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Intracellular emetic signaling evoked by the L-type Ca^{2+} channel agonist FPL64176 in the least shrew (*Cryptotis parva*)

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Abstract

Ca^{2+} plays a major role in maintaining cellular homeostasis and regulates processes including apoptotic cell death and side-effects of cancer chemotherapy including vomiting. Currently we explored the emetic mechanisms of FPL64176, an L-type Ca^{2+} channel (LTCC) agonist with maximal emetogenic effect at its 10 mg/kg dose. FPL64176 evoked c-Fos immunoreactivity in shrew brainstem sections containing the vomit-associated nuclei, nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus. FPL64176 also increased phosphorylation of proteins ERK1/2, PKC α / β II and Akt in the brainstem. Moreover, their corresponding inhibitors (PD98059, GF 109203X and LY294002, respectively) reduced FPL64176-evoked vomiting. A 30 min subcutaneous (s.c.) pretreatment with the LTCC antagonist nifedipine (10 mg/kg) abolished FPL64176-elicited vomiting, c-Fos expression, and emetic effector phosphorylation. Ryanodine receptors (RyRs) and inositol trisphosphate receptors (IP₃Rs) mediate intracellular Ca^{2+} release from the sarcoplasmic/endoplasmic reticulum. The RyR antagonist dantrolene (i.p.), or a combination of low doses of nifedipine and dantrolene, but not the IP₃R antagonist 2-APB, significantly attenuated FPL64176-induced vomiting. The serotonin type 3 receptor (5-HT₃R) antagonist palonosetron (s.c.), the neurokinin 1 receptor (NK₁R) antagonist netupitant (i.p.) or a combination of non-effective doses of netupitant and palonosetron showed antiemetic potential against FPL64176-evoked vomiting. Serotonin (5-HT) and substance P immunostaining revealed FPL64176-induced emesis was accompanied by an increase in 5-HT but not SP-immunoreactivity in the dorsomedial subdivision of the NTS. These findings demonstrate that Ca^{2+} mobilization through LTCCs and RyRs, and subsequent emetic effector phosphorylation and 5-HT release play important roles in FPL64176-induced emesis which can be prevented by 5-HT₃R and NK₁R antagonists.

Keywords

L-type Ca^{2+} channel; FPL64176; ERK; Ryanodine receptor; Serotonin

1. Introduction

Serotonin (5-HT) type 3 receptor (5-HT₃R)- and substance P (SP) neurokinin receptor (NK₁R) are major emetic receptors in chemotherapy-induced vomiting (CIV) (Darmani and Ray, 2009). Antiemetic regimens against high-dose cisplatin-type CIV include a 5-HT₃R antagonist (e.g. palonosetron), an NK₁R antagonist (e.g. netupitant), and dexamethasone (Jordan et al., 2016). Emetic loci include the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus (DMNX) and the area postrema (AP) in the brainstem, as well as the enteric nervous system (ENS) and enterochromaffin cells (EC cells) in the gastrointestinal tract (GIT) (Darmani and Ray, 2009).

Voltage-gated calcium (Ca²⁺) channels include L-type, P/Q-type, N-type, R-type, and T-type, which govern Ca²⁺ influx into cells (Zamponi et al., 2015). Intracellular Ca²⁺ serves as a second messenger for events such as protein phosphorylation (Zurgil and Zisapel, 1983), neurotransmitter release (Catterall, 2000) and Ca²⁺ influx (Katoh et al., 2000). Both extracellular Ca²⁺ entry and intracellular Ca²⁺ release contribute to cytosolic Ca²⁺ increase (Taylor and Tovey, 2010). Recently we provided an overview of how Ca²⁺ mobilization is linked to emesis (Zhong et al., 2017). Direct support comes from the identification of a novel emetogen FPL64176 (a selective L-type Ca²⁺ channel (LTCC) agonist), which causes vomiting in least shrews (*Cryptotis parva*) (Darmani et al., 2014). Moreover, LTCC blockers nifedipine and amlodipine are broad-spectrum antiemetics against diverse emetogens including FPL64176 (Darmani et al., 2014; Zhong et al., 2014a).

The intracellular Ca²⁺ mobilizing agent thapsigargin, is a sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor, which increases cytosolic Ca²⁺ concentration via intracellular Ca²⁺ store depletion followed by extracellular Ca²⁺ entry (Beltran-Parrazal et al., 2012). Thapsigargin is also a potent emetogen since it evokes robust vomiting in least shrews and increases c-Fos expression in brainstem emetic nuclei AP, NTS and DMNX, as well as SP immunoreactivity in DMNX (Zhong et al., 2016). Ca²⁺ release from intracellular sarcoplasmic/endoplasmic reticulum (SER) into cytoplasm is accomplished by inositol trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyRs) (Gomez-Viquez et al., 2003). IP₃Rs and RyRs are differentially involved in vomiting evoked by different emetogens (Zhong et al., 2014b and 2016). Thapsigargin- and FPL64176-like drugs affecting cytosolic Ca²⁺ dynamics are considered as potential targeted apoptotic chemotherapeutics (Cano-Abad et al., 2001; Doan et al., 2015).

Vomit-associated cytosolic Ca²⁺ rise as well as time-dependent increases in phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) occur in the shrew brainstem following 5-HT₃R-mediated vomiting and emesis caused by thapsigargin (Zhong et al., 2014b and 2016). Moreover, protein kinase Cα/βII (PKCα/βII) and ERK1/2 phosphorylation have been observed during cisplatin-induced emesis (Darmani et al., 2013 and 2015).

In the current study, we investigated the mechanisms underlying FPL64176-induced emesis by: i) pharmacologically evaluating the involvement of RyR, IP₃R, NK₁R and 5-HT₃R using their corresponding antagonists, ii) the use of immunohistochemistry to demonstrate whether

c-Fos induction and/or 5-HT- or SP-release in the shrew brainstem emetic nuclei occur following FPL64176-evoked vomiting; and iii) using Western blots to demonstrate if enhanced phosphorylation of brainstem intracellular emetic effectors [PKC α / β II, ERK1/2, and protein kinase B (Akt)] occurs following FPL64176-induced emesis.

