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Original Research

NAPE-PLD regulates specific baseline affective behaviors but is dispensable for inflammatory hyperalgesia

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ABSTRACT

N-acyl-ethanolamine (NAEs) serve as key endogenous lipid mediators as revealed by manipulation of fatty acid amide hydrolase (FAAH), the primary enzyme responsible for metabolizing NAEs. Preclinical studies focused on FAAH or NAE receptors indicate an important role for NAE signaling in nociception and affective behaviors. However, there is limited information on the role of NAE biosynthesis in these same behavioral paradigms. Biosynthesis of NAEs has been attributed largely to the enzyme *N*-acylphosphatidylethanolamine Phospholipase D (NAPE-PLD), one of three pathways capable of producing these bioactive lipids in the brain. In this report, we demonstrate that *Nape-pld* knockout (KO) mice displayed reduced sucrose preference and consumption, but other baseline anxiety-like or depression-like behaviors were unaltered. Additionally, we observed sex-dependent responses in thermal nociception and other baseline measures in wildtype (WT) mice that were absent in *Nape-pld* KO mice. In the Complete Freund's Adjuvant (CFA) model of inflammatory arthritis, WT mice exhibited sex-dependent changes in paw edema that were lost in *Nape-pld* KO mice. However, there was no effect of *Nape-pld* deletion on arthritic pain-like behaviors (grip force deficit and tactile allodynia) in either sex, indicating that while NAPE-PLD may alter local inflammation, it does not contribute to pain-like behaviors associated with inflammatory arthritis. Collectively, these findings indicate that chronic and systemic NAPE-PLD inactivation will likely be well-tolerated, warranting further pharmacological evaluation of this target in other disease indications.

Introduction

Bioactive lipids have been widely recognized as important neuromodulators in the central nervous system. In contrast to traditional neurotransmitters that are stored and released from presynaptic vesicles, lipids signals are synthesized "on demand" and rapidly metabolized to terminate signaling *in vivo*. *N*-acyl-ethanolamines (NAEs) serve as key endogenous lipid mediators as revealed by manipulating fatty acid amide hydrolase (FAAH), the primary enzyme responsible for metabolizing NAEs. Accordingly, global inactivation of FAAH elevates multiple bioactive NAEs including *N*-oleoylethanolamine (OEA), *N*-palmitoylethanolamine (PEA), and the endocannabinoid anandamide (*N*-arachidonoylethanolamine, AEA) (Leung et al., 2006; Mock et al., 2020; Nyilas et al., 2008; Simon and Cravatt, 2010). NAE biosynthesis has been attributed largely to the enzyme *N*-acylphosphatidylethanolamine Phospholipase D (NAPE-PLD), supported by multiple studies showing reduced NAE levels in the brain following genetic or pharmacological inactivation (Leishman et al., 2016; Leung et al., 2006; Mock et al., 2020; Simon and Cravatt, 2010). However, NAPE-PLD represents one of at least three identified enzymatic pathways in the central nervous

Abbreviations: AEA, Anandamide *N*-arachidonoylethanolamine; 1 CB1, cannabinoid receptor; 2 CB2, Cannabinoid receptor; CFA, Complete Freund's Adjuvant; FAAH, fatty acid amide hydrolase; HET, heterozygous; KO, knockout; NAE, *N*-acyl-ethanolamine; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine; NAPE-PLD, *N*-acylphosphatidylethanolamine phospholipase D; PPARα, peroxisome proliferator-activated receptor α; TRPV1, transient receptor potential vanilloid 1; WT, wildtype.

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system that control NAE production (Leung et al., 2006; Mock et al., 2020; Nyilas et al., 2008; Simon and Cravatt, 2010), hence the contributions of NAEs produced specifically by NAPE-PLD to nociceptive and affective behaviors remains unclear. NAEs mediate their effects through a number of receptors in the central nervous system (Gregus and Buczynski, 2020; Mock et al., 2023; Pistis and Melis, 2010). For example, AEA can activate cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), OEA and PEA can activate peroxisome proliferator-activated receptor α (PPARα), and all three of these NAEs can potentiate transient receptor potential vanilloid 1 (TRPV1) activity. Thus, alterations in endogenous NAE levels may influence numerous central nervous system signaling pathways.

Preclinical studies focused on FAAH or NAE receptors indicate an important role for NAE signaling during nociception and affective behaviors. Both rats and mice with elevated FAAH activity subsequently have diminished AEA levels in the amygdala, a critical control hub in the brain for nociception and affective behaviors (Gray et al., 2016; Natividad et al., 2017). Accordingly, long-term FAAH inactivation reduces anxiety-like behaviors in Wistar-Kyoto rats (chronic FAAH inhibitor treatment) and C57BL6/J mice (genetic FAAH knockout) as measured by increased center time in the open field test (Bambico et al., 2010; Vinod et al., 2012). Similar to models of anxiety, elevated FAAH also induces depression-like phenotypes in multiple output modalities including increased sucrose preference and increased forced swim test immobility time (Blanton et al., 2021), while conversely FAAH inhibitors reduce forced swim immobility time in both species (Gobbi et al., 2005; Griebel et al., 2018; Jankovic et al., 2020). These behavioral effects may be mediated by multiple NAE signaling pathways, as pharmacological activation of CB1 and PPARα produces anti-depressant effects in rodents. Administration of synthetic cannabinoids decreases immobility during the forced swim test in rats (15916883), and restores sucrose preference following chronic restraint stress in male mice (Rademacher and Hillard, 2007). Following chronic social defeat stress in male mice, treatment with a PPARα agonist restores sucrose preference and decreases immobility in the forced swim test (Jiang et al., 2017; Jiang et al., 2015). Affective dysfunction exhibits co-morbidity with the emergence of pain hypersensitivity, and systemic inactivation of FAAH produces antinociceptive responses in multiple mouse preclinical pain models including neuropathy, gastrointestinal inflammation, and inflammatory arthritis (Schlosburg et al., 2009). The carrageenan paw inflammation model produces tactile pain hypersensitivity that is reversed by systemic treatment with a FAAH inhibitor in rats and mice (Holt et al., 2005; Sagar et al., 2008). This antihyperalgesic effect of FAAH inactivation is blocked by antagonists of PPARα but not of CB1 in rats (Sagar et al., 2008), and is recapitulated by OEA and PEA in mice (Lo Verme et al., 2005). In the Complete Freund's Adjuvant (CFA) model of inflammatory arthritis, inactivation of FAAH reduces tactile pain hypersensitivity in rats (Ahn et al., 2011) and in mice through CB1- and CB2-dependent mechanisms (Jayamanne et al., 2006). These studies highlight the need to elucidate specific endogenous NAE signaling mechanisms underlying affective and nociceptive behavioral responses.

