Palmitoylethanolamide Reverses Paclitaxel-Induced Allodynia in Mice

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ABSTRACT

Chemotherapy-induced peripheral neuropathy (CIPN) represents a serious complication associated with antineoplastic drugs. Although there are no medications available that effectively prevent CIPN, many classes of drugs have been used to treat this condition, including anticonvulsants, serotonin and noradrenaline reuptake inhibitors, and opioids. However, these therapeutic options yielded inconclusive results in CIPN clinical trials and produced assorted side effects with their prolonged use. Thus, there is an urgent need to develop efficacious and safe treatments for CIPN. In this report, we tested whether the endogenous lipid palmitoylethanolamide (PEA) alone or in combination with the anticonvulsant gabapentin would reduce allodynia in a mouse paclitaxel model of CIPN. Gabapentin and PEA reversed paclitaxel-induced allodynia with respective ED_{50} doses (95% confidence interval) of 67.4 (61.52–73.94) and 9.2 (8.39–10.16) mg/kg. Isobolographic analysis of these drugs in combination revealed synergistic antiallodynic effects. The PPAR-α antagonist receptor antagonist GW6471 [*N*-((2S)-2-(((1Z)-1-methyl-3-oxo-3-(4-(trifluoromethyl)phenyl)prop-1-enyl)amino)-3-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy)phenyl)propyl)propanamide] completely blocked the antinociceptive effects of PEA. In addition, PEA administered via intraplantar injection into a paw, intrathecal injection, and intracerebroventricular injection reversed paclitaxel-induced allodynia, suggesting that it may act at multiple sites in the neuroaxis and periphery. Finally, repeated administration of PEA (30 mg/kg, 7 days) preserved the antiallodynic effects with no evidence of tolerance. These findings taken together suggest that PEA possesses potential to treat peripheral neuropathy in cancer patients undergoing chemotherapy.

Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) represents a dose-limiting side effect of anticancer drugs, including taxanes (paclitaxel and docetaxel), vinca alkaloids (vincristine and vinblastine), platinum-based agents (cisplatin, carboplatin, and oxaliplatin), bortezomib, and thalidomide (Grisold et al., 2012). Paclitaxel (Taxol, Bristol Myers Squibb, New York, NY) is one of the most commonly used anticancer drugs successfully employed as a first line treatment of several solid as well as blood cancers, such as ovarian cancer, breast cancer, cervical cancer, non–small-cell lung carcinomas, and Kaposi sarcoma (Vyas and Kadow, 1995). Unfortunately, paclitaxel induces peripheral neuropathic pain, with an incidence of 30 to 50% after a single dose, increasing to more than 50% after a second dose (Farquhar-Smith, 2011). Thus, CIPN limits the selection of cytostatic drugs and dosage, delays subsequent treatment cycles, and leads to discontinuation of therapy. Analgesic drugs currently used to treat neuropathic pain (e.g., amitriptyline or gabapentin) failed to alleviate CIPN in randomized, placebo-controlled clinical trials (Rao et al., 2007; Kautio et al., 2009). Additionally, long-term gabapentin treatment elicits significant adverse effects, including sedation, dizziness, peripheral edema, and ataxia (Mathiesen et al., 2014). Thus, safe and efficacious treatments are greatly needed to treat CIPN.

Based on recent data demonstrating efficacy of the endogenous fatty acid amide palmitoylethanolamide (PEA) in rat model of oxaliplatin-induced neurotoxicity (Di Cesare Mannelli et al., 2015a), the present study investigated whether the effectiveness of PEA would extend to a paclitaxel mouse model of CIPN. Specifically, we investigated PEA mechanism of action, the effect of PEA in combination with gabapentin, the consequences of repeated PEA administration, and its locus of action. PEA acts as an autocoid local injury antagonist amide because of its negative modulation of mast cell activation (Aloe at al., 1993). It also elicits antinociceptive effects in different animal models of pain, such as spinal cord injury (Genovese at al., 2008), carrageenan-induced acute inflammation (D'Agostino et al., 2009), and complete Freund's adjuvant-induced chronic inflammation (LoVerme et al., 2006). In addition, PEA reduces thermal hyperalgesia

ABBREVIATIONS: AEA, anandamide; ANOVA, analysis of variance; AUC, area under the curve; CIPN, chemotherapy-induced peripheral neuropathy; mPFC, medial prefrontal cortex; PEA, palmitoylethanolamide; PPAR-α, peroxisome proliferator-activated receptor alpha; TRPV1, transient receptor potential channel of the vanilloid type 1.

and mechanical allodynia in the mouse sciatic nerve injury model of neuropathic pain (Costa et al., 2008), as well as reverse allodynia in mouse model of diabetes-induced peripheral neuropathy (Donvito et al., 2015).

Although is recognized that PEA is an endogenous ligand for peroxisome proliferator-activated receptor alpha (PPAR-α), its pharmacological effects may be indirectly mediated by other receptors, including transient receptor potential channel of the vanilloid type 1 (TRPV1), and cannabinoid CB$_1$ and CB$_2$ receptors. In fact, it was shown that PEA potentiated the antinociceptive effects of anandamide (AEA) on cannabinoid or TRPV1 receptors (De Petrocellis et al., 2001; Smart et al., 2002). This so-called "entourage effect" may be mediated by PEA competitive inhibition of AEA hydrolysis by its hydrolytic enzyme fatty acid amide hydrolase (Jonsson et al., 2001) and/or a direct allosteric effect on TRPV1 (Ho et al., 2008).

PPAR-α is expressed in peripheral sensory neurons, throughout the central nervous system, and in immune cells (Braissant et al., 1996). Its activation leads to a downregulation of the nuclear factor κB cascade controlling pain and inflammation (D'Agostino et al., 2009). TRPV1 is distributed in brain as well as in sensory nerve terminals involved in the pain pathway (Tóth et al., 2005). The CB$_1$ receptor is heterogeneously expressed at high levels throughout the central nervous system (Hohmann and Herkenham, 1999) and is sparsely expressed in lymphocytes, splenocytes, and T cells (Schatz et al., 1997). Myeloid, lymphoid mast cells, and macrophages express CB$_2$ receptors (Lu and Mackie, 2016). CB$_1$ and CB$_2$ receptor agonists produce antinociception in several animal models (Rani Sagar et al., 2012). Accordingly, we used selective antagonists of PPAR-α, TRPV1, CB$_1$, and CB$_2$ receptors to elucidate which receptor(s) mediate the antinociceptive effects of PEA in paclitaxel-treated mice.

