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### Permalink

<https://escholarship.org/uc/item/5sn8c9vs>

### Journal

Journal of Neuroscience, 32(37)

### ISSN

0270-6474

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### Publication Date

2012-09-12

### DOI

10.1523/jneurosci.0130-12.2012

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Peer reviewed

Published in final edited form as:

*J Neurosci.* 2012 September 12; 32(37): 12735–12743. doi:10.1523/JNEUROSCI.0130-12.2012.

## PPAR $\alpha$ mediates acute effects of palmitoylethanolamide on sensory neurons

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### Abstract

The amplitude of the depolarization-evoked Ca<sup>2+</sup> transient is larger in dorsal root ganglion (DRG) neurons from tumor-bearing mice compared to that of neurons from naive mice, and the change is mimicked by co-culturing DRG neurons with the fibrosarcoma cells used to generate the tumors (Khasabova et al., 2007). The effect of palmitoylethanolamide (PEA), a ligand for the peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ), was determined on the evoked-Ca<sup>2+</sup> transient in the co-culture condition. The level of PEA was reduced in DRG cells from tumor-bearing mice as well as those co-cultured with fibrosarcoma cells. Pretreatment with PEA, a synthetic PPAR $\alpha$  agonist (GW7647), or ARN077, an inhibitor of the enzyme that hydrolyses PEA, acutely decreased the amplitude of the evoked Ca<sup>2+</sup> transient in small DRG neurons co-cultured with fibrosarcoma cells. The PPAR $\alpha$  antagonist GW6471 blocked the effect of each. In contrast, the PPAR $\alpha$  agonist was without effect in the control condition, but the antagonist increased the amplitude of the Ca<sup>2+</sup> transient suggesting that PPAR $\alpha$  receptors are saturated by endogenous ligand under basal conditions. Effects of drugs on mechanical sensitivity *in vivo* paralleled their effects on DRG neurons *in vitro*. Local injection of ARN077 decreased mechanical hyperalgesia in tumor-bearing mice, and the effect was blocked by GW6471. These data support the conclusion that the activity of DRG neurons is rapidly modulated by PEA through a PPAR $\alpha$ -dependent mechanism. Moreover, agents that increase the activity of PPAR $\alpha$  may provide a therapeutic strategy to reduce tumor-evoked pain.

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Understanding the effect of cancer cells on somatosensory neurons is fundamental to resolving the generation of tumor-evoked pain because these neurons directly innervate the tumor environment, and tumor-evoked pain is a debilitating factor in the quality of life of patients with advanced cancer (Meuser et al., 2001). Although tumor-evoked pain shares some underlying mechanisms with inflammatory and neuropathic pain, dorsal root ganglion

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**Conflict of Interest** DP declares the following conflict of interest: he is lead inventor in a patent application on ARN077 filed by the University of California, Irvine, the Italian Institute of Technology, and the Universities of Parma and Urbino.

(DRG) neurons from tumor-bearing mice have a unique phenotype (Honore et al., 2000). Using an *in vitro* model of DRG cells co-cultured with cancer cell lines, we (Khasabova et al., 2007, 2008) and others (Chizhnikov et al., 2008; Schweizerhof et al., 2009) have shown that medium conditioned by cancer cells causes long-term changes in membrane proteins that underlie the transduction of excitatory stimuli in DRG neurons. Importantly, changes in membrane proteins *in vitro* mirror changes in DRGs that innervate the tumor-bearing region *in vivo*. One change that occurs is an increase in the expression of the enzyme that hydrolyzes the endocannabinoid *N*-arachidonoyl-ethanolamide [anandamide (AEA)].

In DRG neurons, AEA activates the cannabinoid type-1 receptor (CB1R), and activity of this receptor attenuates the excitability of nociceptors (Agarwal et al., 2007). Mechanical hyperalgesia in tumor-bearing mice is associated with a decrease in AEA in DRGs that innervate the region of the tumor (Khasabova et al., 2011). An increase in mRNA for fatty acid amide hydrolase (FAAH), the enzyme that catabolizes AEA, contributes to the decrease in AEA (Khasabova et al., 2008).

Palmitoylethanolamide (PEA) is synthesized from the same class of membrane phospholipids, the *N*-acylphosphatidylethanolamines, that are precursors for AEA (Cadas et al., 1996, 1997). PEA is primarily catabolized by *N*-acylethanolamine-hydrolyzing acid amidase (NAAA, Tsuboi et al., 2007), but is also a substrate for FAAH (Lichtman et al., 2002). PEA is an agonist at the peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) but has no activity at the CB1R (LoVerme et al., 2005, 2006).

PPAR $\alpha$  is a member of the nuclear receptors superfamily. There is keen interest in the ability of PPAR $\alpha$  agonists to inhibit the development of inflammation through PPAR $\alpha$  regulation of gene expression (O'Sullivan and Kendall, 2010). In animal models of peripheral inflammation, PPAR $\alpha$  agonists are both anti-inflammatory and analgesic (Calignano et al., 1998; Calignano et al., 2001; LoVerme et al., 2005, 2006, Sagar et al., 2008). The rapid timecourse underlying the anti-hyperalgesic properties of the compounds suggest PPAR $\alpha$  mediates transcription independent effects, but *in vivo* experiments cannot resolve whether reductions in hyperalgesia are direct effects of the drugs on sensory neurons or secondary to the effects of the drugs on the immune system.

Initially we determined that medium conditioned by fibrosarcoma cells decreased the basal level of PEA in primary cultures of murine DRG cells. A functional consequence of the decrease in PEA was explored by determining the effect of PPAR $\alpha$  ligands on the depolarization-evoked Ca<sup>2+</sup> transient in DRG neurons co-cultured with fibrosarcoma cells. Evidence that PPAR $\alpha$  agonists and antagonists modulated nociception in naive and tumor-bearing mice in parallel with effects *in vitro* supports the utility of PPAR $\alpha$  agonists in the management of cancer pain.

## METHODS

### Animals

Adult, male C3H/He mice (National Cancer Institute, 25–30 g) were used in the studies. This strain is syngeneic to the fibrosarcoma cells used in the co-culture condition (Clohisy et al., 1996). All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. To generate tumor-bearing mice, fibrosarcoma cells ( $2 \times 10^5$  cells in 10  $\mu$ l of phosphate buffered saline, pH 7.3) were injected into and around the calcaneus bone of the animal's left hind paw while the mouse was anesthetized with isoflurane (2%). The development of tumors over the course of 10 days results in bone osteolysis and mechanical hyperalgesia (Wacnik et al., 2001; Khasabova et al., 2007).

### Culture of DRG cells

Following euthanasia, DRGs were dissected from all levels of the spinal cord of naïve adult male mice. After enzymatic and mechanical dissociation, the final cell suspension of neurons and supporting cells was plated on laminin-coated glass coverslips (Fisher Scientific) at a density of 10,000 cells/25 mm<sup>2</sup>. Cells were maintained in 5 ml of Ham's F12/DMEM medium supplemented with l-glutamine (2 mM), glucose (40 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), and DNAase I (0.15 mg/ml, Sigma-Aldrich) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C for 40–48 h before use (Khasabova et al., 2007). DRG samples were similarly prepared for the co-culture model.

### DRG cell-fibrosarcoma cell co-culture

Cells from the murine NCTC clone 2472 fibrosarcoma cell line (American Type Culture Collection) were plated on glass coverslips at a density of 50,000 cells/25 mm<sup>2</sup> in the DRG culture medium (minus the DNAase) and maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. After 3 days *in vitro*, a coverslip with freshly dissociated DRG cells was combined in one Petri dish with a coverslip of pre-plated fibrosarcoma cells. Cells were maintained in 3 ml of fresh Ham's F12/DMEM with 2 ml of fibrosarcoma cell-conditioned medium at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> as previously described (Khasabova et al., 2007).

### Quantification of AEA, PEA and 2-arachidonoyl-sn-glycerol (2-AG)

DRGs L3-L5 (3 DRGs/sample, ipsilateral to tumors in tumor-bearing mice) were removed from mice following euthanasia under a surgical level of isofluorane-induced anesthesia. Samples were rapidly weighed, frozen in liquid nitrogen, and stored at –80°C until the time of processing. Samples of cultured DRG cells (one coverslip/sample) were processed after 48 hr *in vitro*. Processing began with extraction of lipids in 5 volumes of chloroform containing 5 pmol of *d*<sub>8</sub>-AEA, 100 pmol of *d*<sub>4</sub>-PEA and 100 pmol of *d*<sub>8</sub>-2-AG (Cayman Chemical) as internal standards. Extraction of lipids proceeded overnight at 4°C. Mixtures were then homogenized with an equal volume of methanol:Tris-HCl 50 mM (1:1). Homogenates were centrifuged at 3000 × *g* for 15 min at 4°C; the aqueous phase plus debris were collected and extracted again with 1 volume of chloroform. The organic phases were pooled and evaporated with a gentle stream of nitrogen gas. Vials containing the dried samples were stored at –80°C until analyzed. Targeted isotope-dilution HPLC/atmospheric pressure chemical ionization/mass spectrometry was conducted on each sample. A ZORBAX SB-C18 (0.5 × 150 mm) column was used. The column was maintained at 40°C. The mobile phase A was 0.1% formic acid in 2 mM of ammonium acetate, and phase B was 0.1% formic acid in acetonitrile. The flow rate was 10 µl/min with a gradient that began with 50% A:50% B. The AEA, PEA and 2-AG levels in unknown samples were estimated from the ratio of the area of the signals of deuterated and non-labeled standards of AEA (0.02–20 pmol), PEA (0.2–200 pmol) or 2-AG (0.2–200 pmol). Data are expressed as mol AEA, PEA or 2-AG per sample.

