

For primary statistical analysis, flinching was expressed as area under the curve (AUC) for total flinch counts for phase 1 (0–5 minutes) and for phase 2 (6–45 minutes) for each animal. Comparisons were then made between treatments for phase 1 and separately for phase 2. Repeated measures of per-minute flinch were not performed. For conditioned place preference, drug effect was defined as the time spent in the drug-paired chamber.

2.9. Statistics

Previous work has shown that significant intergroup variations can be demonstrated in formalin flinching, and data may fail to meet assumptions of normality. Accordingly, we conservatively performed comparisons in these AUC data sets using non-parametric statistics (Kruskal–Wallis as required). To compare APAP effect across doses, AUC for phase 1 and phase 2 were normalized by dividing the individual AUC for a given mouse by the median of the respective vehicle treatment at each dose. The plotted data for dosing were compared by Kruskal–Wallis test.

For the tactile allodynia end points, comparisons were undertaken using 2-way ANOVA with repeated measures over time as required. The conditioned place preference was analyzed by a two-way ANOVA over time for the 3 drug treatment groups. In either situation, pairwise, post hoc, multiple comparison analysis was performed using Sidak. All analysis and graphics were performed using GraphPad Prism v. 9.4.0.

3. Results

3.1. Acetaminophen and formalin evoked phase 1/2 flinching

Unilateral hind paw delivery of formalin led to a robust biphasic (phase 1 early phase/phase 2 late phase) flinching of the injected paw in both males and female mice (**Fig. 1**, and Supplemental Figure S1 and S2, http://links.lww.com/PR9/A235).

Pretreatment with APAPa and APAPp had no effect upon phase 1 but resulted in a reliable suppression of formalin flinching phase 2 in male mice (see **Fig. 1A–F** and Supplemental Figure S1A, B, http://links.lww.com/PR9/A235). To assess dose dependency in the male mice of the effects for the 3 doses (100, 200, and 300 mg/kg) of the powdered formulation, the scatter gram of the AUC of the flinching for phase 1 and for phase 2 was normalized by dividing by the median AUC of the respective vehicle effect measured for phase 1 and 2 at each dose. These scattergram plots are presented in Supplemental Figure S3, http://links.lww.com/PR9/A235. For each treatment, the median with 95% CI calculated by PRISM is presented. As shown, calculation of the 95% CI shows no overlap with the ratio = 1 for doses of 200 and 300 mg/kg in phase 2 for male mice, indicating no difference from the respective vehicle control, whereas such overlap was reliably observed for all doses in phase 1. Based on the male data, we chose to assess the effects of a single APAP dose on formalin flinching in the female mice (300 mg/kg) and