Neuronal pathways are involved in the development of neuropathic pain but also dorsal root ganglia, Schwann cells, microglia, astrocytes, and immune cells play contributing roles, suggesting that it may be modulated at multiple levels in the neuroaxis as well as in the periphery (Scholz and Woolf, 2007). To examine locus of action, we tested the effectiveness of intraplantar, intrathecal, and intracerebroventricular PEA administration in reversing paclitaxel-induced allodynia. Moreover, to ascertain if the antinociceptive effects of PEA undergo tolerance, we evaluated the consequences of repeated injections of PEA in paclitaxel-treated mice.

Methods

Animals. Adult male ICR mice (18–35 g, Harlan Laboratories, Indiana, IN) served as subjects in these experiments. Mice were housed in plastic cages four per cage in a temperature (20–22°C)- and humidity (55 ±10%)-controlled animal care-approved facility on 12-hour light/dark cycle with standard rodent chow and water available ad libitum. All procedures adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs and Treatment. PEA, gabapentin, and the TRPV1 antagonist capsazepine were acquired from Cayman (Ann Arbor, MI), and paclitaxel and the PPARα receptor antagonist GW6471 [N-((2S)-2-(((1Z)-1-methyl-3-oxo-3-(4-(trifluoromethyl)phenyl)prop-1-enyl)amino)-3-(4-(2-(5-methyl-2 phenyl-1,3-oxazol-4-yl)ethoxy)phenyl)propyl)propanamide] was purchased from Tocris (Minneapolis, MN). Rimonabant, the

CB$_1$ receptor antagonist, and the CB$_2$ receptor antagonist SR144528 (N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide) were generously provided by the Drug Supply Program of the National Institute on Drug Abuse NIDA (Rockville, MD). Gabapentin was dissolved in saline, and all other drugs were dissolved in ethanol (5% of total volume), alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ) (5% of total volume) and saline (0.9% NaCl) (90% of total volume). Drugs were administered via the intraperitoneal route of administration in an injection volume of 10 μl/g body weight. PEA or vehicle was also given via an intraplantar hind paw, intrathecal, or intracerebroventricular route of administrations. PEA was administered in 20 μl for intraplantar injection and in 2 μl for intrathecal and intracerebroventricular injection.

Paclitaxel-Model of CIPN. Mice were injected with paclitaxel (8 mg/kg i.p.) or vehicle every other day for a total of four injections. This protocol has been well characterized to produce bilateral allodynia (Smith et al., 2004).

The von Frey test was used to assess responses to mechanical touch. For acclimation to the test environment (Murphy et al., 1999), mice were positioned on a wire mesh screen (spaces 0.5 mm apart) and habituated for 30 minutes/day for 4 days before paclitaxel administration. The von Frey test consist of a series of calibrated monofilaments, (2.83–4.31 log stimulus intensity; North Coast Medical, Morgan Hills, CA), which are randomly applied to the left and right plantar surface of the hind paw for 3 seconds. Lifting, licking, or shaking the paw was considered a response. Mice were assessed for mechanical allodynia 24 hours after the final paclitaxel injection.

Experimental Design. To assess the time course of the antiallodynic effect elicited by each drug, mechanical allodynia was evaluated prepaclitaxel injection 24 hours after the fourth injection of paclitaxel (0 minute) and 30, 60, 90, 120, 180, and 240 minutes after PEA or gabapentin administration (Fig. 1, A and B). To evaluate the time course of the antiallodynic effects evoked by equieffective doses of each drug in combination, mechanical allodynia was assessed prepaclitaxel injection, 24 hours after the fourth injection of paclitaxel (0 minute) and 30, 60, 90, 120, 180, 240, and 300 minutes after coadministration of PEA and gabapentin (Fig. 1C). A within-subject, Latin square design was employed to assess each the dose-response relationship of each drug, and between each test day, a 72-hour washout period was imposed.

In the antagonism experiments, rimonabant (3 mg/kg), SR144528 (3 mg/kg), capsazepine (5 mg/kg), and GW6471 (2 mg/kg) were administered 10 minutes before PEA (30 mg/kg) administration, and mechanical thresholds were measured 60 minutes after PEA. The doses of rimonabant, SR144528, and capsazepine selected in this study were based on the results of published data (Kinsey at al., 2009; Donvito et al., 2015), whereas the selected dose of GW647 was based on unpublished data (data not shown).

Acute intrathecal injections were performed as previously described (Wilkerson et al., 2016). In brief, at the end of the intrathecal catheter a 27-gauge needle with the plastic hub removed was inserted. At the level of lumbosacral enlargement (L4-L5), an injection containing 2 μl of drug or vehicle was gently infused. A successful intrathecal injection was evident after light tail twitching. Mice were randomly assigned to drug treatment. Once the intrathecal procedure was completed, the 27-gauge needle, as well as the intrathecal catheter, was removed.