2. Material and methods

2.1. Animals

Adult least shrews were bred in the animal facilities of the Western University of Health Sciences. Shrews were housed in groups of 5–10 on a 14:10 light:dark cycle, fed with food and water *ad libitum*. All the shrews used were 45–60 days old and weighed between 4–5 g (Darmani, 1998). All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Western University of Health Sciences. We made all efforts to minimize animal suffering and to reduce the number of animals used.

2.2. Chemicals

The following drugs were used for the present studies: FPL64176 from Tocris Bioscience (Minneapolis, MN), LTCC inhibitor nifedipine from Sigma (St. Louis, MO), RyR antagonist dantrolene, and IP₃R antagonist 2-APB from Santa Cruz (Dallas, TX), PI3K-Akt pathway inhibitor LY294002 and PKC inhibitor GF109203X from Tocris (Minneapolis, MN), MEK-ERK inhibitor PD98059 from Calbiochem (San Diego, CA). PD98059 inhibits MEK and therefore phosphorylation of ERK (Hotokezaka et al., 2002). 5-HT₃R antagonist palonosetron and NK₁R antagonist netupitant were kindly provided by Helsinn Health Care (Lugano, Switzerland). Palonosetron was dissolved in water, FPL64176, nifedipine, dantrolene, 2-APB, PD98059, LY294002, GF109203X in 25% DMSO in water and netupitant was dissolved in a 1:1:18 solution of emulphor™, ethanol and saline. All drugs were administered at a volume of 0.1 ml/10 g of body weight.

2.3. Behavioral emesis studies

On the day of the experiment shrews were brought from the animal facility, separated into individual cages and allowed to adapt for at least two hours (h). Daily food was withheld 2 h prior to the start of the experiment but shrews were given 4 mealworms each prior to FPL64176 injection. Immediately following injection, each shrew was placed in the observation cage and vomiting behavior (number of animals vomiting within groups and frequency of vomits) were observed for 30 min (Chebolu et al., 2010). An administration of FPL64176 at 10 mg/kg (i.p.) has previously been shown to evoke vomiting with a maximal frequency in 100% of tested shrews (Darmani et al., 2014; Zhong et al., 2014a). Thus, this dose was used for subsequent antagonist and biochemical studies. To evaluate drug interaction studies, different groups of shrews were pretreated with varying doses of the: i) LTCC inhibitor nifedipine (0, 10 mg/kg, s.c., n = 6); ii) RyR inhibitor dantrolene (0, 0.5, 1 and 5 mg/kg, i.p., n = 6 per group); iii) IP₃R inhibitor 2-APB (0, 0.5, 1, 5 and 20 mg/kg, i.p., n = 6 per group); iv) PKC inhibitor GF109203X (0, 2, 5 and 10 mg/kg, i.p., n = 6); v) MEK-ERK1/2 pathway inhibitor PD98059 (0, 1, 2.5 and 5 mg/kg, i.p., n = 6 per group); vi) PI3K inhibitor LY294002 (0, 2, 5 and 10 mg/kg, i.p., n = 6); and vii) NK₁R antagonist netupitant

(0, 1, 5, and 10 mg/kg, i.p., n = 6 per group). The injection of corresponding vehicles was expressed as 0 mg/kg. The antiemetic effects of a combination of non-effective doses of nifedipine (s.c.) with dantrolene (i.p.) were also investigated. Thus, different groups of shrews (n = 7 per group) were pre-treated either with their corresponding vehicles (nifedipine 0 mg/kg + dantrolene 0 mg/kg) or varying combinations of different drugs (nifedipine 0.25 mg/kg + dantrolene 0 mg/kg; or nifedipine 0 mg/kg + dantrolene 0.5 mg/kg; or nifedipine 0.25 mg/kg + dantrolene 0.5 mg/kg). Thirty minutes later, each treated shrew received a 10 mg/kg emetic dose of FPL64176 (i.p.). To evaluate the anti-emetic effect of a combination of palonosetron (s.c.) with netupitant (i.p.), different groups of shrews (n = 8 per group) were pretreated with either: palonosetron 0 mg/kg + netupitant 0 mg/kg, palonosetron 0 mg/kg + netupitant 1 mg/kg, palonosetron 0.1 mg/kg + netupitant 0 mg/kg, or palonosetron 0.1 mg/kg + netupitant 1 mg/kg. The number of animals vomiting within groups and the frequency of vomits for each animal were recorded for the next 30 min. Each shrew was used once and then euthanized with isoflurane following the termination of each experiment.

2.4. c-Fos staining and image analysis

Immunohistochemistry was performed as described previously (Chebolu et al., 2010; Darmani et al., 2008). Different shrew groups (n = 3 shrews per group) were treated with nifedipine (10 mg/kg, s.c.), or the corresponding vehicle (25% DMSO); 30 min prior to FPL64176 (10 mg/kg, i.p.) injection. Shrews injected with vehicle of nifedipine followed by vehicle of FPL64176 were used as controls. For non-vomiting animals, i.e. controls and shrews treated with nifedipine followed by FPL64176, shrews were euthanized 110 min after the second injection. Otherwise 90 min after the first emesis induced by FPL64176 occurred, shrews were anesthetized with isoflurane and perfused with ice cold 4% paraformaldehyde in pH 7.4, 0.1 M phosphate-buffered saline (PBS) for 10 min. Brains were removed and cryoprotected with 30% sucrose in 0.1 M PBS overnight. The optimal cutting temperature compound-embedded brainstem block was cut on a freezing microtome (Leica, Bannockburn, IL, USA) into 30- μ m sections, and stored in PBS with 0.03% sodium azide.

Sections were observed under a light microscope and those containing the DVC were subjected to immunostaining using rabbit anti-c-Fos polyclonal antibody (1:10000, PC38, CalBiochem, San Diego, CA) which were performed according to our published studies (Ray et al., 2009a and 2009c). Photomicrographs of the DVC containing AP, NTS and DMNX were taken by Nikon Eclipse E600 microscope. c-Fos-immunoreactive cell nuclei for a given region (AP, NTS, DMNX) were then counted by an observer blind to the animal's treatment. For each region, 3 sections at 90- μ m intervals were counted per animal. The means of each region per animal was further compared. Our lab has well described the circumventricular organ AP and the different cytoarchitectonic detail such as cell size and packing differentiating DMNX from NTS within the DVC, as part of a stereotaxic atlas of the least shrew brainstem (Ray et al., 2009a). This criterion has been well recognized (Chebolu et al., 2010; Darmani et al., 2008; Ray et al., 2009a and 2009c; Zhong et al., 2014b and 2016).

