The endocannabinoid system is a promising target to mitigate pain as the endocannabinoids are endogenous ligands of the pain-mediating receptors—cannabinoid receptors 1 and 2 (CB1 and CB2) and TRPV1. Herein, we report on a class of lipids formed by the epoxidation of N-arachidonoyl-dopamine (NADA) and N-arachidonoyl-serotonin (NASHT) by epoxygenases. EpoNADA and epoNASHT are dual-functional rheostat modulators of the endocannabinoid-TRPV1 axis. EpoNADA and epoNASHT are stronger modulators of TRPV1 than either NADA or NASHT, and epoNASHT displays a significantly stronger inhibition on TRPV1-mediated responses in primary afferent neurons. Moreover, epoNASHT is a full CB1 agonist. These epoxides reduce the pro-inflammatory biomarkers IL-6, IL-1β, TNF-α and nitrous oxide and raise anti-inflammatory IL-10 cytokine in activated microglial cells. The epoxides are spontaneously generated by activated microglia cells and their formation is potentiated in the presence of anandamide. Detailed kinetics and molecular dynamics simulation studies provide evidence for this potentiation using the epoxygenase human CYP2J2. Taken together, inflammation leads to an increase in the metabolism of NADA, NASHT and other eCBs by epoxygenases to form the corresponding epoxides. The epoxide metabolites are bioactive lipids that are potent, multi-faceted molecules, capable of influencing the activity of CB1, CB2 and TRPV1 receptors.
opioids are highly addictive pain medications that are susceptible for abuse. The age-adjusted death rate by opioid overdose was determined to be nearly 20 per 100,000 people in the United States in 2016, according to a report by the Centers for Disease Control and Prevention. Hence, there is a need for therapeutic alternatives to opioids that combat inflammation and the associated pain.

Pain is regulated primarily by sensory afferent neurons and immune cells. Both of these cell types are rich sources of lipid mediators. Lipid mediators are generated via the enzymatic oxidation of dietary omega-3 and omega-6 polyunsaturated fatty acids (PUFAs). The pro-inflammatory lipid mediators contribute to pain sensitivity by activating the GPCRs in the sensory neurons to increase membrane excitability and pain response. Non-steroidal anti-inflammatory drugs (NSAIDs) are used to inhibit cyclooxygenases, leading to a decrease in the synthesis of pro-inflammatory lipid metabolites such as prostaglandin E2 (PGE2), thereby decreasing inflammatory pain. On the other hand, anti-inflammatory and pro-resolving lipid mediators suppress and resolve the inflammatory process, and thus attenuate inflammatory pain. Hence, lipid mediators can fine-tune the pain response and have been at the center for the development of alternative non-opioid pain therapeutics.

Additionally, cannabis has been used for centuries to reduce nociceptive pain either alone or in combination with opioids. The primary components of cannabis interact in the body with cannabinoid receptors 1 and 2 (CB1 and CB2) and other GPCRs. An endogenous class of bioactive lipids, known as endocannabinoids (eCBs), activates CB receptors and suppresses inflammation and pain sensitization. The eCBs are derivatives of dietary omega-3 and omega-6 PUFAs and are generated by damaged neurons and inflamed tissues. CB1 receptors are highly expressed in the central nervous system, mostly in the presynaptic region, and there is substantial CB1 expression in the nociceptive sensory neurons. It has been shown that under different pain conditions there is a concomitant increase in CB1 expression. Hence, there is sufficient evidence that CB1 mediates the psychotrópic effects of cannabinoids such as modulating nociceptive pain, as well as modulating inflammation. CB2 is mostly expressed in immune cells and mediates the anti-inflammatory effects of cannabinoids, which indirectly contributes to the antinociception of acute inflammatory pain. CB2 receptor activation exerts profound anti-nociceptive effects in animal models of acute, inflammatory, and neuropathic pain.

In addition to CB1 and CB2, a subclass of the eCBs act through transient receptor potential vanilloid 1 (TRPV1). TRPV1 is a non-selective cation channel that is activated by noxious temperatures, pH, and inflammatory agents. TRPV1 often spearheads nociceptive pain signaling, and thus antagonizing TRPV1 can reduce pain. Paradoxically, the activation of TRPV1 by small molecules such as capsaicin (CAP), the spicy component of chili peppers, can also alleviate pain by desensitizing TRPV1 signaling. TRPV1 also exhibits pro- and anti-inflammatory effects.

Recently, it has been postulated that there is a crosstalk between CB1 and TRPV1 receptors, which are co-localized in dorsal root ganglion (DRG) and in neuron-enriched mesencephalic cultures, hippocampus, and cerebellum. Therefore, the eCB system and TRPV1 axis provides a promising target to develop pain and inflammation therapeutics. For example, CMX-020 (Patent US8658632B2) is a novel drug based on the structure of eCBs and is in development to alleviate pain by binding to both cannabinoid receptors and TRPV1. Endogenous molecules and their synthetic derivatives may provide insight into effective therapeutic strategies with which to target the eCB-TRPV1 axis.

The best-studied eCB is anandamide (N-arachidonoyl-ethanolamine: AEA), which is derived from the omega-6 PUFAs arachidonic acid (AA) and docosahexaenoic acid (DHA). Besides AEA, other eCBs such as N-arachidonoyl-dopamine (NADA) and N-arachidonoyl-serotonin (NA5HT) are also derivatives of AA (Fig. 1). These dopamine and serotonin derivatives were identified in vivo in brain and intestinal tissues. It was shown that NADA binds with a higher affinity to CB1 than to CB2; however, conclusive evidence for NA5HT binding to CB1/2 has yet to emerge. Additionally, NADA was shown to be an agonist of TRPV1 and NA5HT is an antagonist of TRPV1, which results in analgesia. Hence, NADA and NA5HT are classified as endovanilloids (eVDs) for their actions at the TRPV1 receptor. Interestingly, NA5HT has low-affinity binding to DA receptors25, and the stimulation of degranulation in mast cells by NA5HT suggests that it does not activate the 5HT receptors26. Therefore, NADA and NA5HT do not display typical DA or 5HT responses and are instead regulators of TRPV1.

To add to the complexity of lipid metabolism, cytochromes P450 (CYPs) are known to epoxidize lipids into anti-inflammatory and anti-pain mediators that are more effective than the parent molecules. For example, CYPs convert AA and omega-3 PUFAs into epoxy-PUFAs that have been shown to decrease pain. Recently, the CYP-mediated metabolism of eCBs was shown to produce epoxy-eCBs that exhibit CB2 receptor selectivity and are anti-inflammatory and anti-tumorigenic. For instance, CYPs epoxidize AEA into epoxyicosatrienoic acid ethanamides (EET-EAs) that bind to CB2 receptors.

Herein, we evaluated whether epoxides of eVDs are more effective than parent eVDs at targeting cannabinoid receptors and TRPV1, and whether they are anti-inflammatory. We report a class of dual-functional epoxides of NADA and NA5HT (epoNADA and epoNA5HT, respectively) that reciprocally regulate both cannabinoid receptors (CB1 and CB2) and TRPV1 (Fig. 1).