To test whether supraspinal site of action mediates the PEA-induced antinociceptive effects, PEA or vehicle was injected via acute intracerebroventricular route of administration, as previously described (Wilkerson et al., 2016). Briefly, on the evening before the test day, mice were anesthetized via isoflurane, and their bregma was expose through an incision. A unilateral injection site was prepared in animal using stereotaxic coordinates −0.6 mm rostral, 1.2 mm lateral from bregma, and at a depth of 2.0 mm. To check the right depth of the needle, a portion of a polyurethane tubing was used. On the test day, a 26-gauge needle was used to make the intracerebroventricular injection, and the needle was kept in the same position for 20 seconds to ensure drug delivery. Mechanical allodynia was assessed in ipsilateral
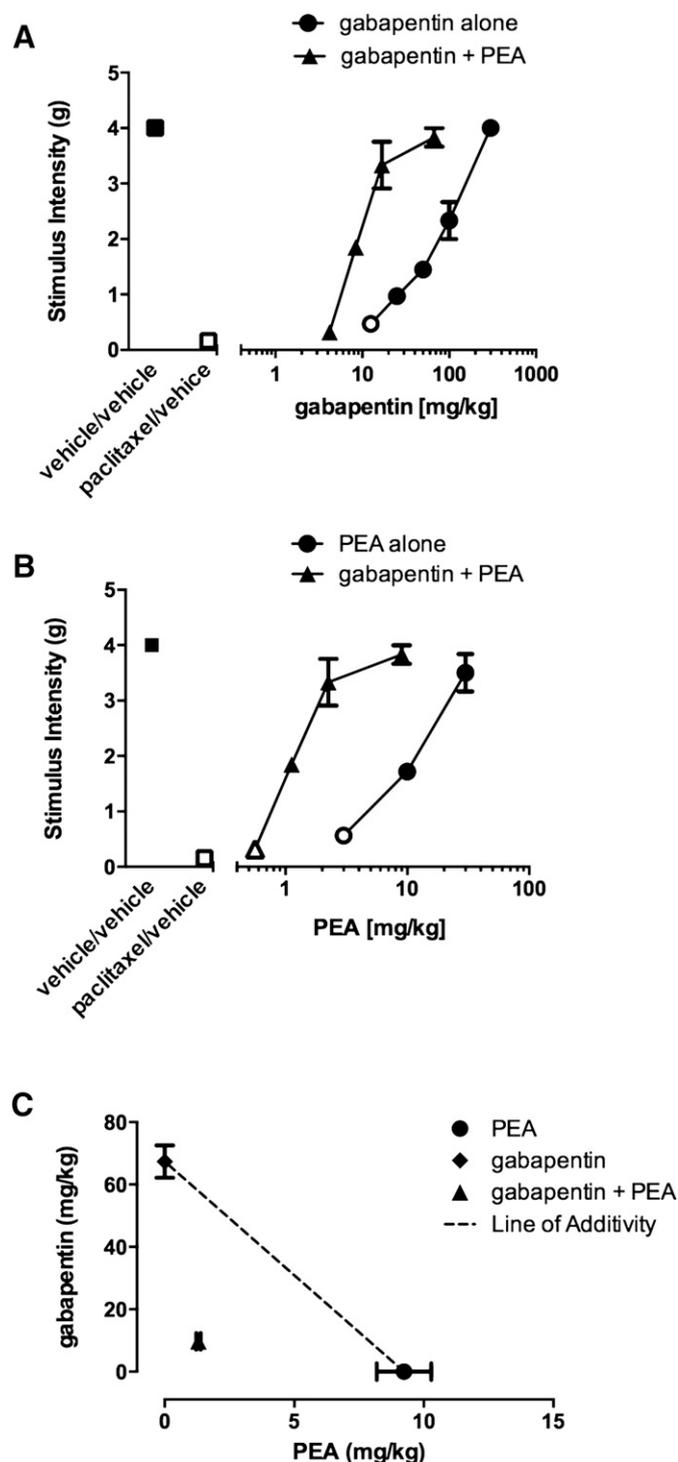
Fig. 1. Systemic gabapentin and PEA reverse paclitaxel-induced allodynia. (A) Intraperitoneal gabapentin dose relatedly reverses paclitaxel-induced allodynia 60 minutes after administration. Equieffective doses of gabapentin and PEA given in combination produces a leftward shift of the dose-response curve. (B) Intraperitoneal PEA reverses paclitaxel-induced allodynia in a dose-related manner 60 minutes after administration. An equieffective combination of gabapentin and PEA produces a leftward shift of dose-response curve. (C) The equieffective combination of gabapentin and PEA produces a synergistic effect as it falls below the line of additivity. For this graph, the derived ED_{50} value of PEA was plotted on the abscissa, and ED_{50} value of gabapentin was plotted on the ordinate. Filled symbols indicate significance from paclitaxel/vehicle ($P < 0.05$) by one-way ANOVA followed by Dunnett's test. Data reflect mean ± S.E.M., $n = 6$ mice/group.

and contralateral paws before paclitaxel injection (prepaclitaxel); after paclitaxel injections but before intraplantar, intrathecal, and intracerebroventricular injections of PEA (0 minute); and 30, 60, 90, 120, 180, and 240 minutes after administration. The intraplantar, intrathecal, and intracerebroventricular doses were selected basing on previous preliminary data (data not shown).

To assess the expression of tolerance, paclitaxel-injected mice were injected with PEA (30 mg/kg i.p., $n = 6$ mice; group repeated PEA) or vehicle ($n = 12$ mice) once a day for 7 days. On day 8, half the mice in the vehicle group were administered vehicle (group vehicle) and the other half of the mice were injected with PEA (30 mg/kg i.p.; group acute PEA). These mice were then tested for allodynia 1 hour after their final injection, while group repeated PEA was tested 24 hours after their final injection.

Data Analysis. Data were analyzed using one- or two-way analysis of variance (ANOVA). For the time course study, we calculated the area under the curve (AUC) using GraphPadPrism (GraphPad Software Inc., San Diego, CA). Dunnett's test was used for post hoc analysis in the AUC evaluation, antagonism study, and repeated administration study. In the dose-response study, intraplantar, intrathecal, and intracerebroventricular injection study data were analyzed using two-way ANOVA followed by Tukey's post hoc.

The ED_{50} dose and lower equally effective dose values as well as 95% confidence limits (Bliss, 1967) were calculated through a standard linear regression analysis of the linear portion of the dose-response curve for gabapentin, PEA or the combination of both drugs that reversed allodynia. The additive ED_{50} value of the combined drugs was calculated from each dose-response curve to determine whether the interaction was synergistic, additive, or subadditive (Tallarida, 2002). The combination was assumed to be the sum of the effects of each drug. In the isobologram, the ED_{50} of PEA was plotted on the abscissa, and the isoeffective dose of gabapentin was plotted on the ordinate. The line that connects the two points in the graph represents the theoretical additive effect of gabapentin and PEA dose in combination. The ED_{50} of the drug in combination was evaluated by linear regression as the mixed dose of gabapentin and PEA. The experimentally derived ED_{50} value (Z_{mix}) from the dose response curves of the ratios was compared with the predicted additive ED_{50} value (Z_{add}). The interaction is considered synergistic if the ED_{50} values of the Z_{mix} are below those of Z_{add} and the confidence intervals do not overlap (Tallarida, 2001, 2006). Fisher's exact test was used to analyzed the statistical difference between the theoretical additive ED_{50} value and the experimental ED_{50} value (Naidu et al., 2009). A P value of <0.05 was considered statistically significant. For all statistical analysis, GraphPad Prism version 6.0 (GraphPad Software Inc.) was employed. All data are expressed as mean ± S.E.M.