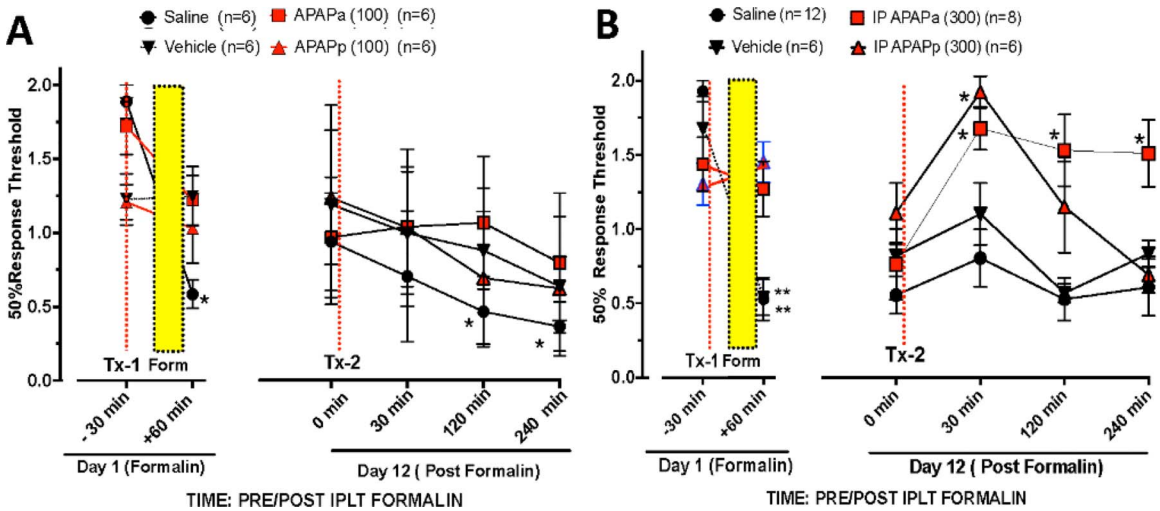

Figure 2. von Frey hair threshold (mean ± SEM) in the male mice receiving intraplantar formalin (Fig. 1) assessed immediately before drug treatment at TX-1(red dashed vertical line: A). IP saline, IP vehicle, IP APAPa (ampoule) (100 mg/kg) or IP APAPp (powder) (100 mg/kg) or (B) IP saline, IP vehicle, IP APAPa (ampoule) (300 mg/kg) or IP APAPp (powder) (300 mg/kg) and formalin on day 1 (formalin) at +30 minutes (indicated by the vertical dotted black line) and after intraplantar formalin (+60 minutes). On day 12, von Frey thresholds were assessed at T = 0 and the animal then administered the same treatment as on day 1 (Tx-2) and then tested at intervals out to 240 minutes. At 1 hour after formalin flinching and then again at 7 and 9 days. Two-way repeated-measures ANOVA were performed with main effects Time and Time × Treatment showing significant ($P < 0.01$). *Post hoc* comparisons between treatments were made with Sidak. * ($P < 0.05$): Day 1 vs Pre Tx1 and Day 12 vs Pre Tx2. APAP, acetaminophen; IP, intraperitoneal.

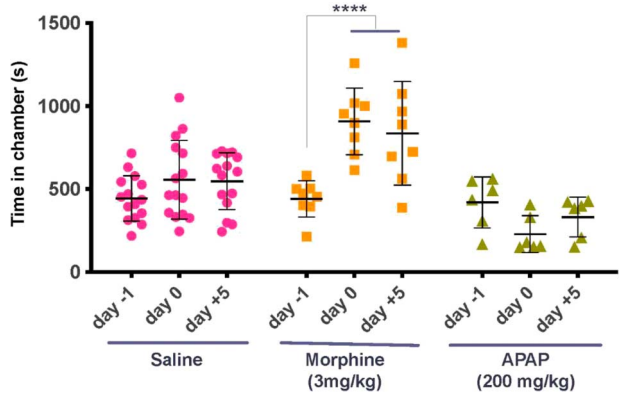

Figure 3. Scattergram showing time in seconds (mean ± SEM) spent in drug-paired chamber for each animal and the drug dosing as assessed before dosing (day 2), on the day after the 4 consecutive days of dosing (day 9) and 5 days later (day 14). Dosing for the 3 groups was saline vs saline, saline vs morphine (3 mg/kg), or saline vs acetaminophen (APAP: 200 mg/kg). Two-way repeated-measures ANOVA was performed with *post hoc* assessment with Sidak multiple comparison. ****$P < 0.001$. APAP, acetaminophen.

observed a significant suppression of phase 2, but not phase 1, with both APAPa and APAPp (Supplemental Figure 2, http://links.lww.com/PR9/A235). Plotting the normalized effect of the single 300 mg/kg dose for female mice showed no overlap in phase 2 behavior, like the results with the male mice at the 300 mg/kg dose (Supplemental Figure 3, http://links.lww.com/PR9/A235).

3.2. Acetaminophen and postformalin late-phase allodynia

Tactile thresholds before formalin were around 1.5 grams. Following formalin, there was a significant reduction in the tactile thresholds in control mice (**Fig. 2A, B**). Treatment before formalin (30 minutes) with APAPa and APAPp at 300 mg/kg (**Fig. 2B**), but not at 100 mg/kg (**Fig. 2A**), resulted in a significant reduction in the allodynia (eg, withdrawal thresholds) during the hour after resolution of formalin flinching.