We synthesized NADA, NA5HT, 14′-epoNADA (Fig. 2a) (Supplementary Figs. 1–5). As shown in Method 1, we developed an liquid chromatography with tandem mass spectrometry (LC-MS/MS) quantitation method in selected reaction monitoring (SRM) mode to quantify NADA, NA5HT do not display typical DA or 5HT responses and are instead regulators of TRPV1. We report a class of dual-functional epoxides of NADA and NA5HT (epoNADA and epoNA5HT, respectively) that reciprocally regulate both cannabinoid receptors (CB1 and CB2) and TRPV1 (Fig. 1). We synthesized NADA, NA5HT, 14′,15′-epoNADA, and 14′,15′-epoNA5HT. Using targeted lipidomics, we were able to identify the eVDs and epoxy-eVDs in porcine brain tissue, but because of the variability of the results we cannot definitively conclude their presence and levels in vivo. We then explored if NADA and NA5HT are potentially epoxidized under inflammatory conditions. We show that epoNADA and epoNA5HT are formed under inflammatory conditions by CYP epoxygenases and show that AEA potentiates the formation of epoNADA in microglial cells. We show that one potential mechanism of this potentiation may be through multiple-ligand binding to CYP-Nanodics using in vitro kinetics methods and molecular dynamics (MD) simulations. We further demonstrate that epoNA5HT is a potent TRPV1 antagonist suppressing intracellular Ca2+ response and membrane currents provoked by the TRPV1 ligand CAP in the primary afferent neurons. Altogether, we show that epoNADA and epoNA5HT act as dual CB1/2 and TRPV1 ligands and exhibit anti-inflammatory activity. These molecules are potential candidates for the development of pain therapeutics.

Results

Biosynthesis of epoxy-endovanilloids (eVDs). We first determined the levels of NADA and NA5HT in porcine brain as they express similar CYP isoforms to humans. As the epoxidation of PUFAs and eCBs by CYPs occurs mainly on the terminal alkene, we synthesized 14′,15′-epoNADA and 14′,15′-epoNA5HT (Fig. 2a) (Supplementary Figs. 1–5). As shown in Fig. 2b Method 1, we developed a liquid chromatography with tandem mass spectrometry (LC-MS/MS) quantitation method in the selected reaction monitoring (SRM) mode to quantify NADA, NA5HT do not display typical DA or 5HT responses and are instead regulators of TRPV1.
NA5HT, 14′,15′-epoNADA, 14′,15′-epoNA5HT, and AEA from brain tissue and microglial cells.

Herein, we measured 1.7 and 1.9 pmol of NADA (0.182 ± 0.081 pmol g⁻¹ wet tissue) and 11.3 and 4.9 pmol of NA5HT (0.686 ± 0.006 pmol g⁻¹ wet tissue) from the cerebella of two pig brains, and the values were very low in two more pig brains that were analyzed. From the hippocampus–thalamus–hypothalamus regions, we recovered 0.4 and 0.5 pmol of NADA (0.039 ± 0.009 pmol g⁻¹ wet tissue) and 0.2 and 0.1 pmol of NA5HT (0.010 ± 0.003 pmo g⁻¹ wet tissue) in two pig brains. The epoxy-eVDs levels were variable and often below detection limit due to the low abundance of their parent molecules. Overall, the levels of NADA and NA5HT are much less than AEA in rat and pig brains32 (Supplementary Note 1).

In the field of lipid metabolites, there is strong evidence that the production of lipid metabolites is localized to the site of action and that there is a rapid subsequent degradation37. This leads to low plasma/tissue levels of most lipid metabolites. Hence, we studied the epoxidation of NADA and NA5HT by CYP epoxygenases and test their effects on cannabinoid receptors and TRPV1.

**Epoxidation of eVDs in the presence of AEA.** The endogenous levels of AEA are much higher than NADA and NA5HT. Furthermore, it has been shown that various brain CYPs such as CYP2J2 and CYP2D6 convert AEA into AEA epoxides (EET-EAs). Therefore, to understand the substrate specificity of CYPs when both substrates (AEA and NADA or NA5HT) are present, we used the activated microglial cells to study the co-metabolism of AEA with NADA or NA5HT (Fig. 3a, b). Interestingly, we observed that AEA potentiated the formation of 14′,15′-epoNADA (~2-fold) in a concentration-dependent manner (Fig. 3a). Contrariwise, AEA inhibited 14′,15′-epoNA5HT formation (~0.5-fold) (Fig. 3b). It is

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**Fig. 1 The endovanilloid (eVD) pathway and structures of 14′,15′-epoNADA and 14′,15′-epoNA5HT.** NADA and NA5HT are synthesized by fatty acid amid hydrolase and other unidentified enzymes by the conjugation of arachidonic acid (AA) with dopamine (DA) or serotonin (5HT), respectively. NADA and NA5HT are known modulators of cannabinoid receptors and TRPV1; however, they do not activate DA or 5HT receptors. Herein, we study the epoxidation of NADA and NA5HT by CYP epoxygenases and test their effects on cannabinoid receptors and TRPV1.
possible that AEA acts as a potentiator of CYP-mediated NADA metabolism and an inhibitor of CYP-mediated NA5HT metabolism, either directly or indirectly. Several ligands have shown to either act as direct potentiators or inhibitors of CYP metabolism through cooperative or allosteric binding. To explore this possibility, we delineated the mechanism of eVD metabolism using a recombinantly expressed CYP.

Of the common CYP epoxygenases in mouse, CYP2J9 and CYP2J12 were shown to be highly expressed in mouse brain tissues while CYP2Cs showed low expression. We confirmed that CYP2J9 and CYP2J12 are expressed in the BV2 microglial cells used for our studies (Supplementary Fig. 6). CYP2J9 and CYP2J12 are highly homologous to human CYP2J2. Hence, we elucidated the biochemistry of eVD metabolism in the presence and absence of AEA by human CYP2J2.

Formation of epoxy-eVDs by human CYP2J2. CYP2J2 is highly expressed in the brain, cardiovascular, and cerebrovascular systems and is one of the major epoxygenases in these tissues. Additionally, CYP2J2 is known to epoxidize several endocannabinoids including AEA. In order to study the direct metabolism of NADA and NA5HT, we incubated CYP2J2 with each eVD and detected the metabolites using UV–Vis high-performance liquid chromatography (HPLC) and LC-MS/MS. All of the oxidized products of NADA (PD1-11) and NA5HT were detected.
PS1-7) are detailed in the Supplementary Figs. 7–18 and Supplementary Table 2. We determined that 14',15'-epoNADA (PD5) is a product based on the fragmentation and co-elution with the synthesized standard. PS2 was confirmed to be 14',15'-epoNA5HT. As a comparison to eVDs, we also investigated CAP as a substrate of CYP2J2, and found headgroup-oxidized products among other oxygenated products (Supplementary Figs. 19–26 and Supplementary Table 2).

Kinetics of eVD metabolism by CYP2J2-CPR-Nanodiscs. We next incorporated CYP2J2 and CPR into Nanodiscs (ND) and proceeded to determine the kinetics of the 14',15'-epoxide formation (Fig. 3). The metabolism of NA5HT by CYP2J2 is in a similar range as the metabolism of AEA and other lipid PUFAs, but the metabolism of NADA is low. Interestingly, the data demonstrate the presence of multiple binding sites, as the kinetics plots strongly deviate from a typical Michaelis-Menten model. The plots resemble the beginning of a sigmoidal curve indicating positive binding interactions. However, saturation was not achieved as the eVDs are poorly metabolized and insoluble beyond 100 µM (Fig. 3d and e). We therefore hypothesized that there are at least two binding sites. To further probe the kinetics

Fig. 3 Potentiation of eVD metabolism by AEA. a Metabolism 10 µM NADA and b 10 µM NA5HT in the presence of increasing concentrations of AEA by BV2 microglia. Metabolism was conducted similarly to data in Fig. 2c. Data shown are the mean ± SEM of n = 6 (two sets of triplicate performed on separate days). Statistical significance is based on a one-way ANOVA with a Tukey’s post-hoc. c Schematic of the CYP2J2-CPR-Nanodisc. d, e Metabolism of NADA and NA5HT by CYP2J2-CPR-NDs. Rates are in pmol_{epoxy-eVD} min^{-1} nmol_{CYP2J2}^{-1} and data represents the mean ± SEM of three independent experiments. f–i Co-substrate metabolism of eVDs with AEA. f AEA metabolism in the presence of NADA and g NA5HT. Rates are shown as the fraction of the V_{max} (135 pmol_{EET-EAs} min^{-1} nmol_{CYP2J2}^{-1}) for AEA metabolism (gray squares) as previously published (see text). h Fold change of NA5HT metabolism and i NADA metabolism in the presence of increasing AEA concentrations compared to the metabolism without AEA. f–i Data represents the mean ± SEM of three independent experiments. All data can be found in the Source Data file.
of eVD metabolism, we measured the rate of NADPH oxidation by CYP2J2-CPR-ND in the presence of the eVDs. CPR shuttles electrons from NADPH to CYPs to facilitate the metabolism of substrates. Therefore, the rate of NADPH oxidation increases in the presence of CYP substrates. In this case, NADPH oxidation in the presence of NA5HT showed biphasic kinetics, which is unique to this substrate as described in the supplementary section (Supplementary Fig. 27).