Results

The Combination of Gabapentin and PEA Reverses Paclitaxel-Induced Allodynia in a Synergistic Manner. Gabapentin (Fig. 1A) and PEA (Fig. 1B) dose dependently reversed paclitaxel-induced mechanical allodynia, with respective ED_{50} values (95% confidence limits) of 67.4 (61.5–73.9) and 9.2 (8.4–10.2) mg/kg. Equieffective doses of gabapentin and PEA given in combination produced a leftward shift in the dose-response curve compared with the curves of either compound given alone (Fig. 1, A and B). In Fig. 1C, the isobolographic analysis showed a synergistic interaction between these drugs. The calculated experimental Z_{mix} value [0.286 (0.270–0.303)] was significantly less than the calculated theoretical Z_{add} value [1.581 (0.891–2.806)] and below the line of additivity.

Figure 2 depicts the antiallodynic effects evoked by gabapentin, PEA, and the combination of both drugs over time expressed as area under curve (AUC). The AUC data show
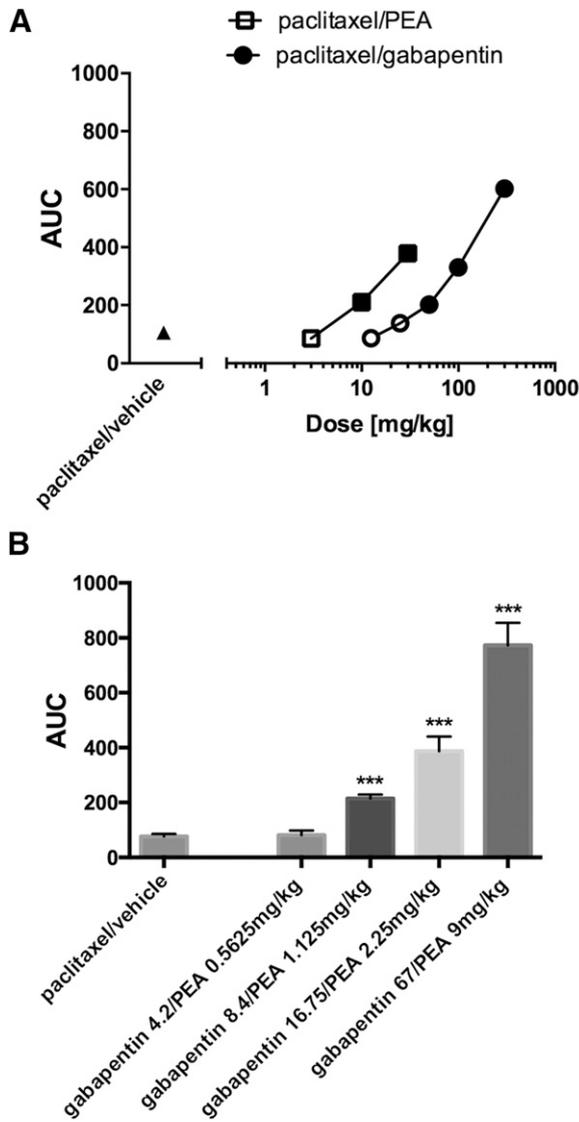
Fig. 2. Administration of gabapentin and PEA reverses paclitaxel-induced allodynia. (A) The area under curve (AUC) shows that an intraperitoneal injection of either gabapentin or PEA reverses paclitaxel-induced allodynia in a dose-related manner. Filled symbols indicate significance from paclitaxel/vehicle ($P < 0.05$). (B) The AUC reveals that intraperitoneal administration of equieffective doses of gabapentin and PEA given in combination reverses paclitaxel-induced allodynia in a dose-related fashion. ***$P < 0.0001$, versus paclitaxel/vehicle by one-way ANOVA followed by Dunnett's test. Data reflect mean ± S.E.M., $n = 6$ mice per group.
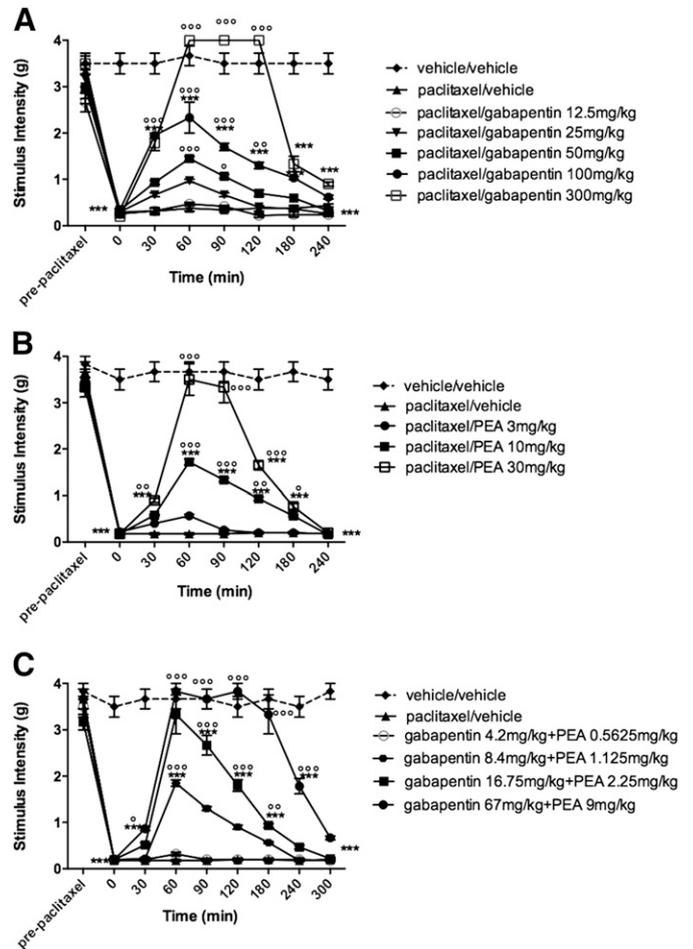