### Measurement of the concentration of free intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>)

The depolarization-evoked Ca<sup>2+</sup> transient was used as the bioassay to measure responses of DRG neurons under experimental conditions. As previously described (Khasabova et al., 2007), the [Ca<sup>2+</sup>]<sub>i</sub> was measured using a dual-emission microfluorimeter (Photoscan, Photon Technology International) to monitor the fluorescence of indo-1 (Molecular Probes) that binds to intracellular Ca<sup>2+</sup>. The preparation was continuously superfused with HEPES buffer at a rate of 1.8 ml/min. The HEPES buffer contained 25 mM HEPES, 135 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 3.5 mM KCl, 1 mM MgCl<sub>2</sub>, and 3.3 mM glucose and 0.1% bovine serum albumin. When the buffer was adjusted to 30 mM and 50 mM KCl, the osmolality of NaCl

was decreased to match the increase in KCl osmolality. The pH of the final solution was adjusted to 7.4, and the osmolality was adjusted to 335–340 mOsm with sucrose.

Only one neuron on each coverslip was assayed. The maximum and minimum diameters of each neuron were estimated using a grid mounted in the eyepiece of the microscope, and the average was used to calculate somal area. Only neurons  $<500 \mu\text{m}^2$  were selected and are referred to as small in this report. Small neurons are most likely to be nociceptive (Hiura and Sakamoto, 1987; Pearce and Duchen, 1994).

During measures of the indo-1 fluorescence counts from the photomultiplier tubes were summed over 1.5 sec and recorded on a computer. Values for the  $[\text{Ca}^{2+}]_i$  were calculated using the equation  $[\text{Ca}^{2+}]_i = K_D \beta (R - R_{min}) / (R_{max} - R)$ , where  $R = 405\text{nm}/485\text{nm}$  fluorescence emission ratio corrected for background fluorescence. The dissociation constant ( $K_D$ ) for indo-1 is 250 nM (Gryniewicz et al. 1985) and  $\beta$  was the ratio of fluorescence at 485 nm in the absence and presence of a saturating concentration of  $\text{Ca}^{2+}$ . Values for the remaining constants were empirically determined in adult murine DRG neurons:  $R_{min} = 0.275$ ;  $R_{max} = 4.73$ .

A stable baseline in the  $[\text{Ca}^{2+}]_i$  for each neuron was obtained during superfusion with HEPES buffer prior to testing responses to chemical stimuli. A coverslip was superfused with only one drug or drug combination before testing the response to 30 mM KCl (10 s). In preliminary studies this concentration of KCl evoked a  $\text{Ca}^{2+}$  transient in 50% of the neurons tested. This frequency of response was chosen to allow the detection of an increase or decrease in the occurrence of a transient. The threshold for defining the occurrence of a  $\text{Ca}^{2+}$  transient was an increase in the  $[\text{Ca}^{2+}]_i > 50\%$  of the basal  $[\text{Ca}^{2+}]_i$ . If the change in the  $[\text{Ca}^{2+}]_i$  did not meet this threshold (defined as no response), the neuron was superfused with 50 mM KCl to check the viability of the neuron. Neurons that did not elicit a  $\text{Ca}^{2+}$  transient in response to 50 mM KCl were excluded from the data set. The frequency of response to 30 mM KCl was defined as a percentage [(number of neurons that responded/number of neurons tested)  $\times 100$  %]. The amplitude of the  $\text{Ca}^{2+}$  transient was defined as the difference in  $[\text{Ca}^{2+}]_i$  measured at the peak minus the average baseline sampled 2 min before superfusion with the test substance. Recordings were generally terminated after reliable determination of a peak response so data for return of the  $[\text{Ca}^{2+}]_i$  to baseline were not routinely obtained.

### Quantification of mRNA by real-time PCR

DRGs L3–L5 (3 DRGs/sample, ipsilateral to tumors in tumor-bearing mice) were isolated from mice, placed in *RNAlater* (Qiagen) and stored at 4°C. Total RNA was isolated from samples using RNeasy Lipid Tissue Mini Kits (Qiagen). RNA was reverse transcribed into cDNA using QuantiTect RT-PCR kits (Qiagen) as per the manufacturer's instructions. Real-time PCR studies were performed with DyNAmo HS SYBR Green Master Mix (Finnzymes) using the DNA engine Opticon 2 (MJ Research) through 45 PCR cycles (94°C for 10 s, 57°C for 20 s, 72°C for 30 s). Each cDNA sample was run in triplicate for murine PPAR $\alpha$ , NAAA, and the reference gene (S15). Primer pair sequences were as follows: PPAR $\alpha$  (GenBank Accession number NM\_011144.3) forward primer 5'-AGT GCA TGT CCG TGG AGA C-3' and reverse primer 5'-TCA AGG AGG ACA GCA TCG T-3'; NAAA (NM\_025972) forward primer 5'-TTC GAA GCA GCT GTC TAC AC-3' and reverse primer 5'-AGA GGG TCA AGA GGC CAA ATG T-3'; S15 (BC094409) forward primer 5'-CCG AAG TGG AGC AGA AGA AG-3' and reverse primer 5'-CTC CAC CTG GTT GAA GGT C-3'. All primers were synthesized by Operon Biotechnologies. Specificity of each amplicon was confirmed by melting curve analysis, evidence of a single band after gel electrophoresis, authenticity of the DNA sequence of the band isolated from the gel, and resolution by BLAST analysis that the sequence of the amplicon was unique to each target. The efficiency

of the primer set was derived from the slope of the line generated from the serial dilution of a sample using the following equation:  $\text{efficiency} = 10^{(-1/\text{slope})}$ . Within each primer set, all DRG samples were assayed in triplicate at the same dilution, and all data were within the linear range used to determine the efficiency of the primers. For each primer set, the change in critical threshold for each sample [ $\Delta C(t)$ ] was calculated by subtracting the average  $C(t)$  for the sample from the mean of the  $C(t)$  for the naive subjects such that the  $\Delta C(t)$  is a positive number when there is an increase in mRNA. Results are expressed as the mean  $\pm$  S.E.M for the treatment group. Using this analysis there was no difference between the naive and tumor-bearing animals in the level of mRNA for the reference gene [ $\Delta C(t)$  naive:  $0.000 \pm 0.417$ ; tumor-bearing:  $-0.059 \pm 0.180$ ,  $p=0.90$ ].

### Measurement of [ $^3\text{H}$ ]PEA hydrolysis

PEA hydrolysis was measured in primary cultures of DRG neurons (approximately 3–4 DRGs/cover slip) after 44–48 hr *in vitro*. Coverslips containing dissociated DRGs were placed in HEPES buffer with vehicle (0.095% ethanol, final concentration) or ARN077 (1  $\mu\text{M}$ ) for 30 min. To model physiological experiments *in vitro*, coverslips were then placed in 0.5 ml of Tris buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mg/ml fatty acid-free bovine serum albumin, pH 7.4). The pH of 7.4 is approximately mid-way between the optimum pH for NAAA (pH 5) and FAAH (pH 9) (Tsuboi et al., 2004). Because AEA can compete with the uptake of PEA (Jacobsson and Fowler, 2001), the contribution of cellular uptake to the measure of exogenous PEA hydrolysis was excluded by subjecting samples to 2 freeze-thaw cycles. Following the second thaw, samples were incubated for 30 min at 37°C with [ $^3\text{H}$ ]PEA (50,000 dpm/sample, American Radiolabeled Chemicals). Preliminary studies confirmed that under these conditions [ $^3\text{H}$ ]PEA hydrolysis was within the linear range with respect to time. The enzyme reaction was stopped by the addition of 2 ml of chloroform:methanol (1:2). After standing at room temperature for 30 min, the phases were separated with the addition of 0.67 ml of chloroform and 0.6 ml of water and centrifugation at  $280 \times g$  for 15 min. The amount of [ $^3\text{H}$ ] in 0.5 ml of each of the aqueous and organic phases was determined using liquid scintillation spectrometry. PEA hydrolysis was expressed as a percentage of total [ $^3\text{H}$ ]PEA taking into account that the radioactivity in the aqueous phase was [ $^3\text{H}$ ]ethanolamine, whereas [ $^3\text{H}$ ]PEA remained in the organic phase.

### Assessment of mechanical sensitivity

Before inclusion in the study, daily baseline measurements of withdrawal frequency to a monofilament that delivered a force of 3.9 mN were obtained for all mice on 3 consecutive days. Mice were isolated under separate glass containers on a mesh platform and allowed to acclimate for 30 minutes prior to testing. The monofilament was applied to the plantar surface of each hind paw 10 times (1–2 sec each). The number of withdrawal responses was determined and expressed as a percentage of the number of stimuli applied (withdrawal frequency). These data were used to screen mice for hypersensitivity, and animals that exhibited baseline withdrawal frequencies  $\geq 50\%$  were excluded from the study ( $<2\%$ ).

The effect of the NAAA inhibitor and PPAR $\alpha$  antagonist on the sensitivity of the hind paws of naive mice was assessed using a repetitive stimulation paradigm (Joseph et al., 2003). Four von Frey monofilaments with ascending bending forces of 3.9, 5.9, 9.8, and 13.7 mN were used. The frequency of withdrawal to each mechanical stimulus was determined in each hind paw before and 1 h after intraplantar (i.pl.) injection of ARN077, GW6471, or vehicle (10  $\mu\text{l}$  volume) into one hind paw. The dose of ARN077 was the maximum dose that was soluble in the vehicle (5% ethanol in saline). The dose of GW6471 was the maximum dose tested that did not have a systemic effect on mechanical sensitivity following intraplantar injection in naive mice.