Relative binding affinities of NADA and NA5HT. As eCBs do not produce substantial Soret shift upon binding, we used an ebastine (EBS) competitive inhibition assay to measure the binding affinity of NADA and NA5HT. Both NADA and NA5HT displayed competitive inhibition of EBS binding (Eq. 2), suggesting that the binding of NADA and NA5HT overlap the binding of EBS (Kᵢ for NA5HT is 71.1 ± 20.0 μM and Kᵢ for NA5HT is 49.3 ± 6.2 μM).

Co-metabolism of AEA with NADA or NA5HT. We next determined the co-substrate kinetics of AEA with the eVDs to determine if we can explain the observed effects of AEA on BV2-mediated metabolism. We developed an LC-MS/MS method to simultaneously measure the four different regioisomers of AEA epoxides (EET-EAs) and either 14′,15′-epoNADA or 14′,15′-epoNA5HT (Fig. 2b, Method 2). We had previously determined the kinetics of AEA metabolism by CYP2J2-CPR-NDs, and we repeated these experiments using two concentrations (25 and 75 μM) of either NADA or NA5HT. NADA inhibited AEA metabolism following a competitive inhibition model (Eq. 2). A 3D global fit of the data (Supplementary Fig. 28) yields a Kᵢ of 7.50 ± 0.88 μM for the inhibition of AEA by NADA, which is among the strongest endogenous inhibitors of CYP2J2 as compared to virodhamine. NA5HT was a noncompetitive inhibitor (Eq. 3) at 25 μM (Kᵢ = 21.4 ± 3.6 μM) and a competitive inhibitor at 75 μM (Kᵢ = 86.6 ± 18.5 μM) (Fig. 3g). NADA and NA5HT also altered the regioselectivity of AEA epoxidation in a concentration-dependent manner (Supplementary Fig. 29). Interestingly, AEA showed a biphasic potentiation of NADA and NA5HT metabolism (Fig. 3h, i) when we measured the epoxy-eVD formation. Overall, the potentiation of eVD metabolism by AEA and the altered AEA regioselectivity in the presence of eVDs demonstrate that eVDs and AEA are binding to CYP2J2 at multiple sites. A further analysis of this multi-site binding is provided in the Supplementary Note 2 and Supplementary Fig S30. Furthermore, these data support the observed crosstalk of AEA and eVDs in microglial cells (Fig. 3a, b), as at similar concentrations within each experiment AEA potentiates NADA and inhibits NA5HT.

Molecular dynamics (MD) simulations of eVD binding to CYP2J2. In order to characterize the molecular basis of the multi-site kinetics observed with the eVDs, we performed MD simulations starting from membrane-bound structures of CYP2J2 in complex with substrates (AEA, NADA or NA5HT). Initial molecular docking was performed with AEA and either NADA or NA5HT in a stepwise manner. These models allowed us to probe the binding mode of a second molecule to CYP2J2 in the presence of another molecule bound in the active site in an unbiased manner (i.e., without any assumptions about location of peripheral binding pockets). Two distinct configurations of peripheral AEA binding, with either NADA or NA5HT in the active site, were identified (Fig. 4a, b). In Configuration 1, AEA was docked in a pocket located below the I-helix, with its ethanolamine group near a residue (R321) that we have previously identified to modulate PUFA binding (Fig. 4a). In Configuration 2, AEA was located closer to the membrane interface (Fig. 4b). For the two identified AEA binding configurations, the initial orientation of NADA or NA5HT in the active site was similar, with the main epoxidation site (carbons C14 and C15) close to the heme moiety (distance <5 Å), and the headgroup (DA or 5HT) pointing away from the heme. These docking results suggested that NADA/NA5HT binding was not modulated by the same PUFA-interacting residues previously reported (T318, R321 and S493). Owing to the larger headgroups of NADA/NA5HT compared to other PUFAs (i.e., DA/5HT vs. carboxylic acid), a different binding orientation (not interacting with the PUFA triad) was necessary to fit these molecules in the active site.

The MD simulations (Fig. 4c, Supplementary Tables 3-6, and Supplementary Movies 1-4) revealed that stable NADA/NA5HT binding (i.e., with the epoxidation site distance to the heme <5 Å) was only achieved when AEA is bound in Configuration 2 (Fig. 4b). When AEA is located in the I-helix pocket (Configuration 1), NADA or NA5HT in the active site gradually move away from the heme, which results in a displacement of its epoxidation site (with the distance to heme between 7.5 and 10 Å during the simulations) (Fig. 4c). In contrast, AEA in Configuration 2 constrains the motion of NADA or NA5HT in the active site, which maintain their potentially productive orientation close to the heme (epoxidation site to heme distance <5 Å) during the simulation (Fig. 4c). In these simulations, the ethanolamine group of AEA interacted with Q228, located near the membrane interface, and remained locked in its binding site. Positioning of AEA in turn constrained the mobility of the molecule in the active site (NADA or NA5HT). NADA/NA5HT are further stabilized by hydrophobic interactions (mainly with F310) and transient electrostatic interactions (i.e., NA5HT serotonin group with D307 and E222). These observations suggest that NADA/NA5HT binding is enhanced by concurrent AEA binding to a peripheral site near the membrane interface and provide insights into the protein residues involved in this binding (e.g., Q228 for AEA and F310 for NADA/NA5HT). Overall, the MD simulations in conjunction with the kinetics data concur with the observations from the cell culture studies that AEA enhances the metabolism of NADA.

Anti-inflammatory action of eVDs in microglial cells. We further proceeded to characterize their pharmacology. Previous studies have demonstrated the anti-inflammatory actions of eVDs, thus, we hypothesize that epoxy-eVDs would also be anti-inflammatory. Microglial cells are strongly activated after injury and release pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. Therefore, there is a significant interest in discovering lipid-based molecules that decrease microglial activation. To investigate the actions of eVDs and epoxy-eVDs, we measured the levels of pro-inflammatory nitric oxide (NO), IL-6, IL-1β, and TNF-α in lipopolysaccharide- (LPS)-stimulated BV2 cells. All eVDs and epoxy-eVDs dose-dependently reduced NO and IL-6 production (Fig. 5a-d) and the IC₅₀ values are tabulated in Table 1. Together, these data demonstrate that the eVDs and the epoxy-eVDs are anti-inflammatory mediators. As determined by MTT and BrdU assays, these compounds were not toxic at their effective concentrations, though NA5HT and 14′,15′-epo-NA5HT increased cell viability in the presence of LPS, suggesting they may be pro-proliferative, which was confirmed using a BrdU assay (Supplementary Fig. 31, 32).