3.3. Acetaminophen and paw incision

Paw incision resulted in a robust tactile allodynia at 24 hours in the operated paw and a similar allodynia, albeit less robust, in the

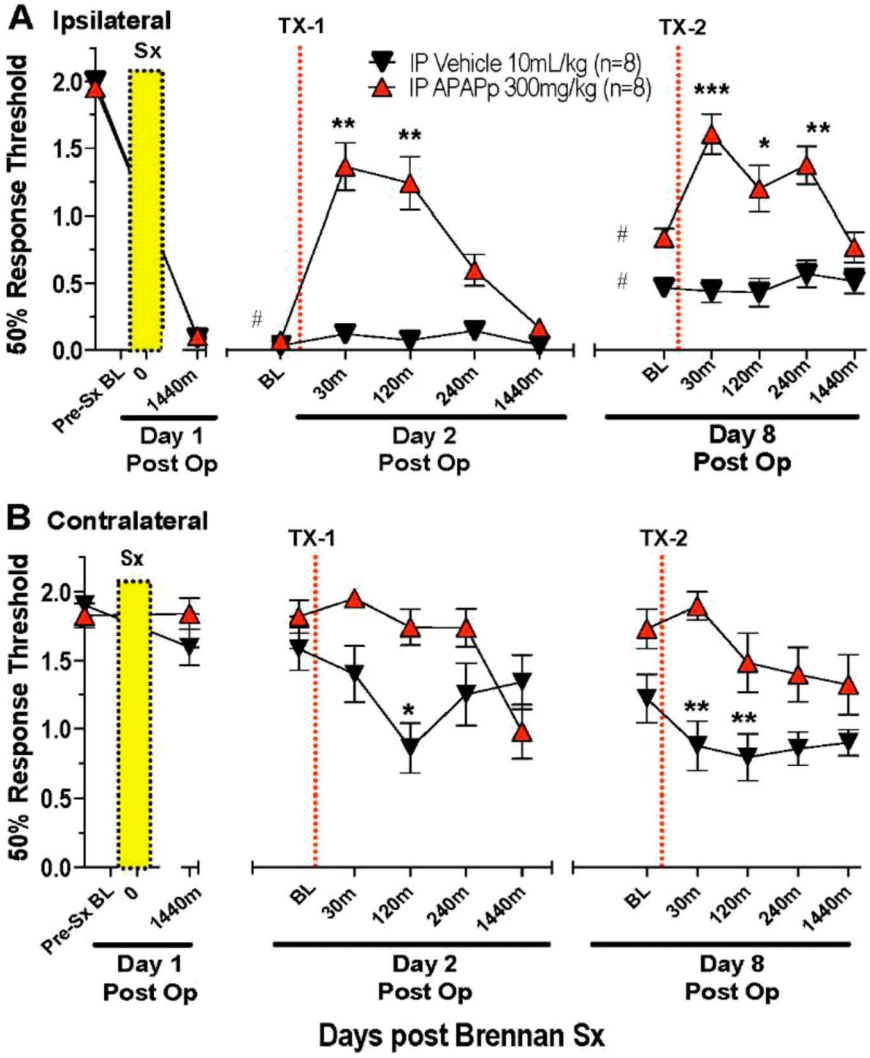

Figure 4. Tactile threshold (mean ± SEM) in male mice measured over time before (baseline: BL) and after unilateral hind paw incision on day 1 (vertical yellow bar) for the (A) ipsilateral and (B) contralateral hind paw in male mice. After 24 hours, on day 2 (Tx-1) again on day 8 (Tx-8), mouse received IP vehicle or acetaminophen (APAP: 300 mg/kg) at vertical dotted line. Two-way repeated-measures ANOVA. Time–treatment main effects were statistically significant ($P < 0.0001$). Post hoc comparisons vs respective presurgical baseline. $P < 0.05$. *Post hoc* comparisons (Sidak) vs respective Pre Tx 1 and Tx2 baselines (BL) *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. APAP, acetaminophen; IP, intraperitoneal.