While many of these studies were performed in males, accumulating evidence indicates that NAE signaling pathways exhibit sex differences in rodents. For example, female rats express higher levels of FAAH activity and lower AEA levels in the amygdala as compared to males (Krebs-Kraft et al., 2010). Conversely, male rodents express higher levels of CB1 receptors than females in this region, which may be attributed in part to the influence of ovarian hormones in the latter (Castelli et al., 2014). Males also express higher levels of PPARα in T-cells (Dunn et al., 2007) and in the hippocampus (Pierrot et al., 2019), where pharmacological activation of PPARα enhances synaptic plasticity in males but not female mice. These findings reflect the established role of sex in the behavioral responses to nociception (Gregus et al., 2021), anxiety (Hodes and Epperson, 2019), and depression (Kropp and Hodes, 2023) suggesting that behavioral evaluations of NAE signaling should incorporate sex as a biological variable.

Despite the importance attributed to NAEs in nociception and affective disorders as revealed by investigation of FAAH, there is limited information on the role of NAE biosynthesis in these same behavioral paradigms. A recent study using acute dosing of the NAPE-PLD selective inhibitor LEI-401 attributes NAEs from this pathway in fear conditioning in male mice (Mock et al., 2020). However, the role of NAPE-PLD in nociceptive and other affective behaviors has not been broadly evaluated. In this paper, we describe the effects of constitutive deletion of *Nape-pld* on multiple nociceptive and affective behaviors in mice of both sexes. We utilized an extensive test battery that includes exploratory (open field, light–dark box), stress-coping (forced swim, splash), natural reward (sucrose preference), nociceptive (tactile paw withdrawal, thermal escape) and motor function (rotarod, locomotor activity). Finally, we measured nociceptive responses following intraplantar CFA including peripheral inflammation (paw edema), tactile sensitization (tactile paw withdrawal), and functional pain-like (grip force) behavioral assessments to evaluate NAPE-PLD as a target for inflammatory arthritis.

Methods

Reagents and consumables

Materials were purchased as follows: Sigma Aldrich: Ultrapure sucrose (#RES0928S-A102X), Fatty acid-free BSA (#A7030), Tween-20 (#P9416), Tris-HCl (#T5941), Complete Freund's Adjuvant (#F5881, Lot number SLCF1289); Viagen: DirectPCR Lysis Reagent (Mouse Tail, #102-T); Integrated DNA Technologies (Bishay et al.): *Nape-pld* primers; Lonza: Seakem-LE Agarose (#50002); Thermo: SuperSignal West Pico PLUS Chemiluminescent Substrate (#34579), Phusion Hi-Fidelity DNA polymerase (#F530S), Bolt 4–12% Bis-Tris gels (#NW04120BOX), iBlot2 PVDF transfer stacks (#IB24002); BioRad: blotting grade blocker (#1706404); Cayman Chemical: anti-NAPE-PLD rabbit polyclonal antibody (#10306); Cell Signaling: goat anti-rabbit HRP-linked secondary antibody (#7074); Braintree Scientific: Iso Pads, 6″x10″ (#ISO); MWI: Isoflurane (#NDC 13985–528-60), Saline (#NDC 0990–7983-02).

Animals

All mice were bred in-house using heterozygous × heterozygous breeding pairs of *Nape-pld* mutant mice kindly provided by Benjamin Cravatt (Leung et al., 2006). These mice were generated from 129SvJ-C57BL/6J and backcrossed for at least 10 generations onto a C57BL/6J background and validated using PCR, qPCR, and Western Blot (Supplemental Information Fig. 1). Wildtype (WT), heterozygous (HET) and *Nape-pld* knockout (KO) weanlings were housed 2 to 5 per cage under a 12-hour reverse light cycle (21:00 on/09:00 off) with ad-libitum access to standard chow and water, except when otherwise stated for specific experimental procedures. A total of 75 (36 male and 39 female) WT or *Nape-pld* KO mice 10–20 weeks of age were entered into the study. All behaviors were performed during the dark cycle and measured under controlled light conditions. All behavioral testing was performed by the same observers who were blinded to the genotype and treatment condition by another investigator, and the observers were unblinded at the conclusion of the experiment. All protocols and experiments were approved by the Virginia Tech (Blacksburg, VA, USA) Institutional Animal Care and Use Committee (IACUC) and complied with the ARRIVE guidelines (Percie du Sert et al., 2020).

Behavioral testing

Affective Behavioral Battery: Mice were evaluated in a behavioral battery to investigate the effects of sex (male vs. female) and genotype (WT vs. NAPE-PLD KO) on baseline affective behavior (Fig. 1), with one test per week over the course of 9 weeks. In order, mice were evaluated

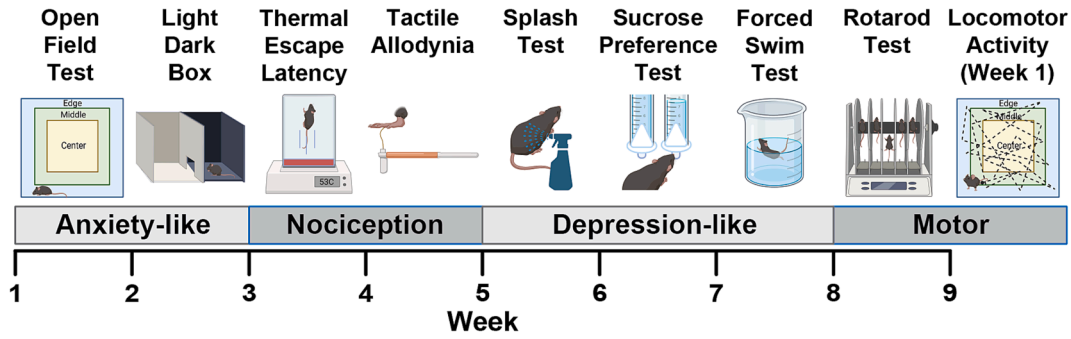


Fig. 1. Experimental timeline for behavioral characterization of male and female *Nape-pld* KO mice. All images were generated using BioRender.

for anxiety-like behavior (Open Field Test, Light-Dark Box), nociceptive responses (Thermal Escape Latency, Tactile Allodynia), depression-like behavior (Splash Test, Sucrose-Preference Test, Forced Swim Test), and motor function (Rotarod Test). Locomotor activity was determined from data collected during Open Field Test.

Open Field Test: Mice were tested as previously described (Johnson et al., 2021). Briefly, mice were acclimated to the testing room in their home cage with cage lids open for a minimum of 30 min. At the start of testing, mice were placed in the corner of an opaque testing arena (43 cm × 43 cm × 43 cm) under illuminated conditions (200 lx) and their movements were recorded for 10 min with an overhead camera. The latency to first enter the center space, amount of time spent in the center space (20 cm × 20 cm) and edge (3.5 cm from each wall) spaces, the total number of entries into the edge and center spaces, and the total distance traveled were measured using ANY-maze tracking software (Stoelting Co., version 5.25).