The effect of drugs on mechanical hyperalgesia in tumor-bearing mice was determined on day 10 after implantation of fibrosarcoma cells using the von Frey monofilament that delivered a force of 3.9 mN. Mechanical hyperalgesia was defined as an increase in the frequency of paw withdrawal responses. Only mice exhibiting mechanical hyperalgesia (> 50% withdrawal frequency) were included in subsequent experiments (~85%). To determine the acute, local anti-hyperalgesic effect of drugs, mice were given an intraplantar injection of drug (10  $\mu$ l volume) into one hind paw. Withdrawal responses evoked by the monofilament were measured in each hind paw before and every 30 min after injection for 2 h. At least two treatment groups were used on each day of behavioral testing; on the following day the treatment groups were switched on the same mice. No mouse received more than 2 drug injections. There were no differences in results for any one treatment between the 2 days. In all behavioral experiments the individual judging behavioral responses was blinded to the treatment of each subject.

### Western blot analysis of PPAR $\alpha$ protein

Mechanical hyperalgesia was confirmed in tumor-bearing mice 10 d after implantation of fibrosarcoma cells in the calcaneus bone and prior to euthanasia by decapitation under a surgical level of isoflurane anesthesia. DRGs L3–L5 from naive and tumor-bearing mice (6 DRGs/sample pooled from 2 mice) and tibial nerve (~1 cm, pooled from 2 mice) were collected, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Samples from tumor-bearing mice were ipsilateral to tumors. Samples were sonicated in single-detergent lysis buffer [50 mM Tris-HCl, pH 8.0 with 1% Triton X-100, 150 mM NaCl, 0.02% Na azide, 100  $\mu\text{g}/\text{ml}$  PMSF, and 1  $\mu\text{g}/\text{ml}$  protease inhibitor mixture (Sigma-Aldrich)], and the particulate fraction of the supernatant was obtained after serial centrifugation at  $800 \times g$  for 10 min and  $14,000 \times g$  for 25 min. Western blot analysis was performed on 15  $\mu\text{g}$  of protein which were loaded onto a 10% SDS-PAGE gel, subjected to electrophoresis and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). Nonspecific binding to membranes was blocked by incubation in phosphate-buffered saline with 3% defatted dry milk for 1 h at room temperature. The membranes were probed with a rabbit anti-PPAR $\alpha$  antibody (1:500, Thermo Fisher Scientific) overnight at  $4^{\circ}\text{C}$ . Specificity of the antibody was determined by pre-absorption of the diluted antibody with 1  $\mu\text{g}/\text{ml}$  PPAR $\alpha$  synthetic immunizing peptide (Affinity BioReagents). Detection was performed using a peroxidase conjugate of goat anti-rabbit IgG (1:10,000; Amersham Biosciences). Immunoreactivity was visualized using a chemiluminescent reagent (Pierce). Loading controls were performed with a mouse anti- $\beta$ -tubulin antibody (1:10,000, Sigma-Aldrich). The optical density of each immunoreactive band was determined using MetaMorph software. The amount of PPAR $\alpha$  protein was defined as the ratio of PPAR $\alpha$ -ir to tubulin-ir within the same sample.

### Drug treatments

PPAR $\alpha$  ligands were purchased from Cayman Chemical, dissolved in ethanol and stored at  $-20^{\circ}\text{C}$ . The stock concentration of the PPAR $\alpha$  agonist GW7647 [2-(4-(2-(1-cyclohexanebutyl)-3-cyclohexylureido)ethyl)phenylthio)-2-methyl-propionic acid] was 20 mM, the concentration of PEA was 33 mM, and the concentration of the PPAR $\alpha$  antagonist GW6471 ([[(2S)-2-[[[(1Z)-1-methyl-3-oxo-3-[4-(trifluoromethyl)phenyl]-1-propenyl]amino]-3-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]phenyl]propyl]-carbamic acid ethyl ester) was 16 mM. Drugs were diluted to their final concentrations in HEPES buffer immediately before use. The agonists were superfused for 5 min prior to stimulation with KCl. The PPAR $\alpha$  antagonist was superfused for 10 min prior to stimulation with KCl. The synthesis and pharmacological characterization of the NAAA inhibitor ARN077 is described elsewhere (Armirotti et al., 2012). A stock solution of ARN077 (10 mM) was prepared in ethanol.

## Statistical Analyses

Data are reported as the mean±S.E.M. when normally distributed. For these data, differences between treatments were determined using Student's *t* test or two-way Analysis of Variance with Bonferroni's post test to determine differences between groups when groups also had equal variance. Data that did not meet these criteria were reported as the median (25<sup>th</sup> – 75<sup>th</sup> percentile range), and effects of treatments were analyzed using the Mann-Whitney Rank Sum test or the Kruskal-Wallis One Way Analysis of Variance by Ranks followed by Dunn's test to determine differences between groups. Data for the frequency of response to 30 mM KCl were analyzed using Fisher Exact Test. A significance level of 0.05 was the threshold for stating a treatment effect in all analyses.

## RESULTS

### Medium conditioned by fibrosarcoma cells decreased the levels of PEA and AEA in DRG cells

To determine the effect of fibrosarcoma cell-conditioned medium on the levels of endocannabinoids and PEA in primary cultures of DRG cells, the amounts of these substances were compared between DRG cultures in the control condition and the co-culture condition (Table 1). The level of AEA in DRG cells maintained for 48 hr in medium conditioned by fibrosarcoma cells was reduced to less than 30% of control, and the level of PEA was reduced to less than 60%. The level of 2-AG was not different between the two conditions, thereby excluding the possibility that the effect of the fibrosarcoma cell-conditioned medium on the levels of AEA and PEA was due to non-specific effects on lipid metabolism. Furthermore, it is not likely that the levels reported for the DRG cells in the co-culture condition were contaminated by the fibrosarcoma cells because the levels of PEA and AEA in fibrosarcoma cells were lower than those of DRG cultures in the control condition.

Importantly, the pattern of changes in fatty acid ethanolamides and 2-AG observed in the co-culture model was consistent with the pattern observed in the levels of PEA and endocannabinoids in L3-L5 DRGs ipsilateral to tumors in tumor-bearing mice (Table 1). The levels of PEA and AEA, but not 2-AG, were lower in DRGs from tumor-bearing mice. To date, each biochemical change in DRG cells co-cultured with fibrosarcoma cells is consistent with the change observed in DRGs from tumor-bearing mice (Khasabova 2007, 2008), thereby validating the relevance of the co-culture model for studying the cell biology of DRG neurons in tumor-bearing mice.

Biochemical analyses were conducted to determine whether DRG cells express NAAA. Data for quantitative RT-PCR confirmed that NAAA mRNA was measurable in DRGs, but there was no difference in the level of mRNA between naive and tumor-bearing mice [ $\Delta C(t)$  for naive:  $0.000 \pm 0.328$ ,  $n=8$ , tumor bearing:  $0.389 \pm 0.175$ ,  $n=9$ ,  $p=0.3$ , Student's *t* test). Experiments with [<sup>3</sup>H]PEA showed a 22% increase in [<sup>3</sup>H]PEA hydrolysis by DRG cells co-cultured with fibrosarcoma cells compared to the control condition (Table 2). The novel NAAA inhibitor ARN077 was used to determine the proportion of [<sup>3</sup>H]PEA hydrolysis attributable to NAAA. ARN077 inhibits human NAAA with an IC<sub>50</sub> of 7 nM and exerts no significant inhibitory effect toward FAAH at concentrations as high as 30  $\mu$ M (Armirotti et al., 2012). ARN077 (1  $\mu$ M) decreased [<sup>3</sup>H]PEA hydrolysis in DRG cells from cultures with fibrosarcoma cells by 15%. These data confirm that DRG cells express NAAA. It is noteworthy that ARN077 decreased PEA hydrolysis by DRG cells without affecting the level of AEA (Table 3), thereby confirming its target selectivity.



### PEA attenuated the evoked $\text{Ca}^{2+}$ transient via $\text{PPAR}\alpha$ in the co-culture condition

The physiological consequence of the change in PEA level in small DRG neurons was explored in a bioassay of the depolarization-evoked  $\text{Ca}^{2+}$  transient. A change in the  $\text{Ca}^{2+}$  transient evoked by 50 mM KCl was noted in our initial characterization of small DRG neurons from tumor-bearing mice and the co-culture model. In both conditions, the evoked  $\text{Ca}^{2+}$  transient was larger in amplitude compared to the transient evoked in the control condition (Khasabova et al., 2007). Similarly, the amplitude of the  $\text{Ca}^{2+}$  transient evoked by 30 mM KCl, a submaximal stimulus in the present study, was more than 3-fold larger in the co-culture condition compared to that evoked in the control condition (Table 4). In addition, the stimulus of 30 mM KCl was almost twice as likely to evoke a  $\text{Ca}^{2+}$  transient in small neurons in the co-culture condition compared to the control condition, paralleling the change in response to 25 mM KCl in small DRG neurons from tumor bearing mice (Khasabova et al., 2007). Since this assay was effective in resolving a change in AEA signaling through CB1R in small neurons in the cancer condition (Khasabova et al., 2008), modulation of the depolarization-evoked  $\text{Ca}^{2+}$  transient was used as a bioassay for acute effects of  $\text{PPAR}\alpha$  ligands on small DRG neurons *in vitro*.

Because the criterion for occurrence of a  $\text{Ca}^{2+}$  transient was dependent upon the basal  $[\text{Ca}^{2+}]_i$ , it was important to determine whether drug treatments altered the basal  $[\text{Ca}^{2+}]_i$ . Among small neurons maintained in the co-culture condition there was no difference in the basal  $[\text{Ca}^{2+}]_i$  during superfusion with the vehicle (0.006 % ethanol) or effective concentrations of  $\text{PPAR}\alpha$  ligands ( $H=3.4$ , 5 df,  $p=0.64$ , Kruskal-Wallis one-way ANOVA on Ranks). For example, the median  $[\text{Ca}^{2+}]_i$  was 96 nM ( $n=20$ ) in the presence of PEA (10  $\mu\text{M}$ ).