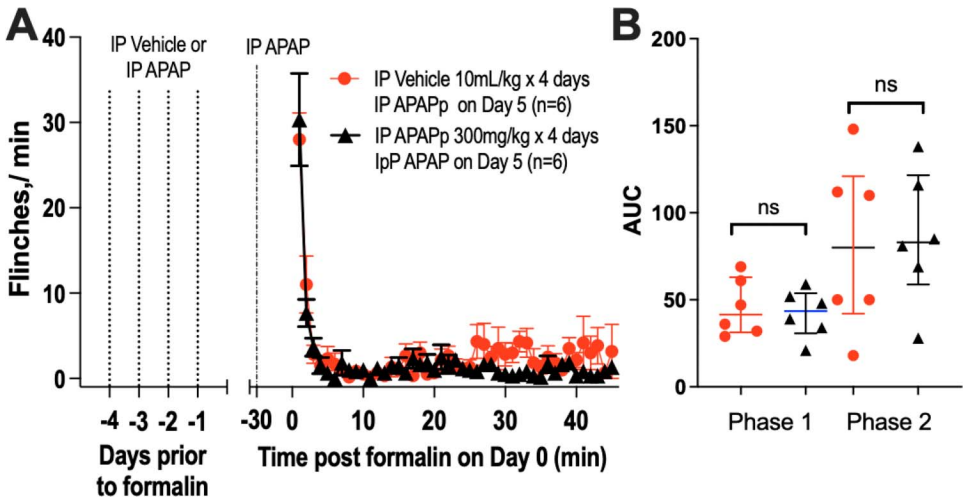

Figure 5. Repeated acetaminophen injections. Male mice were assigned to receive 4 daily injections of either acetaminophen (IP APAP, 300 mg/kg) or vehicle (IP, 10 mL/kg) (days 1–4). On day 5, all animals received IP APAP (300 mg/kg) 30 minutes before intraplantar formalin. (A) Flinch count (mean ± SEM) for mice following intraplantar formalin delivered at time 0. (B) Scattergram for cumulative flinching count (AUC: median/quartiles) for phase 1 (0–10 minutes) and phase 2 (11–45 minutes). AUC analysis for phase 1 and phase 2 flinching between the 2 treatment groups was performed nonparametrically with a rank-sum test; ns: nonsignificant; $P > 0.05$. APAP, acetaminophen; AUC, area under the curve; IP, intraperitoneal.

contralateral paw (**Fig. 3**). As shown, IP APAP (300 mg/kg), but not vehicle, resulted in a reversal that persisted for 2 hours in the ipsilateral paw (injured). After 8 days, a moderately attenuated allodynia was noted in both treatment groups, and this was similarly reversed by APAP, but not vehicle (**Fig. 4A**). The contralateral (uninjured) paw in the vehicle treated mice displayed a modest but significant fall in tactile thresholds when examined on day 2 and day 8 (**Fig. 4B**). By contrast, this fall in threshold in the contralateral paw was not observed in the mice receiving APAP.

3.4. Acetaminophen and Conditioned place preference

Repeated pairing of an analgesic dose of morphine (3 mg/kg) resulted in a significant increase in the time spent in the morphine-paired chamber (**Fig. 3**) when tested on day 5, and this preference continued to be observed on day 10, although no intermediate drug treatment was administered between day 5 and day 10. These observations suggest a positive reinforcing property of morphine. By contrast, the repeated pairing of an analgesic dose of APAP (200 mg/kg) (**Fig. 4**), with a given chamber did not lead to a preference for the APAP-paired chamber at any time.

3.5. Acetaminophen and repeated injection

Mice received 4 daily IP injections of acetaminophen (300 mg/kg) or vehicle. On the fifth day, all animals were assessed for formalin flinching. As shown (**Fig. 5**), APAP on day 5 resulted in similar robust suppressions of phase 2 flinching whether the animal had received prior exposure to vehicle or to the same doses of APAP. Importantly, 5 daily doses of APAPp with 300 mg/kg was well tolerated with no significant change in behavioral assessments or body weight over this 5-day APAP injection protocol (**Table 1**).

3.6. Acetaminophen effects upon behavior

Assessment of behavior after doses prepared in either formulation showed that at the highest APAP dose (300 mg/kg), mice showed some reduction spontaneous activity otherwise observed in formalin-injected mice. However, categorical examination in the formalin-treated groups revealed no loss of behavioral function as assessed by (1) righting, (2) symmetrical ambulation, (3) placing and stepping, (4) pinnae, or (5) blink. In the mice receiving 5 repeated daily injections of APAP (300 mg/kg), no significant changes in body weight were noted over the study periods. All mice completed the study sequence after the acute and the 5 repeated high doses. Mouse behavior during the period after the repeated delivery of an analgesic doses of APAP was unaccompanied by any behavioral signs of dependence or withdrawal (eg, agitation, urination, defecation, piloerection) or weight loss (**Table 1**).

4. Discussion

4.1. Study results

Although a therapeutic with a long history, the present preclinical work sought to focus on the common effect profile of APAP.