Light Dark Box: Mice were tested as previously described (Alkhlaif et al., 2017). Briefly, mice were acclimated to the testing room in their home cage with cage lids open for a minimum of 30 min. A black acrylic rectangular insert (43 cm × 15 cm × 43 cm) was placed into an opaque testing arena (43 cm × 43 cm × 43 cm) to create a division of light (25–30 lx) and dark space (<3 lx), with a small opening in one wall that permitted mice to cross between these spaces. At the start of testing, mice were placed into the dark space and their movements were recorded for 10 min with an overhead camera. The amount of time spent in the light and dark spaces, the number of entries into the light and dark spaces, and the mean light and dark space visit lengths were measured using ANY-maze tracking software (Stoelting Co., version 5.25).

Thermal Escape Latency (hotplate): Thermal thresholds were tested as previously described (Naidu et al., 2010). Briefly, mice were habituated to the testing room in their home cages for a minimum of 1 h with cage lids open. They were then individually acclimated to the hot plate apparatus (IITC, Part #39) within an acrylic cylinder (IITC, Part #39ME, 9 cm diameter, 30 cm height) for a minimum of 10 min while turned off, and baseline measurements were taken once before experimental evaluation. For all testing procedures, the hot plate was set to 53℃ with a cutoff time of 20 s to prevent tissue damage, and then mice were placed into the cylinder. Each mouse was measured three times and then averaged to report mean thermal paw withdrawal latency (*i.e.* shaking, lifting or licking the paw, or jumping) using a hand timer.

Tactile Allodynia: Tactile allodynia was evaluated using manual von Frey filaments with buckling forces between 0.02 and 2 g (Touch Test, Stoelting Co.) applied to the mid-plantar surface of each hindpaw using the up-down method (Chaplan et al., 1994; Gregus et al., 2018). Mice were habituated to the testing room and apparatus once before collecting data. Prior to testing, mice were acclimated to the testing room in a 4-sided acrylic chamber with only one transparent wall (3 × 3 × 7.5 in.) placed on a metal mesh grid under controlled lighting conditions (~100 lx) for a minimum of 60 min (baseline measurements) or 15 min (experimental timepoints). Any mouse with a basal 50% paw withdrawal threshold (PWT) ≤ 0.79 g was excluded from the study. For baseline measurements, PWTs from both hindpaws were averaged; for CFA, PWTs from the hindpaw ipsilateral to injection (left) were reported. Data were expressed as 50% gram thresholds vs time or as area under the curve (hyperalgesic index % change from baseline).

Splash Test: Mice were tested as previously described (Hodes et al., 2015). Briefly, mice were acclimated in a 4-sided acrylic chamber with only one transparent wall (12.5 × 7 × 7.25 in.) containing a mirror opposite to the high-resolution video camera (Logitech C920) for a minimum of 30 min under controlled lighting conditions (25–30 lx). At the start of testing, mice were sprayed three times with water on the backside with a 4 oz spray bottle, then returned to the chamber for evaluation of grooming behavior (20 min). The latency to the first grooming episode and total time spent grooming was recorded.

Forced Swim Test: Mice were tested as previously described (Can et al., 2012). Briefly, mice were acclimated to the testing room in their home cage with cage lids open for a minimum of 30 min. At the start of testing, mice were placed in a 5000 mL beaker filled with 2500 mL of water (25 ± 0.5 ℃) and recorded for 6 min. The latency to the first immobile episode (during all 6 min) and total immobility time amount (during the last 4 min) was recorded.

Sucrose Preference Test: Mice were tested using an open source two-bottle choice apparatus with infrared sensors to detect real-time interactions as previously described (Godynyuk et al., 2019). One sipper tube contained 1% sucrose (1 g of sucrose mixed with 100 g of tap water, made fresh daily) and the other sipper tube contained tap water (prepared fresh daily). Mice were habituated to the two-bottle choice apparatus in their home cages for 48 h, with position of the sucrose tube switched at 24 h. During training, mice were individually placed into a standard home cage with IsoPad bedding and two-bottle choice apparatus for a 2-hour session on three consecutive days. During testing, mice were deprived of water for 24 h and subsequently tested as done during training. Any cage that exhibited substantial liquid below the apparatus indicated a technical malfunction of the sipper tube(s), so these mice were excluded from the final analysis. Each bottle was weighed before and after testing, and the sucrose preference was calculated as the percentage of sucrose consumed relative to the total amount of liquid consumed. Additionally, the total number of sucrose sips during the session was determined by infrared sensor reporting.

Rotarod Test: Mice were tested for motor function as previously described (Brickler et al., 2016). Mice were habituated to the testing room in their home cage with cage lids open for a minimum of 30 min. During testing, mice were placed on the Rotarod apparatus (Economex Rotarod, Columbus Instruments, Part# 0201–003 M) set to an initial velocity (10 rpm) with an acceleration of 0.1 rpm/sec. The latency to fall from the rotarod was measured using a hand timer. All animals were tested 3 times with at least a 2-minute resting period after each test. Any mice that fell initially within 20 s were immediately placed back on the rotarod for continued testing.

Complete Freund's adjuvant model of arthritis

The Complete Freund's Adjuvant (CFA) model of arthritis was induced as previously described (Urban et al., 2011). Mice were briefly anesthetized with isoflurane via low-flow vaporizer (Somnosuite, Kent Scientific) and injected with 10 μl of vehicle (saline) or 100% CFA into the left hind paw (day 0), and subsequently tested for clinical and behavioral signs of arthritis for up to 16 days including grip strength, paw edema, and tactile allodynia. Paw edema was measured using digital calipers (Mitutoyo Corporation Digimatic Caliper #500–196-30) in the ipsilateral and contralateral paw (Ghosh et al., 2013), where each paw was measured three times (reported as the mean of respective paw). Grip strength was evaluated using a grip force meter (BIOSEB #BIO-GS3) using BIO-CIS response analysis software according to a previous report (Montilla-García et al., 2017). Briefly, mice were allowed to grasp a metal grid (BIOSEB #BIO-GRIPGS) and their tails were gently pulled by hand for 3 s to measure the maximal force exerted by the mouse before releasing the grid. Each mouse was measured three times and then averaged to report mean grip force. A systematic review and *meta*-analysis of behavioral outcome measures for intraplantar CFA model of arthritis demonstrates that open field, light–dark box, forced swim test, and sucrose preference test exhibit weak or no correlation with significant effects, so we elected not to examine these parameters in this model (Burek et al., 2022).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 9.4.1). All data are reported as mean ± SEM, and individual data points are indicated where applicable. All baseline behavioral tests were analyzed using 2-way ANOVA (sex × genotype) and Bonferroni post hoc, with all ANOVA statistics and post hoc p-values reported in Table 1. For the CFA experiments, CFA pain study was analyzed by 3-way repeated measures ANOVA (sex × genotype × time) followed by Tukey's post hoc with all ANOVA statistics and post hoc p-values reported in Table 2. Statistical outliers were determined using Grubb's Test. In the event of multiple outliers within the same genotype and sex, the individual with the higher z-value was removed. The criteria for significance were as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Results

Sex-dependent effects on some baseline anxiety-like behaviors

To evaluate the role of both sex and *Nape-pld* genotype in anxiety-like behavior, we examined WT and KO mice of both sexes in the open field and light–dark box tests. The complete statistical results for these experiments can be found in Table 1. In the open field test, two-way ANOVA revealed that there were no significant effects of *Nape-pld* genotype or sex on the time spent in the center of the apparatus (Fig. 2**A**), latency to the first entry into the center (Fig. 2**B**) or the total number of times the mice crossed between the center and edge of the apparatus (Supplemental Fig. 2**A**). In the light–dark box test, there was an effect of sex, but not genotype, in the total amount of time (Fig. 2**C**) and mean visit length in the light side of the box (Fig. 2**D**), with male *Nape-pld KO* spending significantly more time in and making longer visits to the light than female *Nape-pld KO* mice. In contrast, there were no differences in the total number of crossings between light and dark (Supplemental Fig. 2**B**). Collectively, these observations indicate that deletion of *Nape-pld* unmasks a phenotypic sex difference wherein males exhibit less of some anxiety-like behaviors than their female counterparts.