We also measured the mRNA expression of IL-6, IL-10, IL-1β, and Tnf-α to determine the inflammatory phenotype. Whereas IL-6, IL-1β, and TNF-α are pro-inflammatory, IL-10 is an anti-inflammatory cytokine that facilitates the resolution of inflammation. Concentrations around the IC₅₀ of the IL-6 and NO experiments (1 and 2.5 μM of each compound) were tested and
compared to cells with LPS only. All the eVDs and epoxy-eVDs downregulated pro-inflammatory Il-6, Il-1β, and Tnf-α with 14′,15′-epo-NA5HT being the most effective overall (Fig. 5e–g).

NADA and 14′,15′-epoNADA concomitantly upregulated Il-10 (Fig. 5h).

Most eCBs and eVDs mediate anti-inflammatory and anti-pain poly-pharmacologically through CB1, CB2, and TRPV1 receptors. We determined that the mRNA of Cnr1 (CB1 gene), Cnr2 (CB2 gene), and Trpv1 are expressed in the BV2 cells (Supplementary Fig. 33). Since these receptors are known targets of eVDs and mediate inflammation and pain, we proceeded to measure the activation of CB1, CB2, and TRPV1 by epoxy-eVDs.

**Activation of TRPV1 by eVDs and epoxy-eVDs.** Previous studies have shown that NADA mediates anti-inflammation via TRPV134. As TRPV1 is a non-selective cation channel, activation was determined by measuring the relative Ca²⁺ influx (Supplementary Fig. 34 and Table 1). We determined that NADA and 14′,15′-epoNADA are full agonists, with ~80% activation compared to CAP (Fig. 5i). The EC₅₀ of 14′,15′-epoNADA is about 6-fold tighter than NADA. 14′,15′-epoNADA also promoted a broader duration of Ca²⁺ influx opening compared to NADA (Supplementary Fig. 34). The TRPV1-selective antagonist AMG-9810 was able to fully antagonize the signal from CAP, the eVDs, and the epoxy-eVDs, confirming the Ca²⁺ influx is TRPV1-mediated (Supplementary Fig. 35b, c).

On the contrary, 14′15′-epoNA5HT was found to be a partial agonist of TRPV1 up to 250 nM, with a 24% activation compared to CAP (Fig. 5j). This signal was antagonized by AMG-9810, demonstrating it is TRPV1-mediated (Supplementary Fig. 35c). Concentrations above 250 nM, however, resulted in a reduction of the signal, signifying that 14′,15′-epoNA5HT is an antagonist of TRPV1 at higher concentrations (Fig. 5j). Previously, NA5HT had been shown to be an antagonist of TRPV127; therefore, we measured the antagonism of TRPV1 by 14′15′-epoNA5HT and
Fig. 5 Anti-inflammatory actions of eVDs and receptor activation. **a–d** Anti-inflammatory effects of eVDs and epoxy-eVDs were determined by preincubating eVDs and epoxy-eVDs with BV2 cells for 4 h prior to stimulation by 25 ng mL⁻¹ LPS. Data represents the mean ± SEM of n = 3 independent experiments. a Dose-dependent inhibition of nitric oxide (NO) production by NA5HT and 14,15-epoNA5HT as determined by Griess Assay. b Dose-dependent reduction of IL-6 protein expression (ELISA) by NA5HT and 14,15-epoNA5HT. c Dose-dependent inhibition of NO production and (d) IL-6 protein expression by NADA and 14,15-epoNADA. 

**e–h** Anti-inflammation experiments were repeated to measure pro-inflammatory Il-6, Il-1β, and Tnf-α and anti-inflammatory Il-10 mRNA expression in the presence of LPS. Data represents the average of three technical replicates. Data is reported as the mean relative expression. i–k eVD activation to TRPV1-transfected HEK cells was determined using a Fura 2-AM Ca²⁺-influx assay. The B<sub>max</sub> of capsaicin (CAP) activation is defined as 100%. Data represents the mean ± SEM of n = 6 (two sets of triplicate experiments on separate days). k Antagonism was determined by preincubating cells with antagonist prior to stimulating with 250 nM CAP. l–n eVD activation of CB1 and CB2 was determined by the PRESTO-Tango assay. B<sub>max</sub> of CP-55940 is defined as 100%. Data represents the mean ± SEM of n = 6 (two sets of triplicate experiments on separate days). All data can be found in the Source Data file.
compared it to NA5HT. 14′15′-epoNA5HT functioned as an antagonist of CAP at all concentrations, with an IC50 of 250 ± 38 nM (Fig. 5k). Of note, this IC50 correlates to the concentration at which the self-antagonism was observed for 14′,15′-epo-NA5HT (Fig. 5j). From Table 1, we see that 14′,15′-epoNA5HT is a 30-fold stronger antagonist of TRPV1 than NA5HT.

**Activation of CB1 and CB2 by eVDs and epoxy-eVDs.** We next determined the activation of CB1 and CB2 by eVDs and the epoxy-eVDs using the PRESTO-Tango assay32. Herein, NADA, 14′,15′-epoNADA, and 14′,15′-epoNA5HT, but not NA5HT, demonstrated full-agonist activation of CB1 (~100% activation as compared to CP55940) (Fig. 5l, m, Table 1). Of these, NADA activates CB1 2-fold more potently as compared to 14′,15′-epoNADA. 14′,15′-epoNA5HT activated CB1 with an EC50 of 16.5 ± 11.7 nM. Only NA5HT activated CB2. The measured EC50 was 8.80 ± 5.57 nM with 19.2% activation compared to CP55940, which makes it a partial agonist.

We further tested agonism and antagonism of NA5HT, NADA, 14′,15′-epoNADA, and 14′,15′-epoNA5HT on CB1 and CB2 receptors. None of these antagonized 50 nM CP55940 activation of these receptors (Supplementary Fig. 36c). Therefore, NA5HT does not act on CB1, and NADA, 14′,15′-epoNADA, and 14′,15′-epoNA5HT do not act on CB2. This interesting dichotomy could be exploited to design pain therapeutics that specifically target one receptor. Overall, our data shows that 14′,15′-epoNA5HT is anti-inflammatory, is an agonist of CB1, and is an antagonist of TRPV1, thereby making it the most efficacious of the eVDs for the development of pain therapeutics.

**Inhibition of TRPV1 responses in primary DRG neurons.** To further determine if NA5HT and 14′,15′-epoNA5HT could inhibit native TRPV1 expressed in mouse dorsal root ganglia (DRG) neurons, we compared CAP-evoked intracellular free Ca2+ ([Ca2+]i) response in cultured mouse DRG neurons with and without pretreatment of NA5HT and 14′,15′-epoNA5HT using live-cell Ca2+ imaging. Bath application of CAP at 250 nM produced a robust [Ca2+]i increase in 47.12% ± 2.18% of DRG neurons (Fig. 6a, d). However, after pretreatment of 1 µM NA5HT or 1 µM 14′,15′-epoNA5HT for 10 min, 250 nM CAP could only activate 33.64% ± 1.16% and 20.01% ± 2.39% of DRG neurons, respectively (Fig. 6b–d). Consistent with the reduced number of DRG neuron activated by CAP after pretreatment of NA5HT and 14′,15′-epoNA5HT, the amplitude of CAP-induced [Ca2+]i increase was also significantly diminished after pretreatment of NA5HT and 14′,15′-epoNA5HT (Fig. 6b, c, e). We also examined the inhibitory effect of NA5HT and 14′,15′-epoNA5HT on CAP-induced excitation of DRG neurons by using current-clamp recording. Consistent with Ca2+ imaging results, CAP evoked a large membrane potential depolarization and robust action potential firing, which was also significantly inhibited by pretreatment with NA5HT or 14′,15′-epoNA5HT for 10 min (Fig. 6f–i). Of note, inhibition of TRPV1-mediated [Ca2+]i increase membrane potential depolarization by 14′,15′-epoNA5HT was significantly stronger than that produced by NA5HT (Fig. 6i), which is consistent with results in TRPV1-expressing HEK293 cells. Together, these results suggest that both NA5HT and epoNA5HT are potent TRPV1 antagonists suppressing TRPV1 function in both heterologous cells and native DRG neurons.