4.1.1. Acetaminophen dosing

We employed an APAP formulation prepared in DMSO (5%)/ Tween 80 (5%) or in a water-based formulation (APAPa). The 300 mg/kg dose was predicated on employing a maximum concentration in which APAPp could be readily formulated to compare with APAPa. We found that for this 7-day time frame, 5 daily injections of 300 mg/kg were well tolerated, showing no adverse behavioral signs and no loss of body weight. Accordingly, we used the 300 mg/kg as the high dose in these studies. The one exception was the use of 200 mg/kg in the CPP study. Although not the highest dose, it meets the criteria for being a robustly effective dose.

4.1.2. Analgesic profile

This work indicates that water or DMSO/Tween vehicle was well tolerated and resulted in comparable vehicle-only effects in these behavioral models. Both APAP formulations resulted in

Table 1

Body weight before acetaminophen and after 5 daily injections of acetaminophen (300/mg/kg) or 4 daily injections of vehicle and 1 injection of acetaminophen on day 5 and then 2 days after the last acetaminophen dosing.

Treatment group	No. of mice	Body weight		
		Day 0 Pre APAP	Day 5 Post 5th APAP dosing	Day 7 2 d post 5th APAP injection
Vehicle group	6	24.6 ± 0.3 g	24.7 ± 0.2 g	24.1 ± 0.3 g
Repeated 55 APAP injection	6	25.2 ± 0.2 g	25.6 ± 0.2 g	25.4 ± 0.4 g

APAP, acetaminophen.

comparable suppression of formalin flinching in male and female mice, and in male mice, the postformalin allodynia and the incision-induced tactile allodynia.[8,12,42,72,86] This postformalin allodynia is considered to reflect the appearance of a pain phenotype that shows activation of neuraxial epitopes microglial activation and the pharmacology of a neuropathy.[24,25,84] This profile in these models is consistent with previous work in which APAP has been shown in preclinical models to have efficacy in models characterized by facilitated states as revealed by mechanical and thermal hyperalgesic end points in rodent inflammatory models,[1,32,54,67] bone cancer,[70] and in polyneuropathies (chemotherapy)[44] and mononeuropathies.[14,16,34] Importantly, the most robust effects are associated with facilitated states, whether those states are driven by inflammation (as in the incision/irritant models) or by models where inflammation is not considered to be the primary driver (as with formalin/nerve injury).

4.1.3. Tolerance, dependence, and withdrawal

An important observation in these studies was that repeated exposure to a high analgesic dose of APAP (300 mg/kg) did not yield any signs of tolerance (tachyphylaxis) or dependence/withdrawal. Furthermore, in contrast to morphine, APAP showed no signs of a developing preference in the conditioned place preference model and again failed to lead to adverse observations during the 5-day period after the last APAP exposure in the CPP paradigm eg, withdrawal. These results are consistent with clinical use, where APAP is not listed as a controlled substance and is available worldwide as an over-the-counter product, listed as an unscheduled over the counter drug that reveals no rewarding properties in humans[36,61] or animal models in self-administration paradigms[50] or as here in a conditioned place preference (CPP) model, in normal mice[56] at a strongly analgesic doses. A single study has reported a degree of APAP preference, although the reason for this difference is not known.[2] The preference paradigm as employed in this study was robust and validated by its ability to demonstrate the preference for an opiate, but not APAP.

4.2. Utility of acetaminophen

The clinical utility of APAP is based on 5 properties.

(1). *Analgesic efficacy.* Meta-analyses report that oral APAP reduces mild-to-moderate pain.[15,53,69] Repeated-dose, randomized, double-blind, placebo-controlled trials in orthopedic surgery and abdominal hysterectomy patients showed that APAP significantly decreased pain in prospective, randomized, double-blind, multicenter, clinical trials.[9,22,68,74,83] In a Cochrane meta-analysis investigating postoperative pain, APAP (1000 mg) showed numbers needed to treat (NNT) on the order of 3.6,[81] comparing favorably with opiates. In human experimental models, IV APAP reduced evoked hyperalgesia.[39]

(2). *Absence of dependence and abuse liability.* Decades of clinical use reveal no abuse liability or evidence of an intrinsic rewarding potential.[36,50,56,61]