PEA (10  $\mu\text{M}$ ) decreased the amplitude of the depolarization-evoked  $\text{Ca}^{2+}$  transient in small neurons from the co-culture condition by more than 50% compared to vehicle (Fig. 1A), and the effect of PEA was blocked by the  $\text{PPAR}\alpha$  antagonist GW6471 ( $H=14.2$ , 3 df,  $p<0.005$ , Kruskal Wallis one-way ANOVA on Ranks followed by Dunn's test). PEA (10  $\mu\text{M}$ ) had no effect on the occurrence of the evoked  $\text{Ca}^{2+}$  transient in the co-culture condition (75%,  $n=20$ ;  $p=1.0$  compared to the vehicle, Fisher Exact Test).

In order to confirm the physiological relevance of the effect of PEA, an inhibitor of NAAA activity was used to block the degradation of PEA in DRG neurons. Treatment of primary cultures of DRG neurons with ARN077 (1  $\mu\text{M}$ ) for 15 min nearly doubled the level of PEA in the preparation without altering the levels of AEA and 2AG (Table 3). Under the same conditions, pretreatment of DRG neurons with ARN077 mimicked the effect of PEA on the evoked  $\text{Ca}^{2+}$  transient ( $H=21.0$ , 3 df,  $p<0.001$ , Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's test) (Fig. 1B). Together these data support the conclusion that PEA attenuated the amplitude of the evoked  $\text{Ca}^{2+}$  transient through  $\text{PPAR}\alpha$ .

### A synthetic $\text{PPAR}\alpha$ agonist attenuated the evoked $\text{Ca}^{2+}$ transient in small DRG neurons co-cultured with fibrosarcoma cells

In order to confirm a role for  $\text{PPAR}\alpha$  in modulating the evoked  $\text{Ca}^{2+}$  transient, we characterized the response to a synthetic  $\text{PPAR}\alpha$  agonist. GW7647 decreased the amplitude of the  $\text{Ca}^{2+}$  transient evoked in DRG neurons co-cultured with fibrosarcoma cells by more than 50% (Fig. 2A). The effect was concentration-dependent with a minimum effective concentration of 10 nM ( $H=14.5$ , 3 df,  $p<0.0025$ , Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's test). Concentrations greater than 30 nM were not tested because the  $\text{Ca}^{2+}$  transient evoked in the presence of this concentration was near the threshold for a positive response. Examples of the evoked transients in neurons in the presence of vehicle or GW7647 (10 nM) are shown in figure 2B.

To confirm whether the effect of GW7647 on the  $\text{Ca}^{2+}$  transient was mediated through PPAR $\alpha$ , neurons were treated with the PPAR $\alpha$  antagonist GW6471 (1  $\mu\text{M}$ ) in conjunction with GW7647 (10 nM). Treatment with GW6471 blocked the effect of GW7647 on the amplitude of the depolarization-evoked  $\text{Ca}^{2+}$  transient ( $H=13.3$ , 3 df,  $p<0.005$ , Kruskal Wallis one-way ANOVA on Ranks followed by Dunn's test) (Fig. 2C). GW6471 alone had no effect on the amplitude of the  $\text{Ca}^{2+}$  transient compared to vehicle. These data support the conclusion that the effect of GW7647 on the amplitude of the evoked  $\text{Ca}^{2+}$  transient was mediated through PPAR $\alpha$ .

GW7647 (10 nM) had no effect on the frequency at which 30 mM KCl evoked a  $\text{Ca}^{2+}$  transient in neurons in the co-culture condition (66%,  $n=35$ ;  $p=0.64$  compared to the vehicle, Fisher Exact Test).

### **A PPAR $\alpha$ antagonist but not agonist modulated the evoked $\text{Ca}^{2+}$ transient in small DRG neurons in the control condition**

Using the same concentrations that were effective in the co-culture condition, neither the synthetic PPAR $\alpha$  agonist GW7647 (30 nM) nor PEA (30  $\mu\text{M}$ ) altered the amplitude of the depolarization-evoked  $\text{Ca}^{2+}$  transient when compared to the vehicle control for DRG neurons maintained in the control condition [GW7647: median response of 125 (83–216) nM  $[\text{Ca}^{2+}]_i$ ,  $p=0.21$ ,  $n=11$  neurons; PEA: median response of 102 (56–188) nM  $[\text{Ca}^{2+}]_i$ ,  $p=0.90$ ,  $n=7$  neurons compared to vehicle, Mann-Whitney Rank Sum Test]. In contrast, a concentration-dependent effect of the PPAR $\alpha$  antagonist GW6471 on the amplitude of the  $\text{Ca}^{2+}$  transient was observed in the control condition ( $H=14.8$ , 3 df,  $p<0.0025$  Kruskal-Wallis One Way ANOVA on Ranks followed by Dunn's test) (Fig. 3A). The minimum concentration of GW6471 that increased the amplitude of the  $\text{Ca}^{2+}$  transient compared to the vehicle was 1  $\mu\text{M}$ . Examples of the  $\text{Ca}^{2+}$  transients in neurons in the presence of vehicle and GW6471 (1  $\mu\text{M}$ ) are shown in figure 3B. Solubility limited the ability to test concentrations higher than 3  $\mu\text{M}$  in order to determine a maximum effect. Thus, 1  $\mu\text{M}$  GW6471 was used in subsequent experiments. GW6471 (1  $\mu\text{M}$ ) had no effect on the frequency at which 30 mM KCl evoked a  $\text{Ca}^{2+}$  transient in neurons in the control condition (63%,  $n=38$ ,  $p=0.1$ , Fisher Exact Test).

To determine whether the effect of GW6471 on the  $\text{Ca}^{2+}$  transient was mediated through PPAR $\alpha$ , neurons were superfused with GW6471 (1  $\mu\text{M}$ ) in the presence of the synthetic agonist GW7647 (30 nM). Inclusion of GW7647 blocked the effect of GW6471 on the amplitude of the  $\text{Ca}^{2+}$  transient ( $H=19.2$ , 3 df,  $p<0.001$ ; Kruskal-Wallis, One Way ANOVA on Ranks followed by Dunn's test) (Fig. 3C). GW7647 (30 nM) alone had no effect on the amplitude of the  $\text{Ca}^{2+}$  transient compared to vehicle. None of the drugs had an effect on the basal  $[\text{Ca}^{2+}]_i$  compared to the vehicle ( $H=3.1$ , 4 df,  $p=0.54$ , Kruskal-Wallis one-way ANOVA on Ranks). For example, the median  $[\text{Ca}^{2+}]_i$  during superfusion with GW6471 (1  $\mu\text{M}$ ) was 84 nM ( $n=39$ ). These data indicate that the enhancement of the amplitude of evoked  $\text{Ca}^{2+}$  transient by GW6471 was mediated through PPAR $\alpha$ . Moreover, the absence of an effect of pretreatment with PPAR $\alpha$  agonists alone supports the conclusion that the ambient level of PEA or other endogenous ligand saturates PPAR $\alpha$  in the control condition.

### **Effects of PPAR $\alpha$ ligands on mechanical sensitivity *in vivo* paralleled their effects *in vitro***

The effects of drugs on mechanical hyperalgesia in tumor-bearing mice parallel their effects on the amplitude of the depolarization-evoked  $\text{Ca}^{2+}$  transient. Tumor-bearing mice exhibited mechanical hyperalgesia, defined by a withdrawal frequency of 70–80% in response to the 3.9 mN force, prior to drug treatment. Intraplantar injection of the NAAA inhibitor ARN077 (1  $\mu\text{g}$ ) ipsilateral to the tumor decreased mechanical hyperalgesia from 0.5 to 2 h after drug injection ( $F_{12,159}=2.95$ ,  $p=0.01$ , two-way ANOVA) (Fig. 4A). Recovery of mechanical

hyperalgesia to the pre-drug level was evident 24 h after drug injection (withdrawal frequency of  $72.5 \pm 2.5\%$ ,  $n=4$ ,  $p=0.76$ , paired *t* test). No change in mechanical sensitivity occurred in the paw contralateral to the drug injection ( $n=8$ ,  $p=0.52$ , paired *t* test), indicating that the anti-hyperalgesic effect of ARN077 was mediated locally. The effect of ARN077 was blocked by co-injection of the PPAR $\alpha$  antagonist GW6471 (3  $\mu\text{g}$ , i.pl.). These data support the conclusion that the anti-hyperalgesic effect of NAAA inhibition of NAAA was mediated by an endogenous agonist of PPAR $\alpha$ .

Conversely, in naive mice, ARN077 (1  $\mu\text{g}$ , i.pl.) had no effect on the mechanical sensitivity of the hind paw ipsilateral to the injection (Fig. 4B). However, mechanical sensitivity was increased by intraplantar injection of the PPAR $\alpha$  antagonist GW6471 (3  $\mu\text{g}$ ) ( $F_{9,107}=3.4$ ,  $p=0.001$  for interaction between mechanical forces and treatments, two-way ANOVA). Specifically, naive mice showed increased responses to monofilaments that delivered forces of 3.9 and 5.9 mN at 1 h after drug injection ( $p<0.02$ , Bonferroni's *t* test). Recovery to baseline sensitivity was noted 24 h after injection of GW6471 when the withdrawal frequency to the 3.9 mN monofilament was  $22 \pm 2\%$  ( $p=0.69$  compared to the pre-drug response, one-way ANOVA,  $n=5$ ). Importantly, the effect of GW6471 was blocked by co-treatment with ARN077 (1  $\mu\text{g}$ , i.pl.), the inhibitor of PEA hydrolysis. The antagonist did not alter mechanical sensitivity in the paw contralateral to the injection ( $F_{3,47}=1.42$ ,  $p=0.25$  for an interaction between mechanical forces and treatments of vehicle and GW6471, two-way ANOVA,  $n=5-7$  mice/treatment).