Sex-dependent effects on baseline nociceptive behaviors

Next, we evaluated baseline nociception in WT and KO mice of both sexes in the hotplate (thermal) and von Frey tests (tactile). Two-way

Table 1
Statistics for all behavioral evaluations using 2-way ANOVA.

Figure	Behavioral Test	Factor	F-Value	P-value	Bonferroni
2A	Open Field Test (Center Time)	Genotype	F(1, 71) = 0.2378	0.6273	–
		Sex	F(1, 71) = 3.409	0.0690	–
		Genotype*Sex	F(1, 71) = 0.0179	0.8940	–
2B	Open Field Test (Center Latency)	Genotype	F(1, 71) = 0.8624	0.3562	–
		Sex	F(1, 71) = 0.1005	0.7521	–
		Genotype*Sex	F(1, 71) = 0.2163	0.6433	–
2C	Light Dark Box (Light Time)	Genotype	F(1, 68) = 0.4881	0.4871	WT (M vs F) = 0.8147
		Sex	F(1, 68) = 5.049	0.0279	KO (M vs F) = 0.0294
		Genotype*Sex	F(1, 68) = 0.9386	0.3361	
2D	Light Dark Box (Light Mean Visit)	Genotype	F(1, 67) = 0.2080	0.6498	WT (M vs F) = 0.9678
		Sex	F(1, 67) = 5.126	0.0268	KO (M vs F) = 0.0190
		Genotype*Sex	F(1, 67) = 1.417	0.2381	
3A	Hotplate Test (Response Latency)	Genotype	F(1, 70) = 0.7027	0.4047	WT (M vs F) = 0.0007
		Sex	F(1, 70) = 10.29	0.0020	KO (M vs F) = 0.9999
		Genotype*Sex	F(1, 70) = 6.391	0.0137	
3B	Von Frey Test (Tactile Threshold)	Genotype	F(1, 71) = 0.0076	0.9308	–
		Sex	F(1, 71) = 0.0731	0.7876	–
		Genotype*Sex	F(1, 71) = 0.2135	0.6455	–
4A	Splash Test (Grooming Time)	Genotype	F(1, 74) = 2.599	0.1112	–
		Sex	F(1, 74) = 2.516	0.1170	–
		Genotype*Sex	F(1, 74) = 1.445	0.2332	–
4B	Splash Test (Grooming Latency)	Genotype	F(1, 69) = 0.5876	0.4459	WT (M vs F) = 0.0064
		Sex	F(1, 69) = 6.936	0.0104	KO (M vs F) = 0.9999
		Genotype*Sex	F(1, 69) = 4.109	0.0465	
4C	Forced Swim Test (Immobility Time)	Genotype	F(1, 73) = 0.2544	0.6155	–
		Sex	F(1, 73) = 0.3903	0.5341	–
		Genotype*Sex	F(1, 73) = 0.1056	0.7461	–
4D	Forced Swim Test	Genotype	F(1, 70) = 0.1357	0.7137	WT (M vs F) = 0.1868

(*continued on next page*)

Table 1 (*continued*)

Figure	Behavioral Test	Factor	F-Value	P-value	Bonferroni
	(Immobility Latency)	Sex	F(1, 70) = 11.66	0.0011	KO (M vs F) = 0.0032
		Genotype*Sex	F(1, 70) = 0.6553	0.4210	
4E	Sucrose Preference (% Sucrose)	Genotype	F(1, 70) = 5.609	0.0206	M (WT vs KO) = 0.0817
		Sex	F(1, 70) = 0.3316	0.5665	F (WT vs KO) = 0.4439
		Genotype*Sex	F(1, 70) = 0.4918	0.4855	
4F	Sucrose Preference (# of Sips)	Genotype	F(1, 70) = 5.670	0.0200	M (WT vs KO) = 0.0043
		Sex	F(1, 70) = 0.0009	0.9767	F (WT vs KO) = 0.9999
		Genotype*Sex	F(1, 70) = 5.330	0.0239	
S2A	Open Field Test (Center Crossings)	Genotype	F(1, 71) = 0.0031	0.9555	–
		Sex	F(1, 71) = 0.0704	0.7915	–
		Genotype*Sex	F(1, 71) = 0.5486	0.4614	–
S2B	Locomotor Activity (Total Distance)	Genotype	F(1, 71) = 0.1784	0.6740	–
		Sex	F(1, 71) = 1.550	0.2172	–
		Genotype*Sex	F(1, 71) = 0.1597	0.6906	–
S2C	Light Dark Box (Light Dark Crossings)	Genotype	F(1, 67) = 0.0004	0.9849	–
		Sex	F(1, 67) = 1.748	0.1906	–
		Genotype*Sex	F(1, 67) = 0.4477	0.5057	–
S2D	Rotarod Test (Fall Latency)	Genotype	F(1, 71) = 3.657	0.0599	
		Sex	F(1, 71) = 0.3280	0.5687	–
		Genotype*Sex	F(1, 71) = 0.5377	0.4658	–

ANOVA revealed an effect of sex and an interaction between genotype and sex in thermal thresholds at 53 °C, wherein WT males exhibited significantly lower response latencies than WT females, an effect not observed in *Nape-pld* KO mice (Fig. 3**A**). However, there was no significant effect of sex or genotype on tactile withdrawal thresholds (Fig. 3**B**). These results show that WT female mice exhibit increased thresholds to a 53℃ thermal stimulus compared WT male mice, and this effect was lost in *Nape-pld* KOs as males and females had similar thermal response thresholds.