(3). *Tolerability.* Although APAP is widely used and has a record of safety when used at approved doses,[23,30,52] overdoses, which can occur when multiple combination medication may be ingested, can lead to severe liver damage and is a common causes of hepatotoxicity.[31] Furthermore, APAP use during pregnancy has been associated with an increased risk for neurodevelopmental disorders in prenatally exposed individuals.[38,59]

(4). *Cost-effective.* Although not commonly noted, an important metric of the impact of APAP is that with its notable efficacy, it is among the most cost-effective pain medications in the treatment of a variety of clinical pain phenotypes.[11,37] In economically disadvantaged countries, APAP plays a major role as a safe, tolerated, nonaddictive, and affordable pain therapeutic.[37]

4.3. Mechanisms of acetaminophen action

Studies to identify specific APAP/metabolite binding sites have typically revealed little.[28,65] Several mechanisms have been hypothesized to reflect an effect of APAP itself, or an action mediated by primary liver/brain metabolites. P-aminophenol can be converted through fatty acid amide hydrolase (FAAH), to N-(4-hydroxyphenyl)-arachidonamide (AM404) and into N-acetyl-p-benzoquinone imine (NAPQI) through cytochrome P450 (CYP) enzymes. Other metabolic pathways less well characterized include glucuronidation and sulfation.[17,48] Current thinking has suggested several cellular targets for one or more of these products, including (1) *cannabinoid signaling though formation of the AM404 metabolite* acting as an inhibitor of anandamide (endogenous CB1 agonist) uptake, (2) activating *TRP channels*,[45,78,87] as with the metabolite NAPQI activating TRYP-A1 and causing a depolarization block of spinal afferents,[43] (3) *cyclooxygenase inhibition*,[76] or (4) through *serotonin signaling* by brainstem activation of bulbospinal serotonergic projections by an undefined mechanism.[62,80] Although each mechanism is supported by pharmacologic data, the several mechanisms appear distinct for the profiles of APAP action. Cannabinoid mechanisms would imply evidence of sedation and reward, absent even with high doses of acetaminophen. Similarly, the absence of common COX-mediated effect, such as gastrointestinal, coagulation, cardiovascular, kidney, or particularly an anti-inflammatory actions, is inconsistent with a primary COX-targeted action.[5,6,21,41,71,73,76] Regarding a serotonergic mechanism, APAP/metabolites display little or no affinity for 5-HT receptors or actions on neuronal reuptake.[65] Although APAP may exert a brainstem activation of bulbospinal serotonergic projections, by an undefined mechanism,[62,80] bulbospinal serotonin projections appear to *evoke* hyperalgesia, likely through an excitatory 5HT receptor such as 5HT3.[79] Alternately, the activation of

descending pathway may activate a G protein–coupled inhibitory 5HT receptor (eg, 5HT-1 isotypes) or act through an excitatory receptor (5HT2, 3, 7) to activate GABA or enkephalin interneurons.[20,51,63] Although inhibition of several excitatory and inhibitory 5HT-r ligands reduce APAP actions, 5-HT3 receptor antisense had no effect.[4,13,19,40,60]

In summary, although the therapeutic importance of APAP has been disparaged, its wide spread activity has led several groups to seek to recapitulate the APAP profile by creating analogues.[7,29,66,75] Although efficacy has been identified with these analogues, there is typically no affirming data that the effects of those analogues are mediated by an APAP-related mechanism. Although current mechanisms point to several linking systems, there is no defining property, which can be compared across drug structures that can point to rational approaches for creating an APAP-linked therapeutic target through which APAP or its metabolites may act to alter nociceptive processing initiated by tissue and/or nerve injury.[33] Future work in assessing APAP action requires implementation of agnostic platforms to define pain target engagement of the parent compound and/or its metabolites in animals and humans.

Disclosures

Two authors E.D., A.L. are employees of a company that made the water-based acetaminophen and as stated in the paper. They were not involved in the performance of the experiments or in the analysis of the data but contributed to the manuscript proofing. Other authors report no conflict.

In addition, at the point of revision, each author is required to submit the ICMJE disclosure form, which should be uploaded to Editorial Manager and which can be found on the submission site on the Information for Authors page, or at http://www.icmje.org/downloads/coi_disclosure.pdf. In addition to completing the form, all authors must clearly state all relevant conflicts of interest in the Acknowledgements section of the submitted manuscript.

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