Fig. 3. Time course of the antiallodynic effects evoke by gabapentin and PEA. (A) Gabapentin dose dependently reverses mechanical allodynia in paclitaxel-injected mice with a peak effect at 60 minutes after administration. (B) PEA fully reverses allodynia in dose-dependent fashion when administrated in paclitaxel-treated mice, eliciting the peak effect at 60 minutes after administration. (C) The administration of equieffective doses of gabapentin and PEA given in combination dose dependently reverse paclitaxel-induced allodynia, which is longer lasting than by either drug given alone. ***$P < 0.0001$ versus vehicle/vehicle. °°°$P < 0.0001$, °°$P < 0.001$, °$P < 0.05$ versus paclitaxel/vehicle by two-way ANOVA followed by Tukey's test. Data reflect mean ± S.E.M., $n = 6$ mice/group.

that intraperitoneally administered gabapentin [$F(5,30) = 868$; $P < 0.0001$] or PEA [$F(3,20) = 325$; $P < 0.0001$] dose dependently reversed paclitaxel-induced allodynia (Fig. 2A). Moreover, the intraperitoneal equieffective doses of gabapentin and PEA given in combination reversed paclitaxel-induced allodynia in a dose-related manner [$F(4,25) = 250$; $P < 0.0001$; Fig. 2B]. The increased maximal effect for the AUC data in the drug combination condition compared with either PEA alone or gabapentin alone is in accordance with the time course data in which the combination of PEA and gabapentin resulted in a prolonged duration of action (Fig. 3) [$F(7,245) = 236$; $P < 0.0001$; Fig. 3A; $F(7,715) = 197$; $P < 0.0001$; Fig. 3B; $F(8,240) = 229$; $P < 0.0001$; Fig. 3C].

The Antiallodynic Effect of PEA in Paclitaxel-Treated Mice is PPAR-α Mediated. To elucidate which receptors are involved in the antiallodynic effects of PEA, mice were given an intraperitoneal injection of PPAR-α, TRPV1, CB$_1$, or CB$_2$ receptor antagonists 10 minutes before PEA (30 mg/kg) administration. As shown in Fig. 4A, GW6471 (2 mg/kg i.p.), a selective PPAR-α receptor antagonist, completely blocked the antiallodynic effects evoked by PEA [$F(3,20) = 251$; $P < 0.0001$]. Conversely, administration of the TRPV1 antagonist capsazepine [5 mg/kg i.p.; $F(3,20) = 159$; $P < 0.0001$; Fig. 4B], the CB$_1$ receptor antagonist rimonabant [3 mg/kg i.p.; $F(3,20) = 173$; $P < 0.0001$; Fig. 4C], and the CB$_2$ receptor antagonist SR144528 [3 mg/kg i.p.; $F(3,20) = 157$; $P < 0.0001$; Fig. 4D] failed to inhibit the antiallodynic effects of PEA.

PEA-Induced Allodynia: Locus of Action. To evaluate the locus of action mediating the antiallodynic effects of PEA, we examined whether intraplantar, intrathecal, or intracerebroventricular injections of PEA would reverse
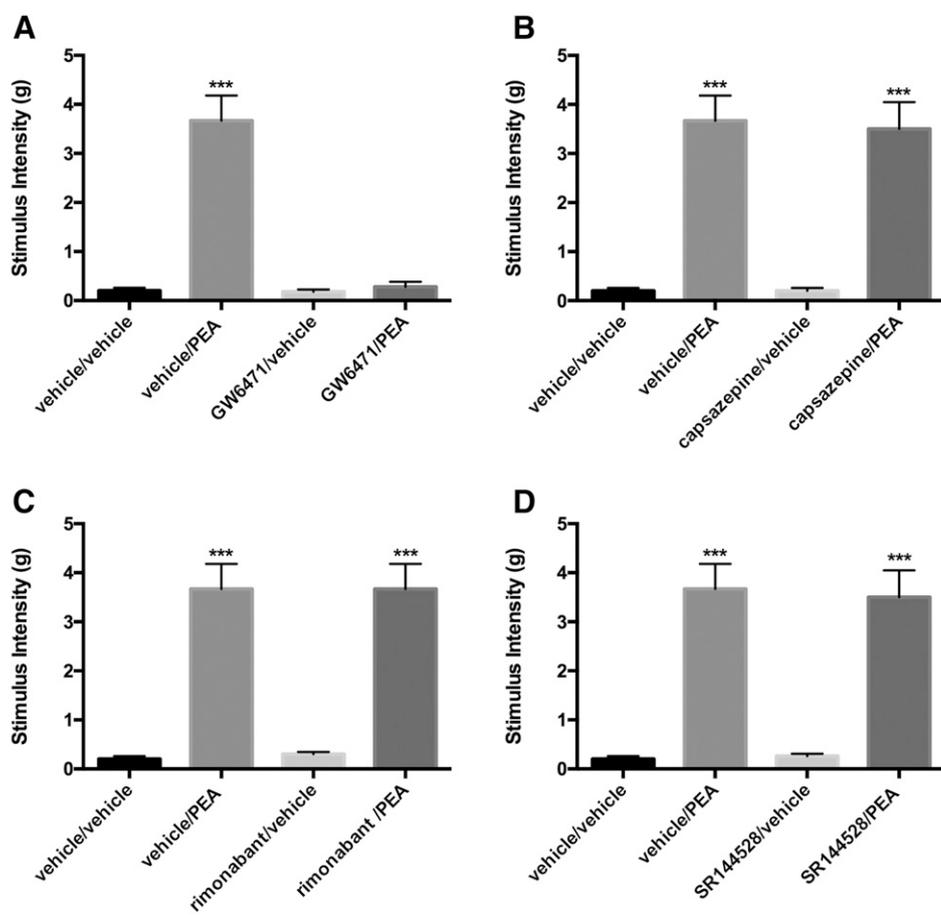
Fig. 4. The antiallodynic effect of PEA (30 mg/kg) requires PPAR-α but not TRPV1, CB_1, CB_2 receptors. (A) GW6471 (2 mg/kg) blocks antiallodynic effects of PEA administration in paclitaxel-induced allodynia, but there is no effect of capsazepin (5 mg/kg) (B), rimonabant (3 mg/kg) (C), or SR144528 (3 mg/kg) (D) in the blockade of mechanical allodynia. ***$P < 0.0001$ versus vehicle/vehicle by one-way ANOVA followed by Dunnett's test. Data reflect mean $\pm$ S.E.M., n = 6 mice per group.