The effect of tumors on the expression of PPAR $\alpha$  in DRGs was estimated using quantitative RT-PCR and Western blots. The amount of PPAR $\alpha$  mRNA tended to be higher in DRGs L3-L5 of tumor-bearing mice [ $\Delta\text{C}(t)$  Naive:  $0.000 \pm 0.361$ ,  $n=9$ ; tumor-bearing:  $0.750 \pm 0.180$ ,  $n=9$ ,  $p=0.08$ , Student's *t* test]. Western blot analysis showed a PPAR $\alpha$ -immunoreactive band at approximately 40 kD that was absorbed by pre-incubation of the diluted antibody with PPAR $\alpha$  protein (Fig. 5A). In comparison to samples from naive mice, there was no difference in tumor-bearing mice in the amount of PPAR $\alpha$ -immunoreactive protein in this band in either DRGs or tibial nerve ( $p=0.16$  for DRGs and  $p=0.38$  for nerve, Student's *t* test) (Fig. 5B).

## DISCUSSION

Tumor-evoked pain may arise from multiple sources: factors released from tumor cells and immune cells as well as nerve injury. Our data provide the first evidence that the level of PEA in DRG cells decreases in response to co-culture with fibrosarcoma cells *in vitro* and to the tumor they generate *in vivo*. The change is associated with decreased fatty acid ethanolamide signaling at PPAR $\alpha$  in small DRG neurons and mechanical hyperalgesia in tumor-bearing mice. In the control condition exogenous PPAR $\alpha$  agonists had no effect on the depolarization-evoked  $\text{Ca}^{2+}$  transient. However, the  $\text{Ca}^{2+}$  transient evoked by a submaximal stimulus occurs more frequently in neurons from tumor-bearing mice and is larger in amplitude compared to neurons from naive mice (Khasabova et al., 2007). These changes were mimicked in the co-culture condition. Increasing PEA in DRG neurons normalized the change in the  $\text{Ca}^{2+}$  transient in the cancer condition by decreasing the amplitude of the evoked transient through a PPAR $\alpha$ -dependent mechanism. Evidence that local inhibition of NAAA, an enzyme that hydrolyzes PEA, reduced mechanical hyperalgesia in tumor-bearing mice through a PPAR $\alpha$ -dependent pathway and local administration of a PPAR $\alpha$  antagonist caused hyperalgesia in naive mice support the conclusion that PPAR $\alpha$  signaling in nociceptors contributes to cellular mechanisms that set the threshold for nociception.

## DRG neurons express NAAA

Several lines of evidence support the conclusion that DRG neurons express NAAA. First, NAAA mRNA was measured in DRGs of naive and tumor-bearing mice. Second, 15% of the hydrolysis of [<sup>3</sup>H]PEA by DRGs co-cultured with fibrosarcoma cells was inhibited by ARN077, a potent and selective NAAA inhibitor (Armirotti et al., 2012). Finally, treatment with the NAAA inhibitor mimicked the effect of exogenous PEA on the depolarization-evoked Ca<sup>2+</sup> transient in single DRG neurons.

Whether an increase in NAAA activity contributes to the reduced level of PEA in DRGs from tumor-bearing mice and the co-culture condition is not clear. Although the level of NAAA mRNA in DRGs did not change in tumor-bearing mice, the data for [<sup>3</sup>H]PEA hydrolysis do not exclude a change in enzyme activity. The data for PEA hydrolysis were generated at a pH of 7.4, and a more accurate quantification of NAAA enzyme activity would occur at its optimal pH of 5 (Ueda et al., 2001). Because FAAH activity in DRGs increases in tumor-bearing mice (Khasabova et al., 2008), it most likely contributed to the increased [<sup>3</sup>H]PEA hydrolysis in the co-culture condition. However, it is not likely that the reduced level of PEA in conditions of our experiments can be attributed to FAAH. Although PEA is a substrate for FAAH (Lichtman et al., 2001; Kathuria et al., 2003), the FAAH inhibitor URB597 does not alter the level of endogenous PEA in DRG cells under the same conditions that produce more than a 2-fold increase in AEA (Khasabova et al., 2012). These data suggest that endogenous PEA is localized to a cellular compartment that protects it from FAAH. Alternatively, a decrease in the capacity to generate fatty acid ethanolamides may contribute to the lower levels observed in DRGs in the cancer condition. Importantly, evidence that the co-culture model mirrored the change in cellular PEA observed *in vivo* supports the utility of the model in studying the role of PEA and other substrates of NAAA in the function of DRG neurons.

## The effect of PEA on DRG neurons was mediated by PPAR $\alpha$

Our conclusion that the effect of PEA on the depolarization-evoked Ca<sup>2+</sup> transient is mediated by PPAR $\alpha$  is supported by evidence that its effect was mimicked by a synthetic PPAR $\alpha$  agonist and blocked by a PPAR $\alpha$  antagonist. The selectivity of the drugs for PPAR $\alpha$  is supported by evidence that each was effective at a concentration near its EC<sub>50</sub> or IC<sub>50</sub> at PPAR $\alpha$ . PEA has an EC<sub>50</sub> of 3  $\mu$ M (LoVerme et al., 2005) and GW7647 has an EC<sub>50</sub> of 6 nM (Brown et al., 2001) at the human PPAR $\alpha$ . The IC<sub>50</sub> of the antagonist GW6471 is 0.24  $\mu$ M (Xu et al., 2002). Moreover, at these concentrations the effect of each agonist was blocked by the antagonist when DRG neurons were prepared in the co-culture condition, and the converse occurred in the control condition.

Inhibition of NAAA increased the level of PEA in DRG cells and mimicked the effect of PEA and PPAR $\alpha$  agonists. In addition, the effect of the NAAA inhibitor on the Ca<sup>2+</sup> transient was blocked by the PPAR $\alpha$  antagonist. These data validate the physiological relevance of PEA as an agonist at PPAR $\alpha$  in DRG neurons. Although AEA is also a substrate for NAAA (Sun et al., 2005), inhibition of NAAA did not result in increased AEA under the conditions tested. However, it is likely that other endogenous fatty acid ethanolamides that activate PPAR $\alpha$ , such as oleoylethanolamide (Fu et al., 2003), were elevated following inhibition of NAAA and contributed to the observed effect.

## Physiological implications

The effect of PPAR $\alpha$  ligands on mechanical hyperalgesia in tumor-bearing mice was consistent with the effect of PPAR $\alpha$  agonists on isolated DRG neurons in the co-culture condition, thereby providing a meaningful physiological context for the effect of PPAR $\alpha$  signaling on the depolarization-evoked Ca<sup>2+</sup> transient. The inhibitory effect of the NAAA

inhibitor on mechanical hyperalgesia in tumor-bearing mice is consistent with the effects of PPAR $\alpha$  agonists in other models of hyperalgesia (Calignano et al., 2001, LoVerme et al., 2006, Sagar et al., 2008). However, the specific factors underlying the change in PPAR $\alpha$  signaling in the cancer condition remain to be resolved. The acute mechanical hyperalgesia produced by the PPAR $\alpha$  antagonist in naive mice is consistent with a recent report of thermal hyperalgesia in PPAR $\alpha$ <sup>-/-</sup> mice (Ruiz-Medina et al., 2012) and supports the conclusion that activity of PPAR $\alpha$  contributes to defining the threshold for activation of nociceptors. The lack of effect of PPAR $\alpha$  agonists in naive mice and control cultures suggests that PPAR $\alpha$  is saturated under normal conditions. Thus, the decrease in the level of PEA is likely one factor contributing to the change in PPAR $\alpha$  signaling. Measures of PPAR $\alpha$  mRNA and protein did not support a change in expression of PPAR $\alpha$  in DRGs of tumor-bearing mice, but the sample size was small. A decrease in PPAR $\alpha$  protein was noted in DRGs ipsilateral to an inflamed hind paw (D'Agostino et al., 2009). Given that PPAR $\alpha$  protein has a half-life of 1 h and ligand binding reduces its turnover (Blanquart et al., 2002), the benefit of inhibition of NAAA in tumor-bearing mice may be that an increase in endogenous PPAR $\alpha$  ligands stabilizes the receptor and promotes its downstream effects. The effect of PPAR $\alpha$  ligands on the evoked-Ca<sup>2+</sup> transient in DRG neurons occurred within minutes, indicating the underlying signaling pathway is independent of gene transcription.

Mechanical hyperalgesia in tumor-bearing mice was associated with increased amplitude of the evoked Ca<sup>2+</sup> transient in small DRG neurons. Calcium is an intracellular messenger that can promote hyperalgesia through multiple mechanisms including increased neurotransmitter release, gene expression and membrane excitability (Berridge et al., 2000). The amplitude of a depolarization-evoked Ca<sup>2+</sup> transient in neurons is an integration of Ca<sup>2+</sup> influx, efflux and release from intracellular stores (Thayer and Miller 1990). The mechanism underlying the PPAR $\alpha$  modulation of the Ca<sup>2+</sup> transient remains to be resolved. The magnitude of the effect of PPAR $\alpha$  agonists in the co-culture condition suggests that PPAR $\alpha$  most likely inhibits Ca<sup>2+</sup> influx through voltage-dependent Ca<sup>2+</sup> channels. Evidence that clearance of intracellular Ca<sup>2+</sup> loads by a plasma membrane Ca<sup>2+</sup> ATPase and sarco-endoplasmic reticulum Ca<sup>2+</sup> ATPase can be increased through an intracellular signaling pathway (Usachev et al., 2006) suggests these proteins may be other downstream targets. Overall, a drug induced decrease in the amplitude of the Ca<sup>2+</sup> transient in tumor-bearing mice would decrease hyperalgesia.