**Discussion**

There is evidence that there is a synergism between the eCB and the opioid system that reduces the need for high doses of opioids7. Hence, it is important to understand the function of eCBs and their metabolites as endogenous and exogenous ligands...
of the receptors that are involved in pain response modulation. It has been previously shown that the activation of CB1 and CB2 is associated with anti-nociceptive and anti-inflammatory actions\textsuperscript{10,54}. Regulation of TRPV1 can also modulate pain by mostly antagonizing TRPV1. While there are ample examples of drugs that target cannabinoid receptors or TRPV1, there is dearth of molecules that are rheostat regulators (varying strength of agonism or antagonism) of both cannabinoid receptors and TRPV1. The primary difference between many drugs and endogenous lipids is that the former are usually structurally rigid and target either CB receptors or TRPV1 whereas the latter are functionally plastic and capable of activating both receptors. NADA and NA\text{5HT} are AA derivatives of neurotransmitters. NADA is an agonist of both CB1 and TRPV1\textsuperscript{26,55}. NA\text{5HT} is an antagonist of TRPV1. PUFAs, through parallel pathways, are converted into epoxide mediators that have been shown to reduce pain\textsuperscript{30}. Overall, there is strong evidence for pain modulation by eCB and PUFA epoxides through multiple receptors.

Fig. 6 Inhibition of CAP-induced response in cultured mouse DRG neurons. a–c Ratiometric Ca\textsuperscript{2+} imaging of cultured wild-type mouse DRG neurons. Each trace corresponds to fluorescence in a single neuron. CAP elicited large [Ca\textsuperscript{2+}]\text{i} responses in cultured DRG neurons (a), which was significantly inhibited by pretreatment of NA\text{5HT} (b) and 14',15'-epoNA\text{5HT} (c) for 10 min, respectively. d Percentage of DRG neurons responding to CAP with or without pretreatment of NA\text{5HT} or 14',15'-epoNA\text{5HT}. (n = 5 from 4 mice; error represents SEM). P-values: 0.0012 (**), NASH\text{t}+CAP vs. Vehicle+CAP, 0.0011 (**), NASH\text{t}+CAP versus 14', 15'-epoNA\text{5HT}+CAP, and < 0.0001 (****). e Quantification of CAP-induced [Ca\textsuperscript{2+}]\text{i}, response with or without pretreatment of NA\text{5HT} or 14',15'-epoNA\text{5HT}. (n = 5 from 4 mice; error represents SEM). P-values: 0.0003 (**), 0.0003 (**), and < 0.0001 (****). (f) Representative traces showing CAP-induced a robust membrane depolarization and action potential firing in cultured DRG neurons, which was significantly inhibited by pretreatment of NASH\text{t} (g) and 14',15'-epoNASH\text{t} (h) for 10 min, respectively. (i) Summarized data showing that CAP-produced membrane depolarization and action potential firing in DRG neurons with or without pretreatment of NA\text{5HT} or 14',15'-epoNASH\text{t}. (n = 5 from 3 mice; error represents SEM). Drug concentration: CAP, 250 nM; NA\text{5HT}, 1 μM; 14',15'-epoNA\text{5HT}, 1 μM; and KCl, 100 mM. P-values: 0.0057 (**), and <0.0001 (****). (one-way ANOVA followed by a Tukey’s post-hoc analysis). All data can be found in the Source Data file.
Using a combined biophysical and cell-based approach, we report the pharmacological characterization of NADA and NA5HT epoxides that are produced by the CYP epoxygenase pathway in microglial cells. These molecules are anti-inflammatory and function through the eCB-TRPV1 axis. These results can potentially inspire new therapeutics that effectively target this axis. The overall findings of this work are outlined in Fig. 7 and discussed below.

The biosynthesis of the epoxy-eVDs is facilitated by CYP epoxygenases as the inhibition of CYP enzymes in microglial cells reduce the levels of these metabolites. In the pig brain, we were able to detect the parent compounds NADA and NA5HT, whose levels were much lower and variable than that of AEA. The epoNADA and epoNA5HT were spontaneously formed by microglia cells in the presence and absence of inflammatory stimulus. As the levels of AEA were high and eVDs were low, we tested the effect of eVD metabolism by CYPs in the presence of AEA. Interestingly, the metabolism of NADA is potentiated by AEA in the microglial cells. This was an interesting observation as there are very few examples where the binding of one ligand at the enzyme active site potentiates the metabolism of another ligand. However, such complicated substrate interactions are common for CYPs with a large active sites such as CYP3A41,42,56. We demonstrate that AEA is a complicated

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Fig. 7 Summary of the eVD metabolism pathway and pharmacology. The eVDs bind in a two-site model to CYP2J2 and other epoxygenases and are metabolized to form epoxy-eVDs. AEA potentiates the metabolism of eVDs as revealed by BV2 metabolism assays, in vitro CYP2J2 kinetics, and molecular dynamics simulations. 14',15'-epoNADA is a better TRPV1 agonist and slightly weaker CB1 agonist compared to NADA. 14',15'-epoNA5HT is a better TRPV1 antagonist compared to NA5HT, and is a CB1 full agonist as opposed to NASHT, which is a partial CB2 agonist. Overall, the eVDs and epoxy-eVDs potently downregulate pro-inflammatory NO production and IL-6, IL-1β, and TNF-α expression while increasing IL-10 expression, thereby demonstrating that they are potently anti-inflammatory.
The effector of eVD metabolism in CYP2J2, which potentially explains the observed potentiation of NADA metabolism in BV2 cells. However, AEA may also be inhibiting unknown enzymes that degrade NADA or 14′,15′-epoNADA.

We then proceeded to investigate the interactions of AEA and the eVDs at the enzymological level to gain better molecular insight into the metabolism. CYP2J2, CYP2J12, and CYP2J2 were all shown to be highly expressed in mouse and human brains. Additionally, CYPs such as CYP2J6 are expressed in DRGs and is involved in the epoxidation of lipids that act on TRPV1 when pacitaxel is administered. In addition, CYP2J4 is found in TRPV1-postive rat trigeminal ganglia, which are also involved in pain-temperature sensing pathways. Comparing the in cellular metabolism data to the in vitro kinetic measurements with CYP2J2-NDs reveals a complex network of substrate-substrate interactions. Previously, we determined that PUFAs bind CYP2J2 at two main sites: the substrate access channel and the substrate interactions. Previously, we determined that PUFAs in cellulo metabolism data to the in vitro kinetic measurements and NA5HT by CYP2J2 in vitro. Therefore, this study provides molecules. CYP2U1 metabolizes NA5HT to a 2-oxo derivative that is a potent antagonist of TRPV1. The epoxy-eVDs are potently activating at TRPV1 and alters their pharmacology at cannabinoid receptors. In particular, 14′,15′-epoNASHT is the most effective epoxy-eVD at reducing pro-inflammatory markers. 14′,15′-epoNASHT is also a potent antagonist of TRPV1 expressed in either HEK293 cells or native DRG neurons and is a potent full agonist of CB1. Lastly, the formation of epoxy-eVDs by CYPs is potentiated by the co-substrate AEA, which is also metabolized by CYPs to form EET-EA that are potent CB2 ligands. Hence, inflammation and related pain response is accompanied by a storm of epoxy-eVDs and epoxy-eCBs that are multi-faceted endogenous molecules capable of influencing the activity of CB1, CB2 and TRPV1 receptors. The discovery of these molecules will serve as templates for new multi-target therapeutic drugs that will prove useful for the treatment of inflammatory pain, as well as of other conditions in which these receptors are targeted in other clinical studies.