2.5. Western blots for phosphorylation analyses

To determine the time-dependent profile of PKC α / β II, ERK1/2, and Akt phosphorylation, different groups of animals ($n = 3$ per time-point group) were euthanized at 5, 10, 15, 20, 25, 30, 60 min following FPL64176 administration (10 mg/kg, i.p.). The lower half of brainstem, mostly medullary structures, was isolated as described elsewhere (Zhong et al., 2014b) and homogenized in lysis buffer. Protein extracts from brainstem lysates were subjected to Western blot.

2.5.1. Antibodies—The following primary antibodies were used for Western blot: phospho-ERK1/2 (1:1000, #9101, Cell Signaling, Danvers, MA), ERK1/2 (1:3000, #9107, Cell Signaling), phospho-Akt (Ser473) (1:2000, #4060, Cell Signaling), Akt (1:2000, #2920, Cell Signaling), phospho-PKC α / β II (Thr638/641) (1:1000, #9375, Cell Signaling), and GAPDH (1:10000, MAB374, EMD Millipore, Temecula, CA). The following secondary antibodies were used for western blot: goat anti-rabbit IRDye 680RD and goat anti-mouse IRDye 800CW (1:10000, LI-COR Biosciences, Lincoln, NE). Blots were visualized using Odyssey imaging system (LI-COR Biosciences, Lincoln, NE) and densitometric analysis of bands was performed using ImageJ software. The ratios of pPKC α / β II (~ 80 kD) to GAPDH (~ 39 kD), pERK1/2 (42/44 kD) to ERK1/2, and pAkt (~ 60 kD) to Akt were calculated. All values were divided by the average value at time-point 0 min which refers to vehicle of FPL64176 treated shrews for normalization and presented as fold change of control.

To identify the role of LTCCs, different groups of least shrews ($n = 3$ per treatment group) were pretreated with either LTCC blocker nifedipine (10 mg/kg, s.c.) or vehicle of nifedipine, 30 min before FPL64176 injection (10 mg/kg, i.p.). Brainstems were collected at 20 min after FPL64176 treatment and subjected to Western blots. Shrews accepting injections of vehicle of nifedipine followed by vehicle of FPL64176 were used as controls.

2.6. 5-HT and SP Immunohistochemistry

Adult least shrews were administered FPL64176 (10 mg/kg, i.p.) and rapidly anesthetized with isoflurane and subjected to perfusion at 15 min post-treatment to examine 5-HT and SP immunoreactivity. Experimental procedure prior to staining was performed as described above in “*c-Fos staining and image analysis*” section. Coronal brain sections (20 μ m) were observed under a light microscope and those containing the DVC subjected to immunostaining. Sections were blocked with 0.1 M PBS containing 10% donkey serum and 0.3% Triton X-100, and then incubated overnight at 4°C with rabbit anti-serotonin (1:500, 20080, Immunostar, Hudson, WI) and rat anti-substance P (1:400, MAB356, EMD Millipore, Temecula, CA) in 0.1 M PBS containing 5% donkey serum and 0.3% Triton X-100. Sections were washed 3 times (10 min each) in PBS and incubated in FITC-conjugated donkey anti-rabbit secondary antibody (1:300, Jackson Immuno Research Laboratories, West Grove, PA) and CYTM3-conjugated donkey anti-rat secondary antibody (1:300, Jackson Immuno Research Laboratories) for 2 h at room temperature. After washing with PBS 4 times, sections were mounted with anti-fade mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) to stain cell nuclei and examined using confocal microscope (Nikon). Images for the DVC were acquired using a confocal microscope with Metamorph software using 20 $\times$ Objective. According to

described subdivisions within the NTS (Phifer and Berthoud, 1998; Pool et al., 2007; Zheng et al., 1999), the border of the dorsomedial NTS and DMNV were defined. Fluorescence intensity values were acquired using ImageJ software. The high magnification images (100×) of interest region were further taken to show the immunoreactive structures.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.04. Except the percentage of animals vomiting, all results are presented as mean values with error bars representing S.E.M. The vomit frequency data were analyzed using the Kruskal-Wallis non-parametric one-way ANOVA and followed by Dunn's post hoc *test*. The percentage of animals vomiting across groups was compared using the Chi-Square test. Numbers of c-Fos-positive cells among groups was compared by one-way ANOVA followed by Dunn's post hoc *test*. Statistical significance for differences of 5-HT/SP fluorescence intensity values between two groups was tested by unpaired t-test. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3. Results

3.1. FPL64176-induced emesis depends on LTCC activity

Administration of the LTCC agonist FPL64176 (10 mg/kg, i.p.) can evoke vomiting in all tested least shrews during a 30-min observation period (Darmani et al., 2014). The evoked vomiting was significantly reduced by LTCC antagonist nifedipine (0.25, 0.5, 2.5, and 5 mg/kg, s.c.) in a dose-dependent manner. Here we confirm that a 10 mg/kg (s.c.) dose of nifedipine administered 30 min prior to FPL64176 injection can completely prevent FPL64176-induced vomiting as indicated by the emetic parameters, vomit frequency ($P = 0.0010$) (Fig. 1A) and the percentage of shrews vomiting ($P = 0.0005$) (Fig. 1B).

3.2. LTCC is required for FPL64176-induced c-Fos expression in brainstem emetic nuclei

To determine central responsiveness to peripheral administration of FPL64176 (10 mg/kg, i.p.), we carried out c-Fos immunostaining and analyzed c-Fos expression in the brainstem DVC containing emetic nuclei AP, NTS and DMNX. As summarized in Fig. 2D, FPL64176 caused significant c-Fos induction by increasing the mean numbers of c-Fos immunoreactivity positive cell nuclei in the NTS ($P = 0.0019$) and DMNX ($P = 0.0085$), when compared to the non-vomiting vehicle control group. However, no difference was found in the AP c-Fos counts between the FPL64176- and control groups ($P = 0.5618$). Likewise, there was no c-Fos activation in the hypoglossal nucleus (XII) below the DMNX (Fig. 2A–B).