paclitaxel-induced allodynia. Intraplantar injection of PEA (10 μg/20 μl) into a hind paw of paclitaxel-treated mice reversed allodynia in a time-dependent manner [$F (7,140) = 68.92; P < 0.0001$; Fig. 5A]. To infer whether the antiallodynic effects occurred because of diffusion from the injection site, we also tested the contralateral paw. As shown in Fig. 5B, PEA did not alter paclitaxel-induced allodynia in the contralateral paw at any time point.

The possibility that PEA would reverse paclitaxel-induced allodynia after central injection was also examined. Intrathecal injection of PEA (1 μg/2 μl) produced full reversal of mechanical allodynia in the right [$F (7,140) = 51.29; P < 0.0001$; Fig. 5C] and left [$F (7,140) = 50.30; P < 0.0001$; Fig. 5D] paws in time-related fashions. Similarly, intracerebroventricular administration of PEA (1 μg/2 μl) time dependently reversed paclitaxel-induced allodynia in the right paw [$F (7,140) = 39.72; P < 0.0001$; Fig. 5E] and left paw [$F (7,140) = 31.79; P < 0.0001$; Fig. 5F]. The peak of antiallodynic effects of PEA occurred at 60 minutes after each route of administration.

PEA Retains Its Antiallodynic Effects after Repeated Administration. To determine whether the antiallodynic effects of PEA after intraperitoneal administration would undergo tolerance, paclitaxel-injected mice were given an intraperitoneal injection of PEA (30 mg/kg; group repeated PEA) or vehicle once a day for 7 days. On day 8, half the mice in the vehicle group were administer vehicle (group vehicle) and the other half of the mice were given an intraperitoneal injection of PEA (30 mg/kg; group acute PEA). All mice were tested on day 8. As shown in Fig. 6, group acute PEA and group

repeated PEA displayed significant antiallodynic effects compared with the paclitaxel-treated mice injected with vehicle, but did not differ from one another [$F (2,15) = 65.1; P < 0.0001$]. Additionally, repeated administration of PEA (30 mg/kg) did not affect the mechanical thresholds in mice not treated with paclitaxel.