Methods
Materials and methods concerning RT-qPCR, IL-6 ELISA kit-based detection, Griess Assay to detect nitric oxide, cell viability assay using MTT, cell proliferation assay using BrdU, protein expression and purification methods, nanodisc assembly, in vitro lipid metabolism, and full-scan mass spectrometry analysis of the metabolites can be found in the supplementary information.

Synthesis of NADA and NASHT. Dopamine hydrochloride (24.9 mg, 0.131 mmol, 2.0 equiv) was dissolved in a mixture of 0.1 M N,N-diisopropylethylamine (7.6 mg, 0.066 mmol, 1.0 equiv) and 4-dimethylaminopyridine (7.6 mg, 0.066 mmol, 1.0 equiv) in an anhydrous CH2Cl2 (1 mL) under argon and stirred at room temperature for 8 h. Afterwards, the reaction was centrifuged and the concentration was reduced under reduced pressure. The product was extracted with ethyl acetate and concentrated under reduced pressure. NADA was shown to be hydroxylated at the ω and ω-1 positions by rat liver microsomes, which were weaker TRPV1 agonists compared to NADA. Therefore, the 2-oxo-NASHT and NADA-OH metabolites may represent a degradation pathway of NADA and NASHT. However, we show that the epoxy-eVDs are potently anti-inflammatory molecules in activated microglial cells. A key observation is that the epoxidation of eVDs increases their activity on TRPV1. 14′,15′-epoNADA is a 6.5-fold stronger agonist than NADA and 14′,15′-epoNASHT is a 30-fold stronger antagonist than NASHT at TRPV1, while also showing partial agonism at lower concentrations. Moreover, both NASHT and 14′,15′-epoNASHT suppressed TRPV1-mediated [Ca2+]i response and membrane potential depolarization in mouse DRG neurons with 14′,15′-epoNASHT exerting a significantly stronger inhibition of TRPV1-mediated responses than that produced by NASHT. Based on the cryo-EM structure of TRPV1, it has been proposed that agonists bind at the CAP-binding pocket facilitate channel opening by promoting the lateral movement of the S4-S5 linker. The epoxide could be forming some interactions with H-bond donating groups such as Tyr or Asp residues that populate this linker, which facilitates the channel opening. Since TRPV1 exists as a tetramer, the binding of 14′,15′-epoNASHT to different sites and perhaps different monomers may help to explain its dual agonism/antagonism. However, since the activation of TRPV1 is self-antagonized at concentrations greater than 250 nM (around the IC50), the 14′,15′-epoNASHT functions overall as an antagonist.