In order to study the role of Ca^{2+} in FPL64176-evoked c-Fos induction, we employed the discussed fully effective antiemetic dose of nifedipine which completely prevented shrews from vomiting in response to FPL64176 (10 mg/kg). Thus, nifedipine (10 mg/kg, s.c.) was injected 30 min prior to FPL64176 administration and immunostaining was conducted to determine c-Fos expression on brainstem sections. In line with behavioral results, Fig. 2C–D demonstrate that the higher c-Fos counts observed in the NTS and DMNX induced by FPL64176 (Fig. 2B) were reversed by nifedipine to basal control levels (NTS, $P = 0.8825$;

DMNX, $P = 0.1323$, Nifedipine 10 mg/kg + FPL64176 10 mg/kg vs. Vehicle + Vehicle). These histochemical observations and behavioral results in Section 3.1 suggest that Ca^{2+} mobilization plays a pivotal role in FPL64176-induced responses.

3.3. Involvement of intracellular Ca^{2+} release channels in FPL64176-induced emesis

In this experiment, we examined the effect of preventing intracellular Ca^{2+} release from the SER through RyRs and IP_3 Rs on FPL64176-evoked emesis. Pretreatment with the RyR antagonist dantrolene (0.5, 1, or 5 mg/kg, i.p.) 30 min prior to FPL64176 challenge (10 mg/kg, i.p.) suppressed the vomit frequency (KW (3, 20) = 10.41, $P = 0.0154$) in a dose-dependent manner with a significant inhibition at its 5 mg/kg ($P = 0.0138$, vs. dantrolene 0 mg/kg + FPL64176) (Fig. 3A). Likewise, dantrolene pretreatment dose-dependently reduced the percentage of shrews vomiting (χ^2 (3, 20) = 10.49; $P = 0.0148$) in response to FPL64176 (Fig. 3B), with substantial reductions at its 1 ($P = 0.0455$) and 5 mg/kg ($P = 0.0034$) doses. In contrast, blockade of IP_3 Rs with various doses of 2-APB (0.5, 1, 5, and 20 mg/kg, i.p.) failed to affect FPL64176-evoked emetic parameters in terms of both the mean frequency (KW (4, 25) = 1.658, $P = 0.7982$) (Fig. 3C) and percentage of shrews vomiting (χ^2 (4, 25) = 3.214; $P = 0.5226$) (Fig. 3D). Moreover, when shrews were pretreated with combined lower doses of nifedipine (0.25 mg/kg, s.c.) plus dantrolene (0.5 mg/kg, i.p.), a near complete blockade of FPL64176-evoked vomiting occurred as shown by significant reductions in both vomit frequency ($P = 0.0038$, vs. Nifedipine 0 mg/kg & Dantrolene 0 mg/kg + FPL64176) (Fig. 3E) and percentage of shrews vomiting ($P = 0.0012$) (Fig. 3F). These results suggest that Ca^{2+} signaling in FPL64176-induced vomiting may involve an initial extracellular Ca^{2+} influx through the LTCC and subsequent intracellular Ca^{2+} release from the SER store mainly through the dantrolene-sensitive Ca^{2+} channels, RyRs.

3.4. LTCC-dependent activation of PKC α / β II, ERK1/2, and AKT in brainstems of FPL64176-treated shrews

To confirm the involvement of emesis-related signaling molecules (Darmani et al., 2013 and 2015; Zhong et al., 2014b and 2016) in FPL64176-evoked vomiting, we determined the effect of FPL64176 (10 mg/kg, i.p.) treatment on the phosphorylation level of some signaling proteins (PKC α / β II, ERK1/2, and AKT) in least shrew brainstems following different exposure periods (5, 10, 15, 20, 25, 30 and 60 min; $n = 3$ per time point) as well as from corresponding vehicle controls which is referred to as 0 min. Thus, Western blots were performed to determine phosphorylation levels of PKC α / β II at Thr638/641, ERK1/2 at Thr202/Tyr204 and Akt at Ser473. As shown in Fig. 4A–B, following FPL64176 (10 mg/kg, i.p.) injection, phosphorylation of PKC α / β II at Thr638/641 peaked at 25 min ($P = 0.0002$ vs. 0 min), and was significantly upregulated at 20 min ($P = 0.0159$), and 30 min ($P = 0.0417$). Phosphorylation status of ERK1/2 ($P = 0.0006$) was significantly elevated at 20 min. In case of Akt, the phosphorylation was significantly increased at the 10 ($P = 0.0371$) and 15 min ($P = 0.0432$) exposure periods, and peaked at 20 min ($P = 0.0001$).

Additional Western blots were conducted to further confirm the role of LTCCs on FPL64176-triggered PKC α / β II, ERK1/2, and AKT activation. Thus, shrews ($n = 3$ per group) were pretreated for 30 min with 10 mg/kg nifedipine (s.c.) or its vehicle and their brainstems were isolated 20 min after FPL64176 (10 mg/kg, i.p.) injection. As expected,

nifedipine completely prevented FPL64176-evoked PKC α / β II phosphorylation ($P=0.0048$, Vehicle + FPL64176 10 mg/kg vs. Vehicle + Vehicle; $P=0.5039$, Nifedipine 10 mg/kg + FPL64176 10 mg/kg vs. Vehicle + Vehicle), ERK1/2 phosphorylation ($P=0.0003$, Vehicle + FPL64176 10 mg/kg vs. Vehicle + Vehicle; $P=0.2984$, Nifedipine 10 mg/kg + FPL64176 10 mg/kg vs. Vehicle + Vehicle), and Akt phosphorylation ($P=0.0015$, Vehicle + FPL64176 10 mg/kg vs. Vehicle + Vehicle; $P=0.1542$, Nifedipine 10 mg/kg + FPL64176 10 mg/kg vs. Vehicle + Vehicle) (Fig. 4C–D). Thus, PKC α / β II, ERK1/2, and AKT activation in response to FPL64176 occur in a time-dependent and LTCC-sensitive manner.