Discussion

CIPN reflects a serious form of neuropathic pain in cancer patients, which adversely impacts treatment. Because of the need for effective pharmacotherapies to treat CIPN, the present study examined whether exogenous administration of PEA would reverse paclitaxel-induced allodynia in mice (Fehrenbacher, 2015). PEA is a bioactive lipid that produces antinociceptive effects in different animal models of neuropathic pain, including spinal cord injury (Genovese at al., 2008), sciatic nerve injury (Costa et al., 2008), and diabetes-induced peripheral neuropathy (Donvito et al., 2015), as well as in inflammatory models of pain (Costa et al., 2002; D'Agostino et al., 2007). Here, we show that PEA dose dependently reverses mechanical allodynia in paclitaxel-treated mice. The positive control gabapentin also dose dependently reversed paclitaxel-induced allodynia. Strikingly, combined administration of gabapentin and PEA produced synergistic antiallodynic effects in paclitaxel-treated mice, with a prolonged duration of action compared with single administration of these drugs. Similarly, PEA and acetaminophen produces synergistic antihyperalgesic
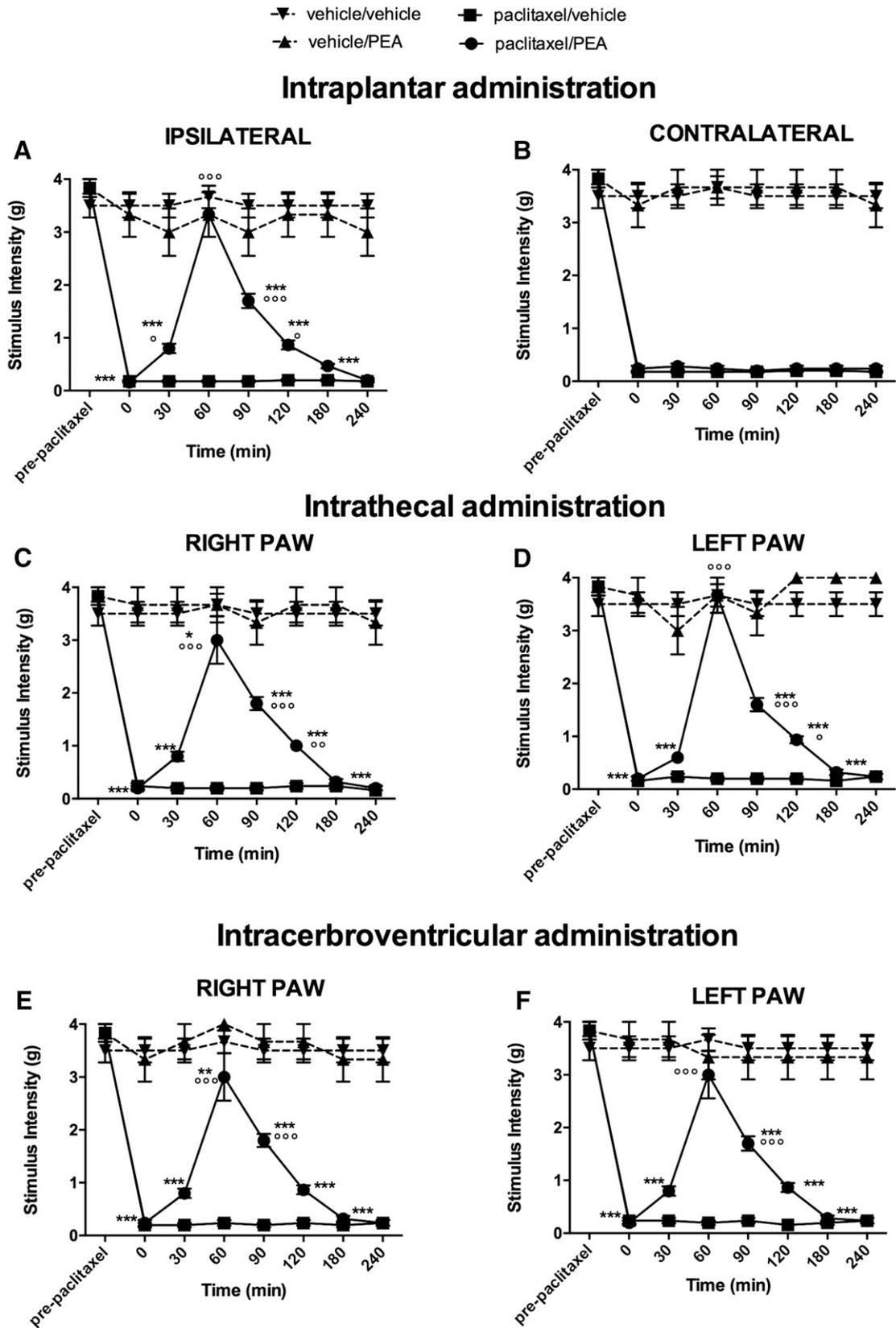
Fig. 5. Locally administered PEA reverses paclitaxel-induced allodynia. (A) Intraplantar PEA (10 μg) fully reversed paclitaxel-induced allodynia 60 minutes after administration. (B) This injection did not affect paclitaxel-induced allodynia in contralateral paw at any time point. PEA (1 μg) administered intrathecally reversed paclitaxel-induced allodynia at 60 minutes after administration in the right paw (C) and left paw (D). PEA (1 μg) administered intracerebroventricularly also reversed paclitaxel-induced allodynia in the right paw (E) and left paw (F) 60 minutes after administration. ***$P < 0.0001$, **$P < 0.001$, *$P < 0.05$ versus vehicle/vehicle. °°°$P < 0.0001$, °°$P < 0.001$, °$P < 0.05$ versus paclitaxel/vehicle by two-way ANOVA followed by Tuckey's test. Data reflect mean $\pm$ S.E.M., $n = 6$ mice/group.
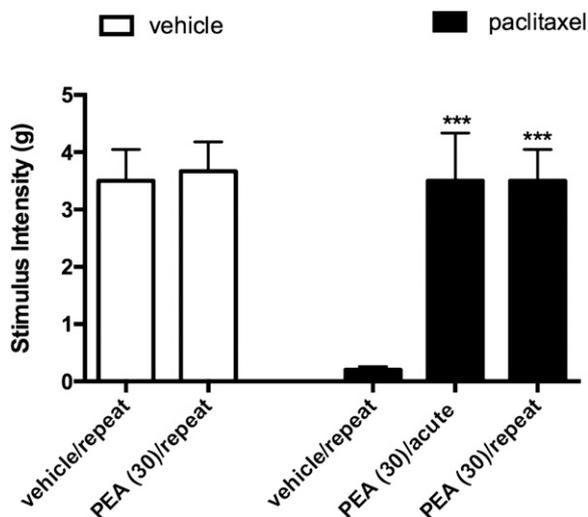
Fig. 6. The antiallodynic effects of PEA (30 mg/kg) are retained after 7 days of repeated administration. Compared with intraperitoneal vehicle paclitaxel-injected mice, the acute administration of PEA (30 mg/kg) produces a total reversal from allodynia in paclitaxel-treated mice, which is not diminished by repeated administration. Repeated administration of PEA (30 mg/kg) did not affect the mechanical threshold in vehicle-treated mice. ***$P < 0.0001$ versus paclitaxel/vehicle by one-way ANOVA followed by Tukey's test. Data reflect mean $\pm$ S.E.M., $n = 6$ mice/group.

effects in the streptozotocin-induced diabetic rat model of neuropathic pain (Déciga-Campos and Ortíz-Andrade, 2015).

Another object of this study was to elucidate which receptor(s) mediate(s) the antiallodynic effects of PEA in paclitaxel-treated mice. Our findings demonstrated that PPAR-α receptors mediated the antiallodynic effects of PEA, because this effect was blocked by the administration of the PPAR-α antagonist GW6471. Conversely, the receptor antagonism studies show that CB_1, CB_2, and TRPV1 receptors do not play a necessary role in PEA-induced reversal of allodynia in paclitaxel-treated mice. These findings are in agreement to the current idea that the primary pharmacological effects of PEA are mediated by activation of PPAR-α, which controls pain and inflammation by switching off the nuclear factor κB signaling pathway, a crucial element in the transcription of genes, leading to the synthesis of proinflammatory and proalgesic mediators (LoVerme et al., 2006; D'Agostino et al., 2009). In this study, we observed that the peak of antinociceptive effects of PEA occurs at approximately 60 minutes regardless of route of administration. This delayed onset of action is consistent with results of Guida et al. (2015) and suggests that PEA-activated PPAR-α receptors may reverse paclitaxel-induced allodynia through a genomic mechanism.

To identify the locus of action of the PEA effect, we tested whether PEA would reverse paclitaxel-induced allodynia given via intraplantar, intrathecal, or intracerebroventricular route of administration. The findings that intraplantar PEA administration reversed allodynia in the injected paw but not in the contralateral paw support a local site of action. Additionally, the observations that PEA reversed allodynia after intracerebroventricular or intrathecal administration suggest supraspinal and spinal sites of action. Accordingly, Jhaveri et al. (2008) found significantly decreased levels of AEA and PEA in the hind paw at the peak of carrageenan-induced hyperalgesia, possibly related to either increased