Because AEA, another fatty acid ethanolamide, is decreased in DRGs in the cancer condition, it is of interest to compare effects of PEA and AEA in parallel experiments. Similar to PEA, AEA decreases the amplitude of the evoked Ca<sup>2+</sup> transient in small DRG neurons and mechanical hyperalgesia in tumor-bearing mice (Khasabova et al., 2008). These actions of AEA are mediated by CB1R coupling to inhibition of voltage-dependent Ca<sup>2+</sup> channels (Ross et al., 2001; Khasabova et al., 2004, 2008). However, the absence of an effect of PPAR $\alpha$  on the occurrence of an evoked Ca<sup>2+</sup> transient differs from CB1R activity in small DRG neurons. Anandamide decreases the occurrence of the Ca<sup>2+</sup> transient evoked under conditions similar to those used in the present experiments (Khasabova et al., 2008) suggesting a divergence in PPAR $\alpha$  and CB1 receptor signaling. A difference in underlying mechanisms most likely contributes to the positive interaction of AEA and GW7647 in promoting antinociception in the formalin assay (Russo et al., 2007).

## CONCLUSION

Medium conditioned by fibrosarcoma cells decreased the levels of PEA and AEA in DRG neurons. The present data in conjunction with an earlier report (Khasabova et al., 2008) support the conclusion that consequences of these changes are decreased activation of PPAR $\alpha$  by PEA and CB1R by AEA. Downstream effects of these receptors converge on

decreasing the depolarization evoked increase in  $[Ca^{2+}]_i$ . Thus, when levels of AEA and PEA decrease in DRG neurons, the amplitude of a depolarization-evoked  $Ca^{2+}$  transient increases, resulting in an increase in the activity of intracellular pathways to enhance nociception. Regardless of whether CB1R and PPAR $\alpha$  are on the same neurons or different populations of neurons, the anti-hyperalgesic efficacy of FAAH inhibitors, which increase the level of AEA, is likely to be enhanced by NAAA inhibitors, which increase the level of PEA, in the management of cancer pain.

## Acknowledgments

This work was supported by grants from the National Cancer Institute CA138684 (VSS), National Institute on Drug Abuse R01 DA012413 (DP) and the University of Minnesota's Undergraduate Research Opportunity Program (YX). The authors are grateful to P. Villalta and B. Mater at the University of Minnesota Cancer Center for assistance in the quantification of endocannabinoids and to N. Burlakova for technical assistance.

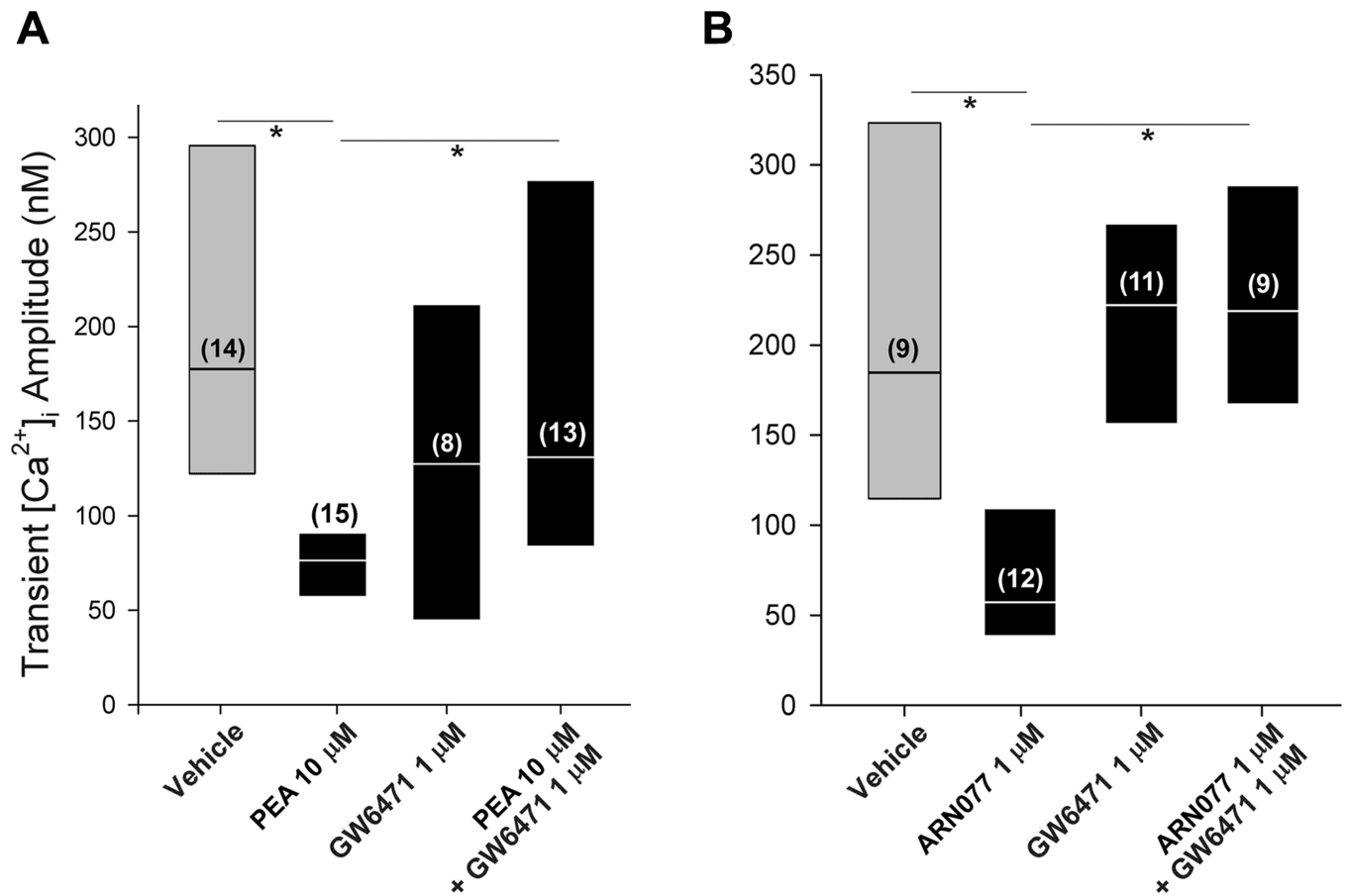
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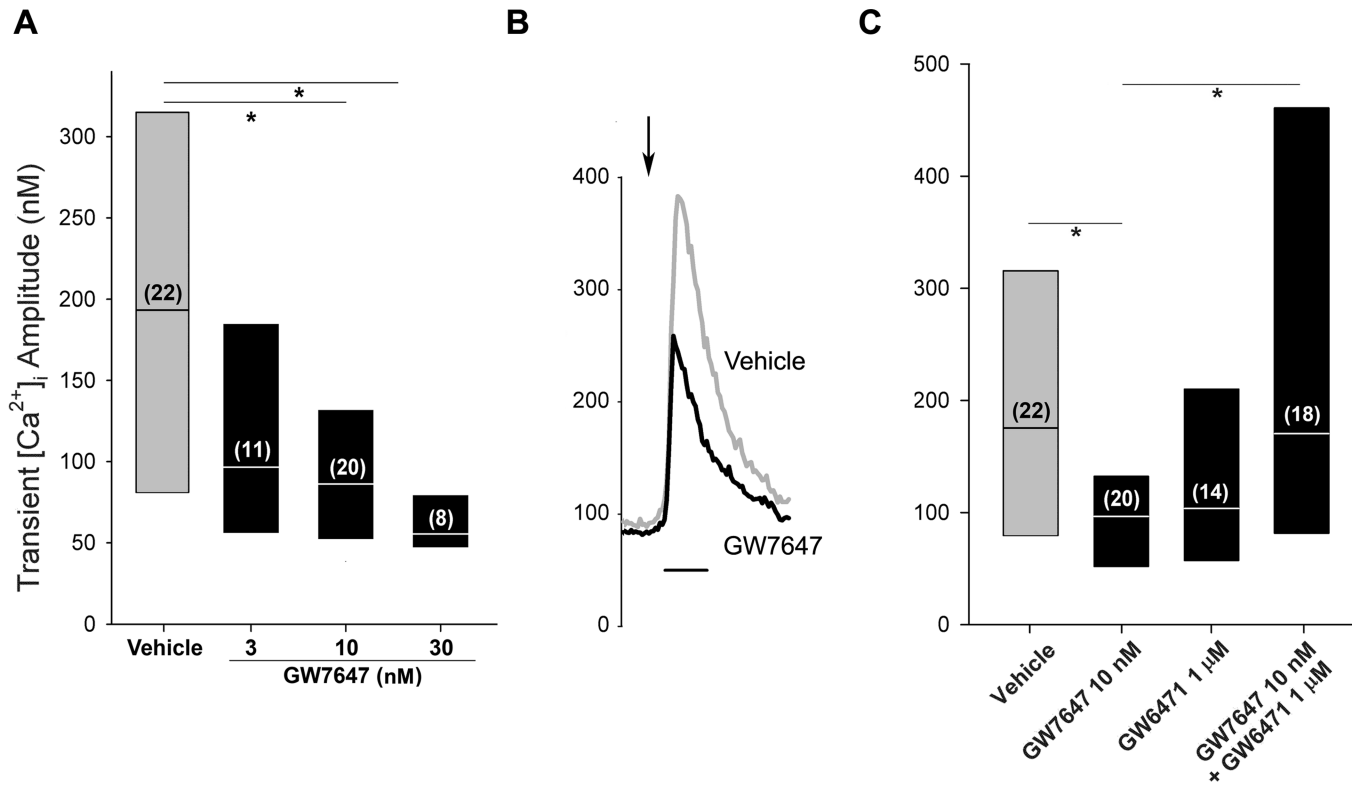
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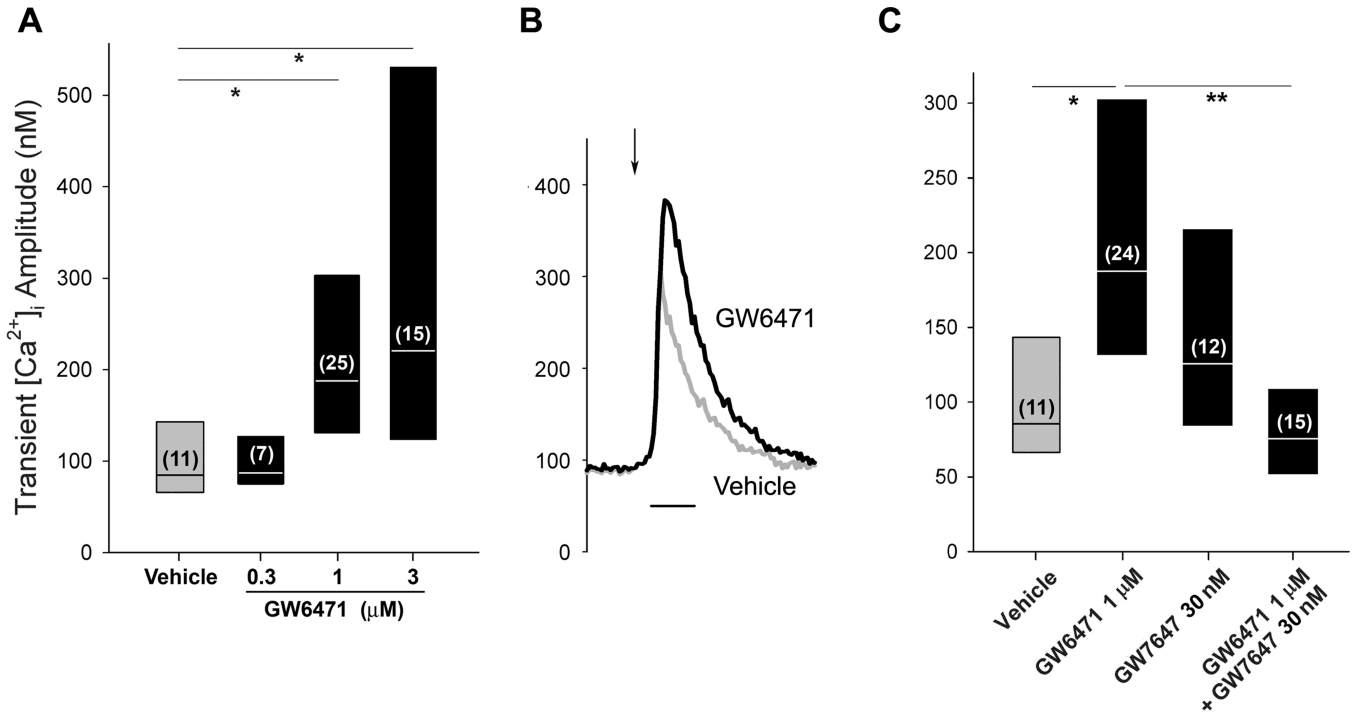
**Figure 1. Pretreatment with PEA decreased the amplitude of the depolarization-evoked  $\text{Ca}^{2+}$  transient in DRG neurons co-cultured with fibrosarcoma cells**