3.5. Effect of PKC, ERK1/2, and PI3K-AKT inhibition on FPL64176-induced emesis

To provide additional evidence for the involvement of PKC α / β , ERK1/2, and AKT in FPL64176-mediated emesis, shrews ($n=6$ /group) were pretreated (i.p.) 30 min prior to FPL64176 challenge (10 mg/kg, i.p.) with either the corresponding vehicle (0 mg/kg) or the following inhibitors: PKC inhibitor GF109203X (2, 5, 10 mg/kg); MEK-ERK1/2 cascade inhibitor PD98059 (1, 2.5 and 5 mg/kg); and the PI3K-Akt pathway inhibitor LY294002 (2, 5 and 10 mg/kg).

In contrast to the vehicle pretreated control shrews, 10 mg/kg dosage of GF109203X significantly suppressed both the frequency ($P=0.0344$, vs. GF109203X 0 mg/kg + FPL64176) (Fig. 5A) and percentage of shrews vomiting ($P=0.0455$) (Fig. 5B). The MEK-ERK1/2 cascade inhibitor PD98059 at 2.5 and 5 mg/kg significantly attenuated the frequency ($P=0.0055$ & 0.0009 , respectively, vs. PD98059 0 mg/kg + FPL64176) and percentage of animals vomiting ($P=0.0143$ & 0.0034) in response to FPL64176 (Figs. 5C and 5D). Similarly, the PI3-Akt inhibitor LY294002 significantly decreased the frequency of induced-emesis at 10 mg/kg ($P=0.0026$ vs. LY294002 0 mg/kg + FPL64176) (Fig. 5E), while significant reductions in the percentage of shrews vomiting occurred at both 5 mg/kg ($P=0.0455$) and 10 mg/kg doses ($P=0.0034$) (Fig. 5F). These data further support a role of intracellular emesis-associated signaling in FPL64176-induced vomiting in least shrews.

3.6. Both 5-HT₃R and NK₁R are involved in FPL64176-induced emesis

In the current study the antiemetic efficacy of the NK₁R antagonist netupitant was tested against FPL64176-induced emesis. Netupitant (0, 1, 5 and 10 mg/kg, i.p.) significantly and dose-dependently reduced both the frequency of vomits (KW (3, 20) = 14.40, $P=0.0024$) as well as percentage of animals vomiting in response to FPL64176 (χ^2 (3, 20) = 10.50, $P=0.0148$). Indeed, the 10 mg/kg dose of netupitant significantly reduced both the mean vomit frequency ($P=0.0006$ vs. netupitant 0 mg/kg + FPL64176) (Fig. 6A) and percentage of animals vomiting ($P=0.0034$) (Fig. 6B). Thereafter, the antiemetic potential of a combination of non-effective doses of palonosetron (0.1 mg/kg, s.c.) and netupitant (1 mg/kg, i.p.) was tested against FPL64176-induced vomiting. As shown in Figs. 6C–D, the low dose of each antagonist by itself failed to significantly affect either emetic parameter. However, relative to the vehicle/vehicle-pretreated group (palonosetron 0 mg/kg & netupitant 0 mg/kg + FPL64176), a combination of 0.1 mg/kg palonosetron and 1 mg/kg netupitant significantly reduced both the frequency ($P=0.0011$) as well as the percentage of shrews vomiting ($P=0.0019$) in response to FPL64176.

3.7. Effect of FPL64176 on 5-HT and SP immunoreactivity in brainstem emetic nuclei

Given that both 5-HT₃R and NK₁R antagonists exerted anti-emetic potential against FPL64176-induced vomiting, we tested the effect of FPL64176 administration (10 mg/kg., i.p.) on 5-HT and SP immunoreactivity in brainstem DVC containing emetic nuclei AP, NTS and DMNX. Least shrews were euthanized at 15 min post FPL64176 treatment and were subjected to immunohistochemistry to label 5-HT and SP. Representative image (Fig. 7A) of the brainstem DVC emetic nuclei after 5-HT immunolabelling shows that in vehicle-treated control group, the 5-HT-immunoreactive profile appears as 5-HT-positive fibers with small beads within the NTS. The density of 5-HT-positive fibers is highest in the dorsomedial subdivision of the NTS. These highly curved strands cross over and intertwine in the same area, forming into a meshwork of 5-HT fibers in the dorsomedial NTS. A lower density of 5-HT-immunoreactive profile is noted in the adjacent subnuclei of NTS. Likewise, the DMNX below the NTS contains noticeably fewer immunoreactive profile. The AP contains a moderate amount of 5-HT-containing neurons. Fig. 7B shows shrews treated with FPL64176 exhibited an increase in 5-HT immunoreactivity in the dorsomedial NTS. The boxed areas referring to the dorsomedial region of the NTS in Fig. 7A and 7B were further magnified and displayed as Fig. 8A and 8B, which exhibits density 5-HT-immunoreactive varicosities (or swelling of the fiber) of different sizes which were noticeably increased upon FPL64176 administration. Following 5-HT and SP labeling, sections were counterstained with DAPI to visualize cellular nuclei (Figs. 7E). Representative images acquired from SP immunolabeling are presented as Figs. 7C and 7D. In the brainstem DVC of vehicle-treated control (Fig. 7C), fibers immunoreactive for SP were found in greatest concentration within the DMNX and to a lesser extent in the NTS, but rarely in the AP. Image analysis indicated that the intensity of 5-HT staining were significantly increased in the dorsomedial NTS of shrews treated with FPL64176 as compared to control shrews treated with the vehicle of FPL64176 ($P=0.0046$). Surprisingly, the intensity of SP staining in DMNX remained unchanged in response to FPL64176 ($P=0.7767$) (Fig. 7F).

4. Discussion

We provide mechanistic evidence for involvement of Ca²⁺ mobilization, intracellular emetic effector phosphorylation as well as participation of 5-HT₃Rs and NK₁Rs in FPL64176 (an LTCC agonist)-induced emesis in least shrews. These findings extend our behavioural observations underlying FPL64176-induced emesis in the least shrew model of emesis (Darmani et al., 2014).