Sex- and Nape-pld-dependent effects on baseline depression-like behaviors

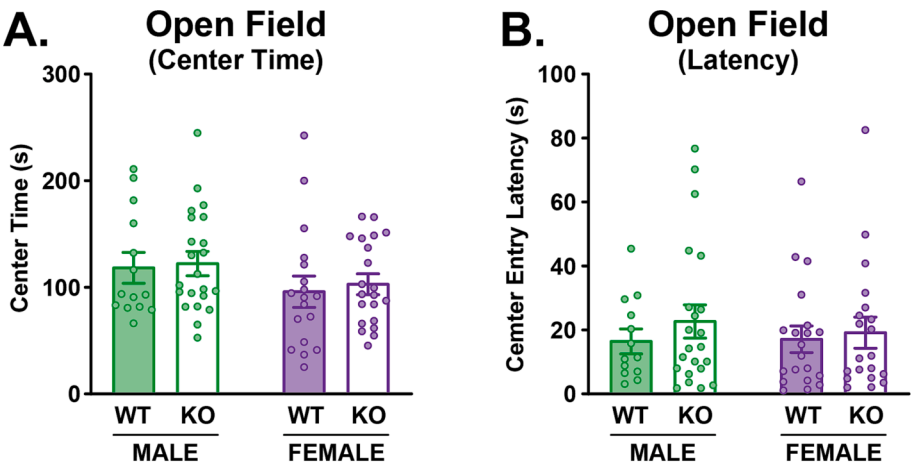
To evaluate the role of NAPE-PLD in depression-like behaviors, we examined WT and *Nape-pld* KO mice in the splash test, forced swim test, and sucrose preference test. In the splash test, two-way ANOVA revealed no significant effect of genotype or sex on the time spent grooming (Fig. 4**A**). However, we observed an effect of sex as well as an interaction between sex and genotype for latency to initiate grooming behaviors, in which WT males exhibited reduced latency relative to WT females. This sex difference is not present in *Nape-pld* KO mice (Fig. 4**B**). For the forced swim test, while there were no significant effects of genotype or sex on total immobility time (Fig. 4**C**), we noted a sex difference in latency to immobility wherein *Nape-pld* KO males initiated floating behaviors earlier than isogenic females (Fig. 4**D**). In contrast, in the sucrose preference test there was a significant effect of genotype (Fig. 4**E**), with male *Nape-pld* KO mice showing a trend towards reduced preference for 1% sucrose compared with male WT mice ($p = 0.08$). Similarly, we observed an effect of genotype as well as an interaction between genotype and sex in the number of sips of sucrose solution (Fig. 4**F**), wherein male *Nape-pld* KO mice took fewer sips of 1% sucrose compared with WT males. Taken together, these results indicate that *Nape-pld* contributes to some motivational depression-like behaviors.

Table 2
Statistics for all behavioral evaluations using 3-way ANOVA.

Figure	Behavioral Test	Factor	F-Value	P-value
5A	Paw Edema (Ispilateral)	Time	F(4.541, 172.6) = 39.66	<0.0001
		Genotype	F(1, 38) = 0.04884	0.8263
		Sex	F(1, 38) = 4.927	0.0325
		Time*Genotype	F(8, 304) = 0.5793	0.7948
		Time*Sex	F(8, 304) = 3.232	0.0015
		Genotype*Sex	F(1,38) = 5.227	0.0279
		Time*Genotype*Sex	F(8, 304) = 2.785	0.0055
5B	Tactile Allodynia (Ipsilateral)	Time	F(8, 552) = 138.4	<0.0001
		Genotype	F(1, 69) = 1.187	0.2796
		Sex	F(1, 69) = 9.040	0.0037
		Time*Genotype	F(8, 552) = 0.5749	0.7988
		Time*Sex	F(8, 552) = 4.678	<0.0001
		Genotype*Sex	F(1, 69) = 0.05516	0.8150
		Time*Genotype*Sex	F(8, 552) = 0.6125	0.7677
5C	Grip Force (change from baseline)	Time	F(6.357, 451.4) = 17.01	<0.0001
		Genotype	F(1, 71) = 0.9521	0.3325
		Sex	F(1, 71) = 0.005372	0.9418
		Time*Genotype	F(8, 568) = 3.647	0.0004
		Time*Sex	F(8, 568) = 1.435	0.1787
		Genotype*Sex	F(1, 71) = 0.07860	0.7800
		Time*Genotype*Sex	F(8, 568) = 0.8642	0.5466

No sex- or Nape-pld-dependent effects on motor function

To evaluate motor coordination and function, we examined WT and *Nape-pld* KO mice for performance in the rotarod test and locomotor activity from the open field test. We observed no *Nape-pld* genotype- or sex-dependent differences in locomotor activity based on total distance



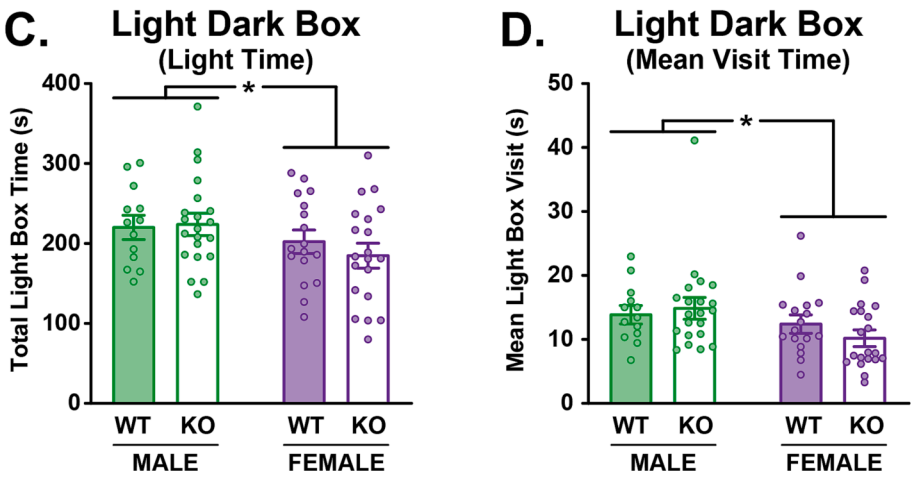


Fig. 2. Contribution of NAPE-PLD to baseline anxiety-like behaviors in males and females. (A) Time spent in the center of the open field test. (B) Latency to first enter the center of the open field apparatus. (C) Total time spent in the light side during the light–dark box test. (D) Mean time spent in a single light side visit during the light–dark box test. Data are presented as mean ± SEM, with WT (filled bars: males in green, n = 14–16; females in purple, n = 14–15) and *Nape-pld* KO mice (open bars: males in green, n = 14–16; females in purple n = 14–15). Statistical significance indicated by *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