The epoxidation of NASHT changes it from being a partial CB2 agonist to a full CB1 agonist. This is intriguing given that 14′,15′-epoNASHT and NASHT differ only by an epoxide. Contrariwise, the epoxidation of NADA does not greatly alter their potency towards CB1 receptor activation. Previously it was shown that the epoxidation of omega-6 and omega-3 eCBs have preferential activation towards CB2. Synthesis of 14′,15′-epoNADA and 14′,15′-epoNASHT was performed using m-chloroperbenzoic acid (m-CPBA) according to our previously established protocol. AA (54.3 µL, 0.165 mmol, 1.0 equiv.) was combined
with mCPBA (0.33 mmol, 2.0 equiv.) in 2 mL dichloromethane (DCM) and allowed to react at room temperature for 1 h. The reaction was terminated with an equivalent solution of 10% NaHCO₃ (aq) and the aqueous layer was re-extracted thrice with DCM. The organic layers were combined and resuspended with acetone for reversed-phase HPLC separation (Mobile Phase A 54:9.0:1 H₂O: acetonitrile:acetic acid; Mobile Phase B 95:4.0:1 acetoni- trile:H₂O:acetic acid; linear gradient of 10% Solvent B to 100% Solvent B in 1 h using a SunFire Prep C₈ OBD™ (see below) of the terminal epoxide (14′,15′-EET) and the other regioisomers. Yield: 4.8 mg of 14′,15-EET (7.6-15% yield). 14′-epi-NPADA ([52±21.21]N-(3,4-dihydroxyphenethyl)-13-(3-pentyl oxiran-2-yl)trideca-5,11-trienamide) and 14′,15-epi-NPASH ([52±21.21]N-(2-(5-hydroxy-1H-indol-3-yl)-ethyl)-13-(3-pentyl oxiran-2-yl)trideca-5,8,11-trienamide) were synthesized by re-dissolving coupling DA and 5HT to 14,15-EET using the above coupling method. Yield from coupling DA and 5HT to 14′,15-EET: 4.03 mm of the soluble epoxide hydrolase inhibitor 4-[[trans-4-[[[tricyclo[3.3.1.1\(^3\),7]dec-1-ylamino]carbonoyl]aminocyclohexyl]oxy]-benzoic acid (t-AUCB) and 1 μL of a solution containing the cerebral cortex) and diced immediately after removal from the pig. Extraction of NADA and NASHT from porcine brain regions. Porcine brains were obtained from freshly slaughtered swine from the Meat Science Laboratory at the University of Illinois at Urbana-Champaign (Ryan Diler Lab). The swine are from a commercial swine line for the swine industry (1050 Camboro line) and were fed a standard diet. The brains were dissected into regions (cerebellum; central core comprising the hippocampus, hypothalamus, and thalamus; and the cerebrum containing the cerebral cortex) and diced immediately after removal from the pig. The tissue was then flash frozen and stored at −80 °C until used. For extraction, the tissue was homogenized in 5 volumes of ice-cold methanol (MeOH) containing 0.03 mm of the soluble epoxide hydrolase inhibitor 4-[[trans-4-[[[tricyclo[3.3.1.1\(^3\),7]dec-1-perfluoro-phenoxoisobutyrylsulfonamide (DFPS) was filtered using a filter column. The extract was then dried under reduced pressure and resuspended in 50% MeOH. The extracts were then loaded onto 1-gm Bond Elut C-18 cartridges (Varian, Harbor City, CA) (one column per gram of tissue), preconditioned with 25% MeOH. Cartridges were washed with 4 mL of 10% MeOH, 4 mL of MeOH containing 1 μL of hygromycin B (in MeCN). The eluant was dried under reduced pressure in amber vials and stored under Ar at −80 °C until resuspended in 150 μL of 180-proof ethanol for LC-MS/MS analysis (the day after). Samples of tissue were spiked with 1 μg of NADA or NASHT standards to test the recovery of this method, and we were able to recover 40% of the material. HPLC analysis of NADA, NASHT, and metabolites. Compounds were analyzed and separated via HPLC consisting of an Alliance 2995 analytical separation module (Waters, Milford, MA) and a Waters 996 photodiode diode array detector (Waters). Synthesis preparation of EETs and epoxy-EETs were separated in reverse-phase using a SunFire Prep C₈ OBD™ 5 μm 19 × 50 mm column (Waters) and a 3.0 mL/min flow rate, and for quantification using a Phenomenex Prodigy® 5 μm ODS-2, 150 × 4.6 mm column (Phenomenex, PN 00F-3300-E0, Torrance, CA) with a 1 mL/min flow rate. Mobile Phase A consisted of 95.5% H₂O (0.1% acetic acid):MeCN and Mobile Phase B consisted of 5% H₂O, 95% H₂O (0.1% acetic acid):MeCN. A full-scan method (Method 1) was developed to investigate all potential products from in vitro enzyme reactions as follows: 0–1 min, 100% A; 1–60 min, linear gradient of 100% A to 100% B; 60–65 min, 100% B. NADA and NA5HT concentrations were confirmed using authentic standards (59.5 mg and 60 min, respectively). A shorter method (Method 2) was developed to analyze the hydrophobic products and for synthesis purification and quantification. 0–30 min: 100% A to 90% A; 30–40 min: 100% B. All wavelengths from 190–600 nm were monitored. 14′,15′-epi-NPADA and 14′,15′-epi-NASHT were quantified at 281 nm and 277 nm wavelengths, respectively, using a NADA and NASHT standard curve, respectively. LC-MS/MS quantitation of NADA, NASHT, and AEA from tissue. Samples were analyzed with the 5500 QTRAP LC/MS/MS system (Sciex, Foster City, CA) in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Software Analyst 1.6.2 was used for data acquisition and analysis. The 1200 Ultimate HPLC system (Agilent Technologies, CA, USA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on an Agilent SB-Aq column (4.6 × 50mm, 5μm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.3 mL min⁻¹. The linear gradient was as follows: 0–1 min, 90% A; 1–8–15min, 0% A; 13.5–18min, 90% A. The autosampler was set at 10 °C. The MS spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage of 5500 V. The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 32 psi, 50 psi, and 65 psi, respectively. Multiple reaction monitoring (MRM) was used for quantification: AEA m/z 348.3 → m/z 282.2; TRPV1 m/z 456.3 → m/z 287.2; TANGO m/z 479.3 → m/z 160.1; NASHT m/z 463.3 → m/z 287.2. Internal standard AEA-d₄ was monitored at m/z 352.3 → m/z 287.2. Cell culture. HEK cells stably transfected with human TRPV1 (HEK-TRPV1) were a gift from Prof. Bradshaw (University of Indiana, Bloomington), which were originally constructed by Merck Research. Cells were grown in Eagle Minimum Essential Media supplemented with l-glutamine (MEM) (ATCC) and 10% fetal bovine serum (FBS) supplemented with penicillin (100 U mL⁻¹) and streptomycin (0.1 mg mL⁻¹) and were incubated at 37 °C with 5% CO₂. Cells were sub-cultured at 80–90% confluency by trypsinization in a 1:6-1:10 ratio. HTLA cells for PRESTO-TANGO and BV2 microglia were grown according to our previously established protocol32. HTLA and BV2 cells were maintained in Dulbecco’s modified Eagle’s media (DMEM) with 10% FBS with penicillin (100 U mL⁻¹) and streptomycin (0.1 mg mL⁻¹) at 37 °C with 5% CO₂ and sub-cultured at 80–90% confluency by trypsinization. HTLA cells were further supplemented with 2 μg mL⁻¹ of purmorphin and 100 μg mL⁻¹ of hygromycin B. Isolation and short-term culture of mouse DRG neurons. Mice were killed by cervical dislocation following CO₂ asphyxia and spinal columns were removed and placed in sterile HBSS. Laminectomies were performed and bilateral DRGs were collected. DRGs were a gift from Prof. Bradshaw (University of Indiana, Bloomington), which were originally constructed by Merck Research. Cells were grown in Eagle Minimum Essential Media supplemented with l-glutamine (MEM) (ATCC) and 10% fetal bovine serum (FBS) supplemented with penicillin (100 U mL⁻¹) and streptomycin (0.1 mg mL⁻¹) and were incubated at 37 °C with 5% CO₂. Cells were sub-cultured at 80–90% confluency by trypsinization in a 1:6-1:10 ratio. HTLA cells for PRESTO-TANGO and BV2 microglia were grown according to our previously established protocol32. HTLA and BV2 cells were maintained in Dulbecco’s modified Eagle’s media (DMEM) with 10% FBS with penicillin (100 U mL⁻¹) and streptomycin (0.1 mg mL⁻¹) at 37 °C with 5% CO₂ and sub-cultured at 80–90% confluency by trypsinization. HTLA cells were further supplemented with 2 μg mL⁻¹ of purmorphin and 100 μg mL⁻¹ of hygromycin B. Metabolism of NADA and NASHT by BV2 microglia. BV2 microglia were plated on 6-well plates at 5 × 10⁵ cells per well and grown to 80–90% confluency. Cell growth media was exchanged for 2 mL of serum-free DMEM and cells were then stimulated with 100 ng mL⁻¹ of LPS for 12 h; control cells were without LPS stimulation. Afterwards, 1 μL of t-AUCB with or without 1 μL of the CYP inhibitor SKF 525A were added for 30 min. 10 μL of HTB prior to stimulating with agonists. Cells were then incubated at 37 °C for 20 min to allow for the de-acetylation of the dye. Fluorescence readings were performed on a SpectraMax Gemini EM ( Molecular Devices, San José, CA) plate reader using the following settings: bottom-read, channel 1—340 nm excitation, 510 nm emission; channel 2—380 nm excitation, 510 nm emission; 2-s mix before experiment; read every 14 sec; 5-min experiment. The assays were performed at room temperature (25 °C). 100 μL of agonists were transferred in triplicate via multi-channel pipette to initiate the assay and the fluorescence intensities of both channels were measured over 5 min. The intensity from channel 1 (Ca²⁺-bound Fura-2) was divided by the intensity from channel 2.
(Ca²⁺-free Fura-2) to achieve the Fluorescence Ratio \(\frac{F_{440} - F_{510}}{F_{440} + F_{510}}\). The Fluorescence Ratio was then plotted over time. Owing to variations in the activation, the AUC of the Fluorescence Ratio from 84–252 s was used to determine activation. The average AUC of DMSO from 84–252 s was considered baseline and subtracted from each data point. The AUCs were plotted as a function of concentration and fitted to a dose-response curve (Eq. 5) using OriginPro. CAP was used as a full agonist positive control, and the \(B_{\text{max}}\) of the CAP was defined as 100% activation. Antagonism experiments for NASHT, 14′,15′-epoNASHT, and AMG-9810 were determined by adding varying concentrations of antagonist in 100 μL of HTB 15 min prior to stimulating with 250 nM CAP.

**Animals.** Male and female C57BL/6J mice were ordered from Jackson Laboratories and were used at the age of 7–8 weeks. Mice were housed in a humidity- and temperature-controlled environment under a 12-h light/12-h dark cycle with free access to water and food. The mice were randomly used and all experiments were done blind to genotypes of animals. All experimental procedures and animal care were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Washington University School of Medicine.

**Live-cell Ca²⁺ imaging.** Cultured DRG neurons were loaded with 4 μM Fura-2 AM (Life Technologies) in culture medium at 37°C for at least 60 min before use. Cells were washed three times and incubated in HBSS at room temperature for 30 min. Fluorescence at 340 nm and 380 nm excitation wavelengths was recorded on an inverted microscope equipped with 340-, 360-, and 380-nm excitation filter wheels with NIH-Elements imaging software (Nikon Instruments Inc.). Fura-2 ratios (F340/F380) reflect changes in [Ca²⁺]i upon stimulation. Values were obtained from 50–100 cells in time-lapse images from each coverslip. Threshold of activation was defined as 3 SD above the average (~20% above the baseline).

**Whole-cell Patch-clamp recording.** Whole-cell patch-clamp recordings were performed using an Axon 700B amplification system (Axon Instruments Inc.) with Patchmaster software (HEPES, pH 7.3 and 320 mM NaCl). Osmolarity was adjusted to 7.4 with NaOH and the osmolarity was adjusted to 320 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl, 10 mM glucose and 10 mM HEPES, pH 7.4 adjusted to 7.4 with NaOH and the osmolarity was adjusted to 320 mM. With sucrose. Data were analyzed and plotted using Patchmaster 10 (Molecular Devices).