4.1. Role of RyRs and IP₃Rs in vomiting

Ca²⁺-induced Ca²⁺ release is a process that extracellular Ca²⁺ influx via activation of voltage-operated Ca²⁺ channels mobilizes intracellular Ca²⁺ release from SER stores, resulting in an increase in the magnitude of the intracellular Ca²⁺ signal (Homma et al., 2006; Ziviani et al., 2011). A functional linkage between LTCCs and RyRs appears to play a major role in RyRs-mediated Ca²⁺ release from the SER into the cytoplasm following extracellular Ca²⁺ entry via LTCCs (Berrouit and Isokawa, 2009; Katoh et al., 2000; Resende et al., 2010). In addition, SER Ca²⁺ release through IP₃Rs also couples with extracellular Ca²⁺ influx via LTCCs in: i) non-excitable cells such as Jurkat human T lymphocytes (Wang

and Wu, 2011), ii) drosophila S2 cells (Wang et al., 2015), and iii) submucosal neurons in the rat colon (Rehn et al., 2013). Currently, we explored the role of RyRs and IP₃Rs in FPL64176-induced vomiting via pharmacological manipulation of their respective antagonists, dantrolene and 2-APB. FPL64176-induced emesis was insensitive to 2-APB, but both the frequency and percentage of shrews vomiting were dose-dependently suppressed by dantrolene. Furthermore, a combination of ineffective doses of the LTCC antagonist nifedipine with dantrolene potentiated the efficacy of either drug being tested alone. These *in vivo* findings are consistent with *in vitro* cellular studies demonstrating FPL64176 initially evokes extracellular Ca²⁺ entry which then triggers such Ca²⁺ release from intracellular stores in a RyRs-sensitive manner (Li et al., 2014). Similar to FPL64176-evoked vomiting, the 5-HT₃R-induced emesis is insensitive to 2-APB (Zhong et al., 2014b), and was suppressed by dantrolene. Furthermore, a combination of non-effective doses of amlodipine (another LTCC antagonist) plus dantrolene was more efficacious than each antagonist being tested alone. These behavioral findings suggest that 5-HT₃R stimulation drives extracellular Ca²⁺ through both 5-HT₃Rs and LTCCs, which subsequently triggers Ca²⁺ release via RyRs from SER stores, which greatly amplifies free Ca²⁺ levels in the cytoplasm (Zhong et al., 2014b). However, both RyRs and IP₃Rs are involved in vomiting caused by the intracellular Ca²⁺ releaser thapsigargin since pretreatment with either dantrolene or 2-APB leads to significant reductions in the frequency of the induced vomiting (Zhong et al., 2016). Moreover, a combination of low doses of 2-APB, dantrolene and nifedipine, completely protected shrews from thapsigargin-induced emesis. Thus, RyRs and IP₃Rs can be differentially modulated by different emetogens.

4.2. Activation of central and peripheral emetic loci is required for FPL64176-induced emesis

Activation of 5-HT₃Rs by 5-HT can evoke vomiting (Darmani and Ray, 2009; Johnston et al., 2014). In this study, 5-HT-immunoreactive fibers are most concentrated in the area within the NTS defined as the dorsomedial region (Phifer and Berthoud, 1998; Pool et al., 2007; Zheng et al., 1999). In serotonergic fibers, 5-HT is concentrated in specialized varicosities with variable dimensions (Bjarkam et al., 2003; Janušonis, 2017; Ruda et al., 1982). In the current study, a 15-min exposure to FPL64176 resulted in a moderate enhancement of 5-HT immunoreactivity within the dorsomedial subdivision of the NTS, marked by numerous varicosities of varying sizes, suggesting subdivision-specific response to administration of FPL64176. This NTS region is also termed the subnucleus gelatinosus (Leslie et al., 1982; Gwyn et al., 1985) and receives gastrointestinal vagal afferent input which can be activated following administration of emetogens or stimulation of vagal afferents (Babic and Browning, 2014). Exposure to the 5-HT₃R antagonist palonosetron prevents FPL64176-evoked vomiting (Darmani et al., 2014) which could be the result of blockade of activation of local 5-HT₃Rs in the brainstem. With respect to SP, FPL64176 failed to alter SP-immunoreactivity in all three emetic nuclei is surprising. This selective alteration in 5-HT immunoreactivity in the AP-NTS-DMNX suggests that 5-HT and SP may be differentially involved in neural circuits that mediate emesis. Induction of c-Fos immunoreactivity has been used to evaluate differential neuronal activation (Seoane et al., 2009). Our lab has utilized c-Fos induction in the brainstem DVC emetic nuclei to demonstrate central responsiveness to peripheral administration of diverse emetogens

(Chebolu et al., 2010; Ray et al., 2009a and 2009c; Zhong et al., 2016). In the current study participation of central emetic neurons in FPL64176-induced vomiting is further indicated by upregulated c-Fos expression in brainstem emetic nuclei NTS, a key site for integration of diverse emetic signals (Ray et al., 2009a and 2009c), and DMNX, which receives axonal projections from NTS (Travagli and Anselmi, 2016). Thus, the blood-brain barrier permeable agent FPL64176 (Jinnah et al., 1999; 2000 and 2003) could excite emetic neurons directly in the NTS and DMNX. The absence of expected increase in Fos-immunoreactivity in the AP may be explained by findings that systemic administration of some emetics may not involve the AP (Miller and Leslie, 1994; Ray et al., 2009a).