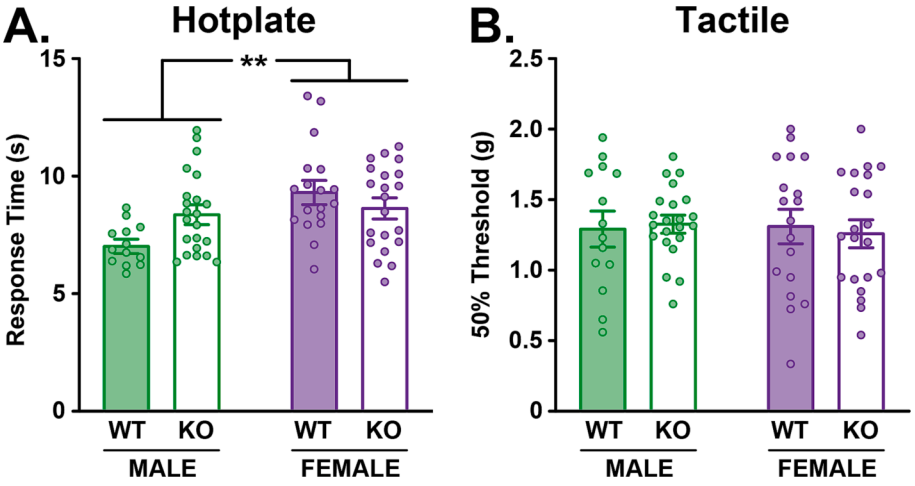


Fig. 3. Contribution of NAPE-PLD to baseline nociceptive thresholds in males and females. (A) Baseline average latency to withdrawal response in the hot plate test at 53 °C. (B) Baseline 50% tactile paw withdrawal threshold in the von Frey test. Data are presented as mean ± SEM, with WT (filled bars: males in green, n = 14; females in purple, n = 18) and *Nape-pld* KO mice (open bars: males in green, n = 22; females in purple n = 21). Statistical significance indicated by **P < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

traveled in the open field arena (Supplemental Fig. 2**C**) or in total time spent on the rotarod (Supplemental Fig. 2**D**) as revealed by two-way ANOVA. Overall, these results indicate intact motor function in all groups regardless of sex or expression of NAPE-PLD.

Sex-specific differences in peripheral inflammation and pain-like behaviors

To evaluate the role of NAPE-PLD in inflammatory hyperalgesia, CFA-induced arthritis was examined in WT and *Nape-pld* KO mice of both sexes. The complete statistical results for these experiments can be found in Table 2. We measured local inflammation (paw edema), von Frey (tactile allodynia), and grip strength deficit (grip force), a common rheumatological measure of functionality. Measurements of paw edema revealed a significant effect of time and sex (but not genotype), in which maximal swelling was evident on Days 1 and 3 in all groups post-injection by 3-way repeated measures ANOVA (Fig. 5**A**). Additionally,