metabolism of AEA and PEA or their decreased synthesis. Importantly, inhibition of fatty acid amide hydrolase or COX-2 was associated with antinociceptive effects, which were blocked by GW6471, a PPAR-α antagonist, consistent with this receptor involvement in pain transmission at the peripheral level (Jhaveri et al., 2008). On the other hand, carrageenan led to a reduction in expression of PPAR-α receptors in DRGs (dorsal root ganglia), which was restored to basal levels by intracerebroventricular administration of PEA, suggesting that supraspinal administration of PEA modulates PPAR-α expression in DRG (D'Agostino et al., 2009). Interestingly, PPAR-α receptors expressed in brain appear to play opposing roles on nociception. In contrast to the well-described anti-inflammatory/antinociception effects of PEA, the medial pre-frontal cortex (mPFC), which is involved in supraspinal affective and cognitive modulation of pain and highly expresses PPAR-α (Moreno et al., 2004), may play a facilitatory role in nociception. Specifically, intraplantar injection of formalin in rats led to reduced levels of PEA and oleoylethanolamide in the mPFC and a PPAR-α receptor antagonist delayed the onset of phase 2 nociception (Okine et al., 2014). These findings suggest that PPAR-α receptors in the mPFC play a permissive role in formalin-induced nociception. In contrast, Guida et al. (2015) observed increased PEA levels in the mPFC at 15 days in the spared sciatic nerve injury mouse model of neuropathic pain and found that exogenously administered PEA elicited antinociceptive effects in this assay. These findings suggest that PEA production might be an adaptive response to neuropathic pain development aimed at counteracting pain transmission or maintenance (Guida et al., 2015).

Another notable finding in the present study is that the antinociceptive effects of PEA (30 mg/kg) were maintained after 7 days of repeated administration, suggesting diminished tolerance. In fact, daily injections of PEA for 7 days produces an antiallodynic effect that persists for at least 24 hours. These results are consistent with other studies showing no tolerance after repeated administration of PPAR-α receptor agonists. For example, Guida et al. (2015) found that 30 days of repeated administration of PEA (10 mg/kg) fully reversed allodynia in the model of spared nerve injury of the sciatic nerve (Guida et al., 2015). Di Cesare Mannelli et al. (2015a) reported that PEA (30 mg/kg) retained its antinociceptive effects after repeated administration for 20 days in a rat oxaliplatin model of CIPN. They also found that repeated PEA injections prevented morphologic derangements in DRGs and oxaliplatin-induced increases in ATF3 expression in Schwann cells and DRG neurons of peripheral nerves. In particulars, ex vivo histologic and molecular analysis of peripheral nerves, spinal cord, and dorsal root ganglia, showed neuroprotective effects and the PEA-induced blockade of glia activation after repeated administration. The normalization of the electrophysiological activity of the spinal nociceptive neurons suggests protective effects of PEA (Di Cesare Mannelli et al., 2015a). Moreover, PEA delayed tolerance to morphine-induced antinociception in rats through a decrease of cytokines released by astrocytes (Di Cesare Mannelli et al., 2015b).

The mechanisms by which coadministration of PEA and gabapentin produced enhanced antiallodynic effects remain to be determined. PEA produces anti-inflammatory, analgesic, and neuroprotective effects through the activation of PPAR-α

receptors throughout the central and peripheral nervous system (LoVerme et al., 2005; D'Agostino et al., 2009). Furthermore, the antinociceptive activity of PEA may be associated with a direct action on mast cells, via autocoid local injury antagonist mechanism (Aloe et al., 1993), combining a dual activity of neurons in nociceptive pathways and nonneuronal cells, such as mast cells in the periphery and glia in the spinal cord. In contrast, the mechanism(s) by which gabapentin evokes its antinociceptive effects in paclitaxel-induced allodynia remains to be elucidated. One possibility is based on the work of Xiao et al. (2007), who reported that paclitaxel increased expression of the $\alpha_2\delta$-1 subunit in the dorsal horn of the spinal cord. Strikingly, they found that repeated administration of gabapentin produced an inhibitory effect on $\alpha_2\delta$-1 subunit of voltage-dependent calcium channels in the spinal cord (Xiao et al., 2007). Because selective pharmacological agents targeting this calcium channel subunit are not currently available, future studies could investigate the underlying mechanisms mediating gabapentin-induced antinociception using genetic approaches (e.g., gene knockdown or overexpression). Considering this multitude of effects, the combination of PEA and gabapentin may produce synergistic antinociceptive effects through simultaneous activation of PPAR-α receptors that are expressed in diverse nervous system and peripheral regions and cell types and decreased glutamate release via gabapentin dampening of paclitaxel-activated presynaptic calcium influx.

The present study focused on PEA reversal of paclitaxel-induced allodynia; however, another important unmet clinical need is preventing the development of CIPN. Thus it will be important in future studies to determine whether PEA offers neuroprotection from paclitaxel-induced allodynia. Consistent with this notion, PEA administration restored nerve function in patients diagnosed with CIPN who were undergoing thalidomide and bortezomib treatment of multiple myeloma. In particular, Truini et al. (2011) demonstrated that PEA exerted a positive action on myelinated fibers through the regulation of mast cell hyperactivity, providing significant restoration of nerve function. Thus, PEA may exert a similar effect to prevent paclitaxel-induced peripheral neuropathy. Moreover, other evidence shows that exogenous administration of PEA can enhance the beneficial effect that endogenous PEA spontaneously exerts in case of damage. In fact, PEA has been reported to act as a protective mediator produced on-demand during inflammation, neuronal damage, and pain. Accordingly, several studies demonstrate that PEA levels, as well as endocannabinoid levels, in tissue are altered in different pathologic conditions in either experimental models or in the clinic, such as after ischemia and stroke in animals (Moesgaard et al., 2000; Berger et al., 2004), in the skin of mice with streptozotocin-induced diabetic neuropathy in biopsies from patients with ulcerative colitis (Darmani et al., 2005), one clinical case of stroke (Schabitz et al., 2002), and in the blood of back pain patients.

In conclusion, the present study demonstrates the endogenous lipid PEA reverses allodynia in a mouse paclitaxel model of CIPN through multiple routes of administration. The antiallodynic effects shown here are mediated by PPAR-α receptors and do not undergo tolerance after 7 days of repeated administration. Strikingly, the combination of gabapentin and PEA reverses paclitaxel-induced allodynia in a synergistic manner and for a prolonged duration of action compared with administration of either drug alone. Overall, these results indicate that PEA represents a potential therapeutic option to treat CIPN in cancer patients.

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