The EC cells lining intestinal mucosa synthesize 95% of 5-HT in the body and release 5-HT in response to mechanical or chemical stimulation (Linan-Rico et al., 2016). 5-HT release from guinea-pig and human EC cells is dependent on external Ca^{2+} entry through LTCCs whose blockade can reduce release event frequency (Raghupathi et al., 2013). FPL64176 can evoke a 12-fold increase in 5-HT release from EC cells (Lomax et al., 1999). FPL64176 (10 mg/kg, i.p.) may also release 5-HT from shrew intestinal EC cells which in turn could increase vagal afferent activity via stimulation of 5-HT₃ receptors, thereby indirectly triggering emetic signals in the brainstem (Darmani and Ray, 2009; Minami et al., 2001). 5-HT₃ receptor antagonists are thought to act through blockade of peripheral 5-HT₃Rs present on vagal afferents (Hornby, 2001). NK₁R antagonists exert their antiemetic effects via both peripheral and central mechanisms (Darmani et al., 2008; Darmani and Ray, 2009; Hornby, 2001; Ray et al., 2009b; Rojas et al., 2014). Based on the above discussion and our current findings that netupitant exerted antiemetic effect against FPL64176-induced vomiting, we suggest that both central and peripheral emetic sites are probably involved in FPL64176-evoked vomiting.

4.3. 5-HT₃/NK₁ receptor crosstalk

We have previously demonstrated that a 15 to 30-minute exposure to thapsigargin (0.5 mg/kg, i.p.), can increase the SP tissue content up to ~3 times over the basal levels in the shrew DMNX (Zhong et al., 2016). With lack of effect on SP-immunoreactivity in the NTS and DMNX following FPL64176 administration in the current study, the antiemetic efficacy of NK₁R antagonist netupitant against FPL64176-evoked emesis and a combination of non-effective doses of netupitant and palonosetron exhibiting synergistic antiemetic efficacy appears to be an enigma. This probably can be explained with the proposed crosstalk hypothesis that occurs between NK₁R and 5-HT₃R emetic signaling pathways. Indeed, NK₁R antagonism has previously been shown to block 5-HT responses (Minami et al., 2001) and netupitant can potentiate palonosetron effects (Stathis et al., 2012; Thomas et al., 2014).

4.4. Ca^{2+} -dependent signaling in emesis

To elucidate intracellular effector signaling in shrew brainstem in response to LTCC agonist FPL64176, we tested the degree of phosphorylation of PKC α /βII, ERK1/2 and Akt via Western blot. Our data demonstrate that FPL64176 increases phosphorylation of these intracellular emetic signals in the brainstem in a time-dependent manner. Moreover, the LTCC antagonist nifedipine reversed these molecular changes, paralleling nifedipine's

blockade of both FPL64176-induced emetic behavior and c-Fos expression. In fact, both the vomit frequency and the degree of PKC α / β II, ERK1/2 and Akt activation appear to have a temporal relationship. Indeed, within 20 min of systemic injection, FPL64176 not only caused maximal number of vomits, but also induced maximal increase in PKC α / β II, ERK1/2 and Akt phosphorylation in the brainstem. In addition, in the presence of inhibitors of either PKC (GF109203X), or MEK-ERK1/2 cascade (PD98059), both the frequency and percentage of FPL64176-induced vomiting were decreased. To evaluate the significance of Akt phosphorylation in FPL64176-induced vomiting, we also determined the anti-emetic effect of LY294002, an inhibitor of its upstream enzyme PI3K. Indeed, LY294002 has been used to study the role of PI3K-Akt pathway and inactivation of PI3K has been shown to inhibit the phosphorylation of Akt at Ser473 (Jiang et al., 2010). Our results reveal that phosphorylation of Akt also contributes to FPL64176-evoked vomiting. Activation of the discussed emetic signals is under regulation of Ca²⁺ mobilization which acts as one of the earliest emetic events in the signal transduction pathways. Of note, phosphorylation of ERK1/2 in brainstem has been shown to be associated with 5-HT₃R-mediated emesis (Zhong et al., 2014b), thapsigargin-induced vomiting (Zhong et al., 2016) and cisplatin-evoked acute and delayed vomiting (Darmani et al., 2013 and 2015). PKC activation is also associated with increase of the 5-HT₃Rs trafficking to the cell surface (Sun et al., 2003) as well as inhibition of SP signaling and further desensitization of the NK₁Rs (Déry et al., 2001), suggesting its role in 5-HT₃R/NK₁R crosstalk (Rojas et al., 2014).

5. Conclusion

Based upon our current and previously published findings, we propose that the LTCC activator FPL64176 is a potent emetogen which induces vomiting indirectly via activation of 5-HT₃Rs and NK₁Rs in a way that increases intracellular Ca²⁺ mobilization involving extracellular Ca²⁺ influx through LTCCs and intracellular Ca²⁺ release from the SER through RyRs. The evoked increase in cytosolic Ca²⁺ induces Ca²⁺-dependent signaling to regulate PKC α / β II, ERK1/2 and Akt activation in brainstem. Altogether our studies provide important preliminary evidences to support the notion that strategies targeting specific emetic mechanisms linking initial Ca²⁺ mobilization to downstream intracellular signaling systems may provide emesis protection. We further suggests that targeted chemotherapeutics that generally enhance intracellular Ca²⁺ mobilization may also concomitantly evoke vomiting as it seen with conventional anticancer drugs such as cisplatin (Darmani and Ray, 2009; Zhong et al., 2017). Thus, a better understanding of their anticancer and emetic signaling mechanisms may lead to development of better chemotherapeutics.

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Figure 1. The L-type Ca^{2+} channel antagonist nifedipine blocks FPL64176-induced emesis Least shrews were pretreated subcutaneously (s.c.) ($n = 6$ per group) with either the LTCC antagonist nifedipine (10 mg/kg) or its vehicle (0 mg/kg) for 30 min, followed by an intraperitoneal (i.p.) FPL64176 injection at its fully effective emetic dose, 10 mg/kg. Each shrew was placed in the observation cage and frequencies of vomiting were recorded separately for the next 30 min. A) The vomiting frequency data between groups were analyzed using unpaired t-test and expressed as mean $\pm$ S.E.M. $**P < 0.01$ vs. 0 mg/kg. B) The percentage of animals vomiting at different doses was compared using Chi-square test. $***P < 0.001$ vs. 0 mg/kg.