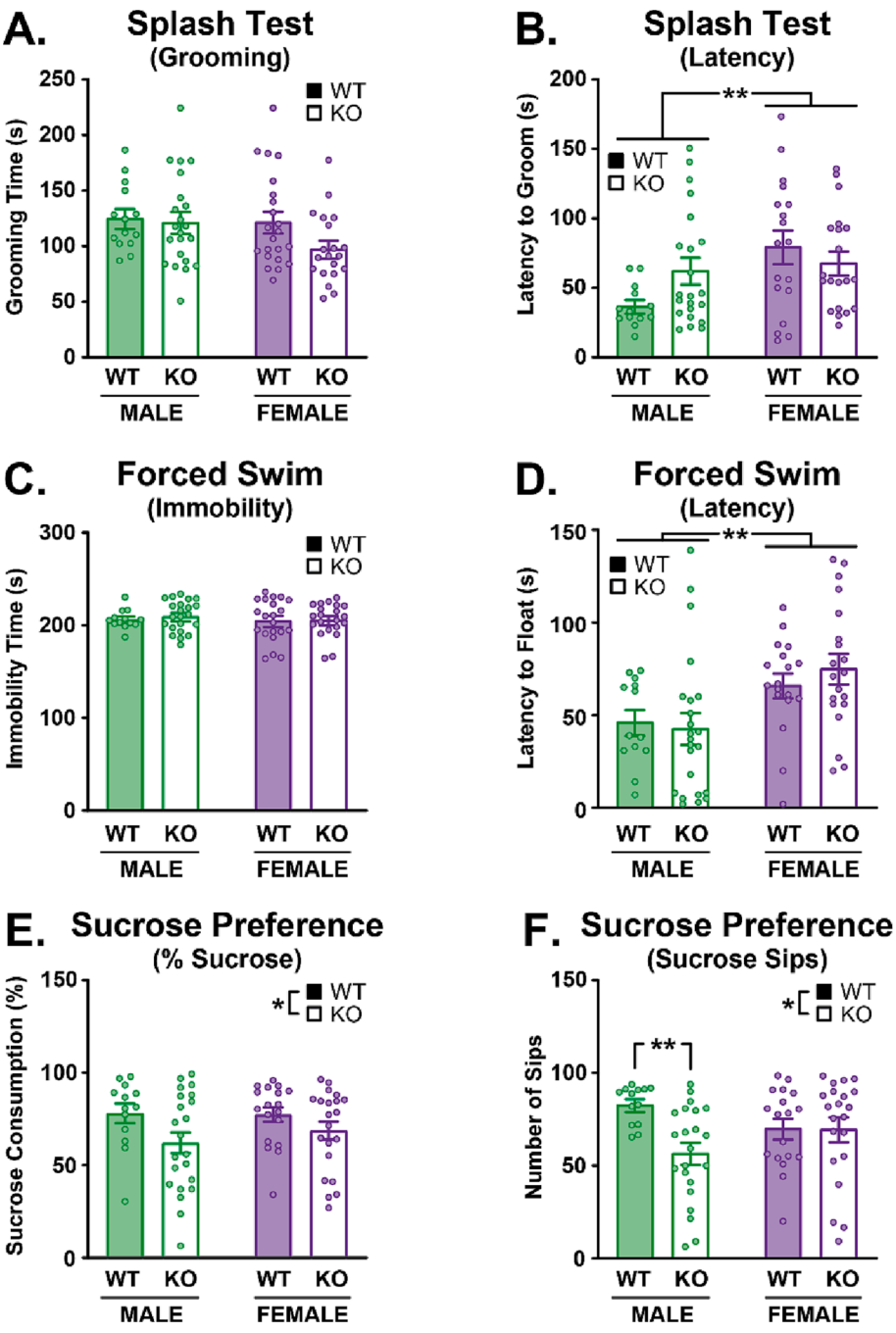


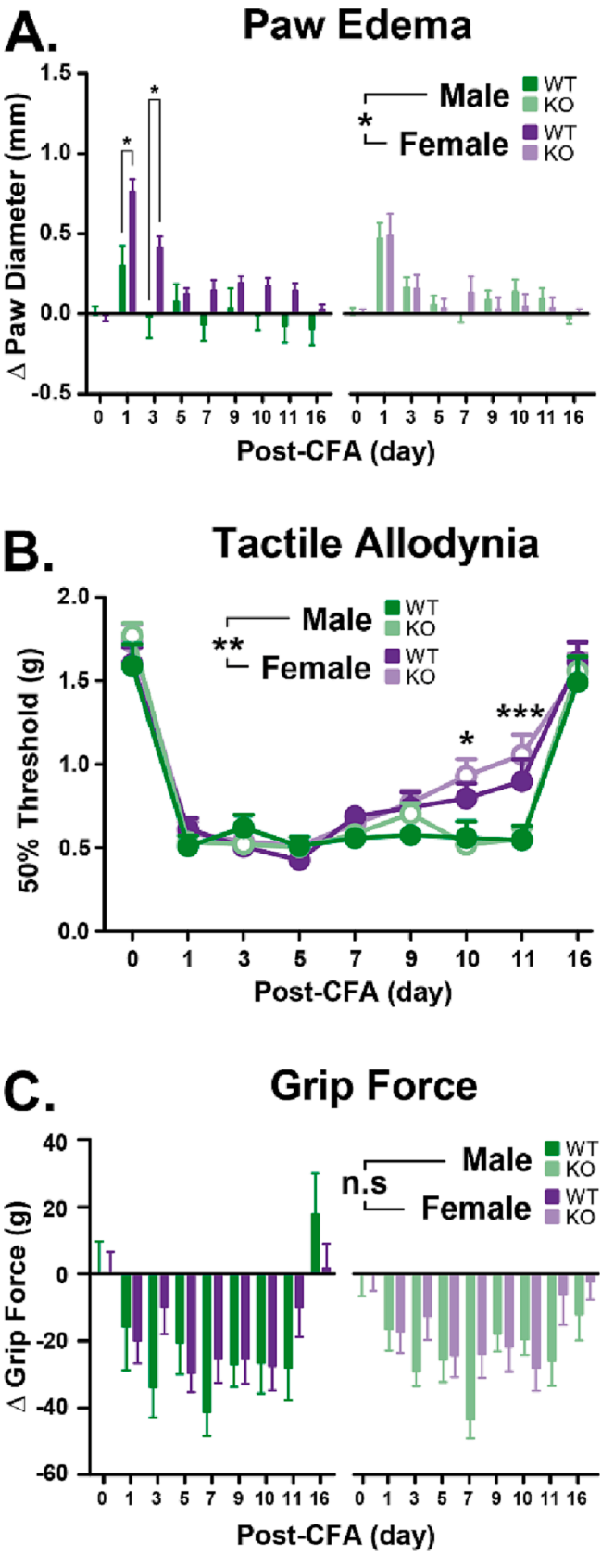
Fig. 4. Contribution of NAPE-PLD to depression-like behaviors in males and females. (A) Time spent grooming (s) and (B) Latency to first grooming (s) in the splash test. (C) Total immobility time (s) and (D) Latency to first immobility time (s) in the forced swim test. (E) Sucrose preference based on total grams consumed and (F) Number of sips of 1% sucrose solution during the sucrose preference test. Data are presented as mean ± SEM, with WT (filled bars: males in green, n = 13–14; females in purple, n = 18) and *Nape-pld* KO mice (open bars: males in green, n = 22; females in purple n = 20–21). Statistical significance indicated by *P < 0.05, **P < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

there was a significant interaction between time and sex, as well as genotype and sex, showing that inflammation in WT females was more pronounced and lasted longer compared with WT males, and this sex-dependent response was lost in *Nape-pld* KO mice.

Next, we determined the effects of *Nape-pld* on inflammatory hyperalgesia in both sexes using the clinically relevant rheumatological outputs of tactile allodynia and grip force. There was a significant effect of time and sex (but not genotype) on the development of CFA-induced tactile allodynia in which WT and *Nape-pld* KO mice of both sexes exhibited reduced paw withdrawal thresholds within 1 day following CFA (Fig. 5**B**). In addition, we observed an interaction between time and sex, as the resolution of allodynia is expedited in *Nape-pld* KO females versus isogenic male littermates. Similarly, we detected a significant effect of time as well as an interaction between time and genotype on the development of grip force strength deficits, which emerged on Day 1 post-CFA and peaked at day 7 in males and trended toward a peak between days 5 and 10 for females (Fig. 5**C**). Collectively, these results demonstrate that while paw inflammation in *Nape-pld* KO mice is not different between sexes, pain-like behaviors began to resolve more quickly in females regardless of genotype.

Discussion

Multiple studies have investigated the physiological roles of NAE signaling by targeting metabolism via FAAH inactivation or downstream receptor activity, but the current work is the first to comprehensively evaluate the behavioral effects of long-term inactivation of NAE biosynthesis by *Nape-pld* KO in mice. Accordingly, our findings reveal that *Nape-pld* KO mice exhibit a subset of behavioral changes previously identified as a result of manipulation of FAAH. Specifically, deletion of



(caption on next column)

Fig. 5. Evaluation of arthritis pain model using CFA in *Nape-pld* KO male and female mice. Time courses of changes in (A) Ipsilateral paw diameter (mm), (B) 50% tactile withdrawal threshold (g) and (C) Grip force (g) from the mean following induction of CFA. Data are presented as mean ± SEM, with WT (left: males in dark green, n = 10–23; females in dark purple, n = 12–18) and NAPE-PLD KO mice (right: males in light green, n = 13–22; females in light purple n = 17–21); the lower # in the range represents n for paw edema experiment. Statistical significance indicated by *P < 0.05, **P < 0.01, ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nape-pld reduced sucrose preference, but not other baseline anxiety-like or depression-like behaviors in naïve mice. In addition, we observed sex differences in multiple baseline measures in WT mice that were absent in *Nape-pld* KO mice. We did not identify any genotypic changes on baseline motor function. Finally, while CFA-induced inflammatory arthritis developed differently in male and female mice, we did not observe any specific effect of *Nape-pld* KO.

The principal genotypic effect of *Nape-pld* inactivation we observed was altered sucrose preference behavior. We found that *Nape-pld* KO mice exhibited less preference for sucrose and less sucrose drinking behavior, consistent with previous studies implicating endocannabinoid signaling in anhedonic aspects of depression-like behavior. Exogenous administration of AEA increased sucrose consumption, whereas systemic administration of a CB1 receptor antagonist decreased sucrose intake in male rats (Higgs et al., 2003). Non-cannabinergic receptor signaling is unlikely to facilitate this aspect of anhedonic behavior as neither TRPV1 (male or female) nor PPARα (male) knockout mice exhibited a difference in sucrose preference (Ellingson et al., 2009; Middleton et al., 1988). Anhedonic differences may also be mediated by stress, as systemic inactivation of FAAH failed to alter sucrose preference in unstressed animals (Bortolato et al., 2007; Rademacher and Hillard, 2007), suggesting that basal endogenous NAE tone is sufficient to support typical hedonic behaviors in naïve mice. However, FAAH inhibitors reduced sucrose preference following social isolation (Carnevali et al., 2020) or stress (Bortolato et al., 2007; Rademacher and Hillard, 2007). Collectively, these studies suggest that anxiogenic or depressive states may be mediated by other NAEs, since exogenous OEA and PEA also prevent stress-induced changes in sucrose preference (Jin et al., 2015; Li et al., 2019). Despite our findings that NAPE-PLD inactivation had no effect on immobility time during the forced swim test, multiple lines of evidence suggest that pharmacological activation of NAE signaling elicits anti-depressive effects during this task. FAAH inhibitors elevate endogenous NAE levels, and subsequently reduce forced swim immobility time in mice (Gobbi et al., 2005; Griebel et al., 2018; Jankovic et al., 2020). These effects can be recapitulated by activation of CB1 with HU-210 in rats (Hill and Gorzalka, 2005) as well as central activation of PPARα with fenofibrate (Jiang et al., 2017). Thus, pharmacological activation of NAE signaling reduces depression-like behavior during the forced swim test that is not induced by inactivation of NAPE-PLD and thus may rely on other NAE biosynthetic pathways. Collectively, these findings support an important role for *Nape-pld* in mediating the interaction between stress and anhedonic behavior.