**PRESTO-TANGO binding with CB1 and CB2.** Binding of eVDs to CB1 and CB2 was performed in HTA cells using the PRESTO-TANGO assay as previously described[32]. HTLA cells, a HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene) were a gift from Brian Roth’s lab. Cells were seeded at 20,000 cells per 100 μL into a poly-l-lysine coated 96-well plate. After 24 h, cells were transfected with cART or cART2 plasmids (0.1 μg per well) using Calfectin (0.4 μL) in a 4:1 reagent to plasmid ratio (final volume 100 μL per well). Transfection media was replaced after 18 h with fresh serum-media and maintained for 48 h. On the day of the assay, serum-media was replaced with 100 μL media containing 1% diazylated FBS for 4 h. Cells were then incubated with 1 μM of each eVD for 30 min and then compound dissolved with media containing 1% diazylated FBS and was added in a log dose manner (final volume 200 μL per well) and incubated for 8 h. Media was then exchanged for 40 μL of 20x-diluted Bright-Glo (Promega, Madison, WI) solution and incubated in the dark for 20 min and analyzed by luminescence readings. CP-55940 was used as a full agonist positive control for both receptors, and its \(B_{\text{max}}\) was defined as 100% activity. For antagonism experiments, analogues were co-administered at varying concentrations with 50 nM CP-55940.

**Quantitation of eVDs using LC-MS/MS.** Samples were analyzed with the 5500 QTRAP LC/MS/MS system (Sciex, Framingham, MA) in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Software Analyst 1.6.2 was used for data acquisition and analysis. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on an Agilent Agilent Eclipse XDB-C18 (4.6 x 150mm, 5 μm) with mobile Phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in MeCN). The flow rate was 0.4 mL min⁻¹. The linear gradient was as follows: 0–2 min, 90%; 8 min, 55%; 13–25 min, 40%; 30 min, 30%; 35 min, 25%; 36–44 min, 0%; 45–50 min, 90%. The autosampler was set at 10°C. The injection volume was 10 μL. Mass spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage of +5000 V. The source temperature was 450°C. The curtain gas, ion source gas 1, and ion source gas 2 were 32, 65, and 55, respectively.

**Multiple reaction monitoring (MRM) was used for quantification: 14:15-epoNASHT \(m/z\) 479.3 → m/z 160.0; 14:15-epoNASHT \(m/z\) 456.3 → m/z 137.1. Internal standards Anadamide-d4 and 14:15-ETE-d8 were monitored at \(m/z\) 352.3 → m/z 287.2 and \(m/z\) 372.2 → m/z 63.0, respectively.

**Binding equations.** The general one-site binding equation used was Eq. 1 below

\[
B = B_s + B_{\text{max}} \left( \frac{[S]}{K + [S]} \right)
\]

where \(B_s\) is the baseline response, \(B_{\text{max}}\) is the maximum response, \(K\) is the binding parameter, and \([S]\) is the substrate concentration. For kinetic experiments, \(B\) and \(K\) represent velocity and \(K_m\) respectively; for Soret binding experiments, \(B\) and \(K\) represent \(\Delta V\) and \(K_d\), respectively. For metabolism and Soret experiments, \(B_s\) = 0; for NADPH metabolism \(B_s\) is nonzero value.

Inhibition experiments were described by either a competitive model (Eq. 2) or noncompetitive model (Eq. 3)

\[
\frac{B}{B_i} = \frac{B_{\text{max}}[S]}{(K + [S])} \left( \frac{1 + \frac{I}{K_i}}{1 + \frac{I}{K_i}} \right) \frac{1}{K_{i}}
\]

where \(K_i\) is the affinity of the inhibitor and \([I]\) is the concentration of the inhibitor.

A general two-site binding equation (Eq. 4) was used to describe the metabolism of NAD and NASHT

\[
B = \frac{B_i \left( \frac{B_{\text{max}}}{B_{\text{max}}_{1}} \right)}{1 + \frac{I}{K_{i1}}} + \frac{B_{\text{max}}_{2}}{1 + \frac{I}{K_{i2}}}
\]

where \(I\) is the concentration of the ligand, \(p\) is the Hill coefficient, \(B_{\text{max}}_{1}\) is the half-maximal response (EC₅₀ for agonism and IC₅₀ for antagonism experiments), \(B_{\text{max}}_{2}\) is the baseline response (bottom asymptote), and \(B_{\text{max}}_{2}\) is the maximum response (top asymptote).

**Statistical analysis.** Statistical significance was determined by either a two-tailed t-test with equal variance or a one-way ANOVA followed by a Tukey post-hoc analysis as indicated in each figure legend. P-values < 0.05 were considered statistically significant.

**Modeling and simulation of CPY2J2.** Initial structural models of membrane-bound CPY2J2 bound to AEA and NADA or NASHT were generated with molecular docking performed with AutoDock Vina in a stepwise manner as described below. A grid box of dimension 22 Å in x, y, and z and centered in the active site of CPY2J2 was employed for docking. First docked NADA or NASHT to our previous membrane-bound models of CPY2J2[36]. From this docking step, configurations of AEA/NADA/NASHT in which the main epoxidation site (carbons C14 and C15) were close to the heme moiety (with distance <5 Å) and with a high docking score, resulting in over 100 initial structures of each eVD were generated with CYP2J2. The resulting structures were then docked into computer-aided docking (CADD) docking sites were used as input for docking of a second molecule (AEA for NADA/ NASHT in active site, or NADA/NASHT for AEA in active site). This second step allowed us to explore potential peripheral binding sites (i.e., outside the central active site cavity of CPY2J2), which were hypothesized from the experimental. From docking, two potential peripheral binding sites were identified. For each peripheral binding configuration, the corresponding CPY2J2 complexes with two molecules were sorted by docking score, and the models
with the highest score were employed as starting configurations for MD simu-
lations. Each simulation system was minimized for 2000 steps, and equilibrated for
1 ns of the C-α atoms of CYP2J2 and the heavy atoms of the ligands (AEA, NADA, and
NASHT) harmonically restrained (with force constant k = 1 kcal per mol per A²). Following this preparation step, the 2-molecule systems were
simulated for 50 ns.

Simulation protocol. The simulations were performed using NAMD24. The
CHARMM27 force field with CHARMM365,66 force field corrections was used for CYP2J2. The
CHARMM365,66 force field was used for lipids. Force field parameters for AEA,
NADA, and NASHT were generated by analogy from the CHARMM General
Force Field67. The TIP3P model was used for water48. Simulations were performed
with the NPT ensemble with a time step of 2 fs. A constant pressure of 1 atm was
maintained using the Nosé-Hoover Langevin piston method69. Temperature was
maintained at 310 K using Langevin dynamics with a damping coefficient of
0.5 ps⁻¹ applied to all atoms. Nonbonded interactions were cutoff at 12 Å, with
smoothing applied at 10 Å. The particle mesh Ewald (PME) method70 was used for
long-range electrostatic calculations with a grid density of -1 A⁻³.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analyzed during this study are included in this published article
(and its supplementary information files). Other material is available from the
Corresponding author on reasonable request. Material availability statement: All plasmids
used in the study are available from Addgene or can be obtained from the corresponding
author on request. All other data or resources are available from the corresponding
author upon reasonable request. Source data are provided with this paper.

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. W.R.A. conceived the project, wrote the manuscript, designed and performed the experiments (synthesis of the compounds, metabolism studies, in vitro kinetics, mass spec, receptor activation assays, TRP channel assays, and extractions), made figures, and analyzed the data. L.N.C. and A.D. designed and performed experiments (synthesis of the compounds, NMR characterization, inflammation assays, metabolism studies, and extractions), wrote the corresponding manuscript sections, prepared figures, and analyzed the data. I.B. and E.T. designed, performed, analyzed, made figures, and wrote sections of the manuscript pertaining to the MD simulations. X.Z. and H.H. designed, performed, and analyzed the Ca2+ imaging and electrophysiology experiments in DRG neurons, prepared figures, and wrote the corresponding section. A.D. conceived the project, designed the experiments, coordinated the collaborations, and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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