(A) The effect of exogenous PEA (10  $\mu\text{M}$ ) was blocked by pretreatment with the PPAR $\alpha$  antagonist GW7647. (B) An inhibitor of NAAA, the enzyme that degrades PEA, mimicked the effect of PEA. The label for the Y-axis is the same as for A. Results are reported as the median and the 25<sup>th</sup>–75<sup>th</sup> percentile range; the numbers in the parentheses indicate the number of neurons that responded to 30 mM KCl. \*Different at  $p < 0.05$  (Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's test).



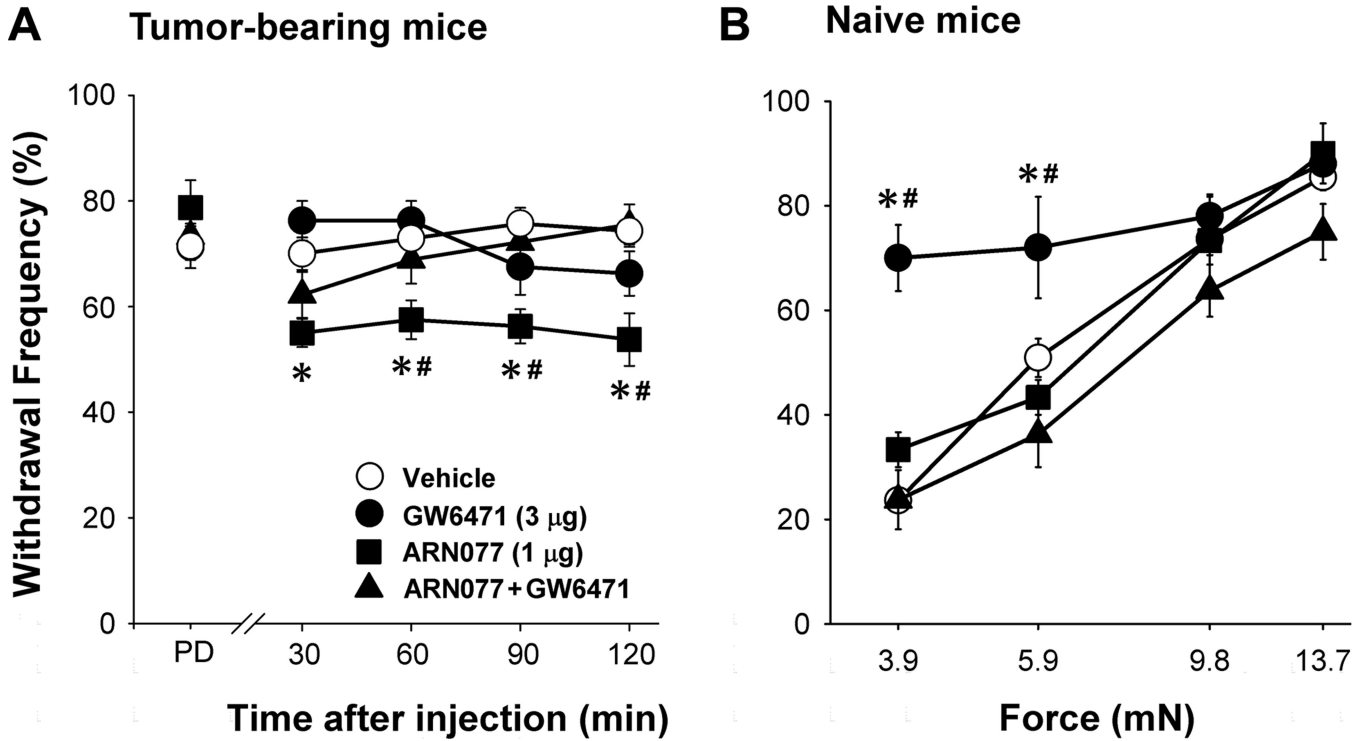
**Figure 2. A synthetic PPAR $\alpha$  agonist decreased the amplitude of the evoked Ca<sup>2+</sup> transient in DRG neurons co-cultured with fibrosarcoma cells**

(A) Pretreatment with GW7647 (5 min) decreased the amplitude of the Ca<sup>2+</sup> transient in a concentration-dependent manner. The label of the Y-axis is the same for B and C. (B) Examples of Ca<sup>2+</sup> transients evoked in small neurons maintained in the co-culture condition during superfusion with vehicle (gray) or 10 nM GW7647 (black). The arrow indicates the time of superfusion with 30 mM KCl; the scale bar = 30 s. (C) GW6471 blocked the effect of GW7647 on the Ca<sup>2+</sup> transient. Results are reported as the median and the 25<sup>th</sup>–75<sup>th</sup> percentile range; the numbers in the parentheses indicate the number of neurons that responded to 30 mM KCl. \*Different at  $p < 0.05$  (Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's test).