Additionally, our findings showed that WT male mice were more sensitive to noxious heat than females in the hotplate test, corroborating findings in rats (Vierck et al., 2008) but in contrast with previous work in mice showing no sex differences in hotplate response latency (Leo et al., 2008). This sex-dependent thermal response sensitivity is lost in *Nape-pld* KO mice, suggesting that NAEs contribute to basal thermal nociception. Males have higher NAE levels and lower expression of FAAH in the amygdala, a key hub for nociceptive processing (Gray et al., 2016; Krebs-Kraft et al., 2010; Natividad et al., 2017). Thus, enhanced thermal sensitivity in males may result from increased NAE activation of pronociceptive signaling via TRPV1, as TRPV1 KO mice (Bolcskei et al., 2005) and rats (Huda et al., 2018) both have increased thermal latency in the hotplate test, and FAAH KO mice unmask endogenous NAE-

dependent TRPV1 thermal nociception (Carey et al., 2016). It is also possible that this sex-dependent thermal response could be explained by decreased CB1 and/or CB2 sensitivity, as multiple studies indicate that the analgesic effect of cannabinoids on thermal response latency may have more efficacy in female rats using the tail flick assay (Craft and Leitl, 2008; Craft et al., 2012; Marusich et al., 2015). However, it is less likely that this sex difference results from disparities in PPARα-mediated signaling, as multiple studies assessing the role of sex indicate that this receptor produces pro-nociceptive responses mainly in male rodents. Chemotherapy-induced peripheral neuropathy results in persistent allodynia in male mice that can be reversed by systemic PEA administration, an effect that is blocked by co-administration of the PPARα antagonist GW6471 (Donvito et al., 2016). In a mouse model of spinal nerve injury, fenofibrate produces anti-allodynic effects in males – but not females – that are reversed by administration of the PPARα antagonist GW6471 (Sorge et al., 2015). However, since these studies evaluated tactile pain hypersensitivity, a role for PPARα in baseline thermal response latency cannot be ruled out.

Our findings suggest that NAPE-PLD does not play a critical role in the development of inflammatory arthritis. We found no impact of *Nape-pld* KO on clinically relevant evoked (tactile) and functional (grip force) measures of pain-like behaviors during inflammatory arthritis, as genetic inactivation did not alter the intensity or duration of allodynia following induction of CFA arthritis. In contrast, acute treatment with a FAAH inhibitor following CFA arthritis increases NAE levels to produce robust anti-allodynia in male mice (Holt et al., 2005) and rats (Ahn et al., 2009). Similar effects were seen in the Collagen-Induced Arthritis model, where long-term chemical or genetic inactivation of FAAH attenuates allodynia in male mice (Kinsey et al., 2011). These studies all indicate that CB1, and possibly CB2, mediate therapeutic actions of FAAH-produced NAE signaling, as FAAH actions are mitigated by treatment with CB1 antagonists. In the Collagen-Induced Arthritis model, daily administration of PEA reverses allodynia after five days of treatment, suggesting that PPARα may also help mitigate arthritis-induced nociception (Impellizzeri et al., 2013). Accordingly, multiple studies indicate that the PPARα agonist fenofibrate may have clinical efficacy in treating pain in patients with rheumatoid arthritis (van Eekeren et al., 2013). In the carrageenan model, the anti-allodynic effects of FAAH inhibitor are blocked by GW647 suggesting a potential role for NAE signaling via PPARα during treatment of arthritis (Jhaveri et al., 2008). These findings indicate that NAEs produced by NAPE-PLD have a therapeutic but not causative role in the development of nociception during arthritis.

We did observe sex-dependent effects in CFA-induced arthritis, wherein females exhibited greater edema during arthritis induction, yet their allodynia resolved earlier than male counterparts. These results are congruent with clinical studies reporting that women with rheumatoid arthritis develop more severe swelling than men (Intriago et al., 2019), yet similar studies examining mice of both sexes during arthritis show mixed results. A murine model of psoriatic arthritis produces more significant paw edema in females (Haley et al., 2021), whereas other studies in mice using either CFA- (Bryant et al., 2019; Chillingworth et al., 2006) or K/BxN-induced arthritis (Woller et al., 2019) displayed no sex differences in mice. Our study used higher animal numbers per group with greater statistical power than previous studies, which may explain the difference in our findings. We observed no effect of NAPE-PLD inactivation on paw edema, consistent with findings showing that FAAH inhibitors also fail to alter paw swelling following intraplantar carrageenan injection in rats (Okine et al., 2012). Taken together, these results suggest that endogenous NAEs do not play a critical role in the development of global inflammation during arthritis. Likewise, prior literature examining time courses of arthritis-induced allodynia in male and female rodents also reports conflicting findings. The K/BxN model of arthritis produces longer lasting allodynia in male mice (Woller et al., 2019), whereas similar studies using CFA in mice show no significant sex differences in nociception (Bryant et al., 2019; Cook and Nickerson, 2005). Our study showing that allodynia resolves faster in wild-type female mice incorporated greater statistical power, and replicated these results in NAPE-PLD knockout mice that showed the same sex-specific effect. While these findings indicate that endogenous NAEs do not play a critical role in the resolution of pain hypersensitivity during CFA arthritis, they suggest that future studies using this model should be performed with appropriately powered male and female cohorts instead of a mixed-sex design.

Taken together, our findings suggest that NAPE-PLD likely would not be a high value target for treating inflammatory arthritis. However, our results do not preclude involvement of NAPE-PLD in the development of neuropathic pain states following chemotherapy (Noya-Riobó et al., 2023) or nerve injury (Bishay et al., 2010; Jee Kim et al., 2018; Palazzo et al., 2012). It also is possible that deletion of *Nape-pld* may alter other aspects of pain hypersensitivity including spontaneous, affective, or cognitive functions using additional output measures, which could be a focus of future research. Importantly, the lack of deficits in motor function or anxiety-like behaviors in KO mice indicates that chronic pharmacological inactivation of NAPE-PLD likely will be well-tolerated, warranting further evaluation of this target in other appropriate disease indications (Mock et al., 2020).

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Author contributions

MWB. and AMG conceived of the project and designed experiments. IC, LBM, CM, YD, and AMG performed experiments. IC, LBM, CM, YD, AMG, and MWB analyzed data. IC, LBM, AMG, and MWB wrote the first draft of the manuscript, and all authors edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynpai.2023.100135.

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