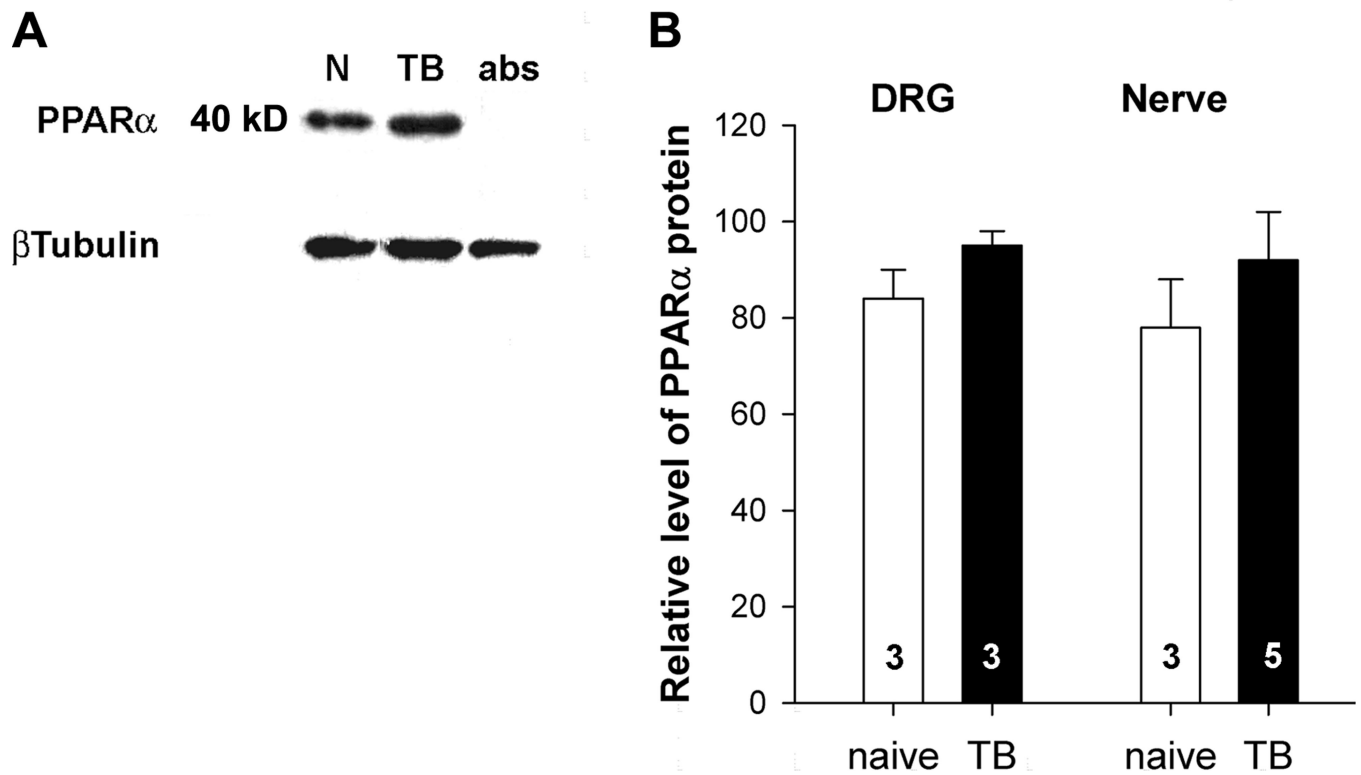


**Figure 3. The PPAR $\alpha$  antagonist increased the evoked Ca<sup>2+</sup> transient in the small DRG neurons in the control condition**

(A) Pretreatment with GW6471 increased the amplitude of the Ca<sup>2+</sup> transient in a concentration-dependent manner in small DRG neurons maintained in the control condition. The label of the Y-axis is the same for B and C. (B) Examples of Ca<sup>2+</sup> transients evoked in small neurons maintained in the control condition during superfusion with vehicle (gray) or 1 μM GW6471 (black). The arrow indicates the time of superfusion with 30 mM KCl; the scale bar = 30 s. (C) Co-treatment with the PPAR $\alpha$  agonist GW7647 blocked the effect of GW6471 on the amplitude of the Ca<sup>2+</sup> transient. Results are reported as the median and the 25<sup>th</sup>–75<sup>th</sup> percentile range, the numbers in the parentheses indicate the number of neurons with a positive response to 30 mM KCl. \*Different at  $p < 0.05$  (Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's test).



**Figure 4. The NAAA inhibitor and the PPAR $\alpha$  antagonist had differential effects on mechanical sensitivity in tumor-bearing and naive mice**  
 (A) Mechanical hyperalgesia occurred in tumor-bearing mice prior to drug injection (PD, pre-drug). The NAAA inhibitor ARN077 (1  $\mu$ g, i.pl., ipsilateral to the tumor) decreased mechanical hyperalgesia, and its effect was blocked by co-administration of the PPAR $\alpha$  antagonist GW6471 (3  $\mu$ g i.pl.).  $n=4-9$  mice/treatment. (B) In naive mice, mechanical hyperalgesia was measured ipsilateral to intraplantar injection of the PPAR $\alpha$  antagonist GW6471 (3  $\mu$ g) at 1 hr after drug injection. The mechanical hyperalgesia was blocked by co-injection of GW6471 with ARN077 (1  $\mu$ g). The label for the Y-axis is the same as for A. Results are expressed as the mean $\pm$ SEM.  $n=3-11$  mice/treatment. \*Significantly different from vehicle and #different from GW6471+ARN077 at  $p<0.02$  (two-way ANOVA with Bonferroni  $t$  test).



**Figure 5. The tumor condition did not change the expression of PPAR $\alpha$  protein in DRGs or tibial nerve**

(A) Representative images of PPAR $\alpha$ - and  $\beta$ -Tubulin-immunoreactive bands from a western blot prepared of tibial nerves from naive (N) and tumor-bearing (TB) mice. (B) Quantification of PPAR $\alpha$  protein in DRGs and tibial nerves from naive and TB mice. The optical density of PPAR $\alpha$  band was divided by the optical density of  $\beta$ -tubulin band and multiplied by 100. Data represent the mean  $\pm$  S.E.M., the numbers inside the bars represent the sample size.

**Table 1**

Levels of PEA and endocannabinoids in DRG cells and fibrosarcoma cells.

| Substance                           | PEA<br>(pmol)      | 2-AG<br>(pmol)   | AEA<br>(fmol) |
|-------------------------------------|--------------------|------------------|---------------|
| DRG cells in culture                |                    |                  |               |
| Control                             | 60.6 (46.0–76.8)   | 17.4 (13.7–20.5) | 32 (15–57)    |
| Co-culture                          | 35.6 (28.6–38.4) * | 18.8 (14.0–20.4) | 9 (8–9) *     |
| DRGs L <sub>3</sub> -L <sub>5</sub> |                    |                  |               |
| Naive                               | 31.6 ± 3.0         | 4.74 ± 0.88      | 273 ± 42      |
| Tumor-bearing                       | 22.6 ± 1.7 **      | 4.35 ± 2.39      | 151 ± 25 **   |
| Fibrosarcoma cells                  | 7.9 ± 2.6          | 417 ± 31         | 7 ± 1         |

One sample for DRG cells in culture was equivalent to cells dissociated from 5–6 DRGs. The data represent the median (25<sup>th</sup>–75<sup>th</sup> percentile range, n=6).

\* Significantly different from control at  $p < 0.005$  (Mann-Whitney Rank Sum Test). DRGs L<sub>3</sub>-L<sub>5</sub> from one side of a mouse were pooled to make one sample at the time of euthanasia. Samples were ipsilateral to the tumor in tumor-bearing mice. Data represent the mean ± S.E.M. (n=5).

\*\* Significantly different from naive at  $p < 0.05$  (Student's *t* test). Values for fibrosarcoma cells represent the mean ± S.E.M. (n=3).

**Table 2**

PEA hydrolysis increased in DRG cells co-cultured with fibrosarcoma cells

| Culture condition | Treatment | % hydrolysis of [ <sup>3</sup> H]PEA |
|-------------------|-----------|--------------------------------------|
| Control           | vehicle   | 18.0 ± 1.1 (5)                       |
| Co-culture        | vehicle   | 23.0 ± 0.5* (6)                      |
|                   | ARN077    | 19.5 ± 0.4** (6)                     |

Each sample was equivalent to cells dissociated from 3–4 DRGs. See Methods for additional experimental details. Values represent the mean ±S.E.M. of hydrolysis of [<sup>3</sup>H]PEA, the number in parentheses is the sample size.

\* Significantly different from control/vehicle at  $p < 0.001$ ,

\*\* different from co-culture/vehicle at  $p < 0.01$  (One-way ANOVA with Bonferroni  $t$ -test).

**Table 3**

Effect of ARN077 on PEA and endocannabinoid levels in DRG cultures from naive mice.

|         | PEA<br>(pmol) | AEA<br>(fmol) | 2AG<br>(pmol) |
|---------|---------------|---------------|---------------|
| Control | 80 ± 8        | 40 ± 2        | 17.6 ± 1.6    |
| ARN077  | 142 ± 2 *     | 42 ± 11       | 19.3 ± 1.9    |

Each sample was equivalent to cells dissociated from 5–6 DRGs. Samples were maintained in vitro for 40–48 hr. Culture medium was replaced with HEPES buffer prior to treatment with 1  $\mu$ M ARN077 for 15 min. DRGs and medium were extracted for measures of PEA and endocannabinoids. Values represent the mean  $\pm$  S.E.M. (n=3).

\* Significantly different from control at  $p < 0.005$  (Student's *t*-test).



**Table 4**

Co-culture of DRG neurons with fibrosarcoma cells increased the response of neurons to 30 mM KCl.

|            | Basal $[Ca^{2+}]_i$<br>(nM) <sup>I</sup> | Amplitude of $Ca^{2+}$ transient<br>(nM) <sup>I</sup> | Frequency of response<br>to 30 mM KCl | n  |
|------------|--|---|---------------------------------------|----|
| Control    | 86 (72–100)                              | 85 (58–143)   | 41%                                   | 27 |
| Co-culture | 94 (70–104)                              | 290 (106–376) *                                       | 71% **                                | 55 |

All data were collected from small neurons during superfusion with vehicle (0.006% ethanol).

<sup>I</sup>Data for  $[Ca^{2+}]_i$  re expressed as the median (25<sup>th</sup>–75<sup>th</sup> percentile range).

\* Different from control at  $p < 0.005$  (Mann-Whitney Rank Sum test).

\*\* Different from control at  $p < 0.02$  (Fisher Exact Test).