Figure 2. FPL64176 triggers c-Fos induction in brainstem emetic nuclei in a LTCC antagonist (nifedipine)-sensitive manner

A–C): Examples of c-Fos immunoreactivity (Fos-IR) in the brainstem. Immunolabeling was performed on free-floating coronal tissue sections using avidin–biotin–peroxidase amplification and nickel-enhanced diaminobenzidine visualization. The area postrema (AP), nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMNX) constitute the brainstem dorsal vagal complex (DVC) emetic nuclei. Note the different cytoarchitectonic details such as cell size and packing, which differentiate each nucleus from each other. A) Relatively few Fos-IR nuclei are found in the DVC of control shrew injected with nifedipine vehicle 30 min prior to the vehicle of FPL64176. B) In FPL64176 (10 mg/kg, i.p.)-injected shrew pretreated with nifedipine vehicle (Veh), shows increased Fos-IR throughout the NTS and DMNX, but only infrequently in AP and none in hypoglossal nucleus (XII). C) In the presence of LTCC antagonist nifedipine (10 mg/kg, s.c.), the ability of FPL64176 (10 mg/kg, i.p.) to induce Fos-IR was abrogated. Scale Bar, 100 μ m. D) Summary data of groups as demonstrated in A, B and C. n = 3 shrews per group. 3 sections from each shrew were used for analysis. Fos-IR positive nuclei (dark and filled) but not weakly labeled nuclei were quantified. Data for each region was expressed as mean number

of Fos-IR nuclei $\pm$ S.E.M. One-way ANOVA followed by Dunn's post hoc *test*. ** $P < 0.01$ vs. control (i.e. Veh+Veh).

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Figure 3. Effect of Ca^{2+} modulators on FPL64176-induced emesis

Varying doses of the intracellular Ca^{2+} release channel RyR inhibitor dantrolene (A–B) ($n = 6$), the IP_3R inhibitor 2-APB (C–D) ($n = 6$) or corresponding vehicles (i.e. 0 mg/kg; $n = 6$) were injected (i.p.) to different groups of shrews. Following 30 min exposure animals were injected with FPL 64176 (10 mg/kg, i.p.) and observed for next 30 min. The vomiting frequency data were analyzed using the Kruskal-Wallis non-parametric one-way ANOVA followed by Dunn's post hoc *test* and expressed as mean $\pm$ S.E.M. The percentage of animals vomiting within groups at different doses was compared using Chi-square test. A–B) Dantrolene dose-dependently reduced both emetic indices in response to FPL64176. $*P < 0.05$, $**P < 0.01$ vs. 0 mg/kg. C–D) 2-APB at all tested dosages failed to affect FPL64176-induced emesis. E–F) Combination of nifedipine with dantrolene at ineffective doses exerted additive anti-emetic efficacy against FPL64176. Nifedipine (0.25 mg/kg, s.c.) plus dantrolene (0.5 mg/kg, i.p.) or corresponding vehicles (i.e. 0 mg/kg) were administered to different groups ($n = 7$) of shrews 30 min prior to FPL 64176 (10 mg/kg, i.p.) challenge. $**P < 0.01$ vs. Nifedipine 0.25 mg/kg + Dantrolene 0.5 mg/kg.

Figure 4. FPL64176 upregulates PKCα/βII, ERK1/2 and Akt phosphorylation in brainstem in a LTCC antagonist nifedipine-sensitive manner

A–B) Time-courses of FPL64176-induced PKCα/βII, ERK1/2 and Akt phosphorylation the least shrew brainstem. Shrews ($n=3$ per group) were injected with either vehicle (0 min) or FPL 64176 (10 mg/kg., i.p.). Brainstems were collected at 0, 5, 10, 15, 20, 25, 30 and 60 min. Phospho-PKCα/βII at Thr638/641 (pPKCα/βII), GAPDH, phospho-ERK1/2 at Thr202/204 (pERK1/2), ERK1/2, phospho-Akt at Ser473 (pAkt) and Akt of protein samples extracted from individual brainstems were determined by Western blots. Panel A shows representative blots. Panel B shows summarized data. Bands were quantified using ImageJ software. Ratios of pPKCα/βII (~ 80 kD) to GAPDH (~ 37 kD), pERK1/2 (42/44 kD) to ERK1/2 and pAkt (~ 60 kD) to Akt were calculated and expressed as fold change (mean $\pm$ S.E.M.) of control (0 min). * $P < 0.05$, *** $P < 0.001$ vs. 0 min, One-way ANOVA followed by Dunnett's test. C–D) Nifedipine abolished PKCα/βII, ERK1/2 and Akt phosphorylation evoked by FPL64176. Following a 30 min prior treatment with either vehicle (Veh) or nifedipine (10 mg/kg., s.c.), shrews were given FPL64176 (10 mg/kg., i.p.). Shrews injected with vehicle of nifedipine followed by vehicle of FPL64176 were used as controls (Ctl). Following 20 min after the second injection, collected brainstems were subjected to Western

blots. Panel C shows representative blots. Panel D shows summarized data. $N = 3$ shrews per group. $**P < 0.01$, $***P < 0.001$ vs. Ctl. One-way ANOVA followed by Dunnett's test.

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Figure 5. Effects of inhibitors of PKC, ERK1/2 and PI3K-Akt on FPL64176-induced emesis Varying doses of inhibitors of PKC (GF109203X), MEK-ERK1/2 cascade (PD98059), PI3K-Akt (LY294002) or corresponding vehicles (i.e. 0 mg/kg) were administered (i.p.) to different groups of shrews 30 min before to FPL64176 (10 mg/kg, i.p.) injection (n = 6 shrews/group). Vomiting was recorded for the next 30 min post FPL64176 treatment. The vomiting frequency data were analyzed using the Kruskal-Wallis non-parametric one-way ANOVA followed by Dunn's post hoc *test* and expressed as mean $\pm$ S.E.M. The percentage of animals vomiting within groups at different doses was compared using Chi-square test. GF109203X (10 mg/kg) (A–B), PD98059 (2.5 and 5 mg/kg) (C–D) and LY294002 (10 mg/kg) (E–F) significantly decreased both emetic indices (i.e. Vomit frequency & Percentage of shrews vomiting) in response to FPL64176. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0 mg/kg.

Figure 6. Effect of the NK₁ receptor antagonist netupitant on FPL64176-induced emesis
 A–B) Netupitant dose-dependently reduced both emetic parameters in response to FPL64176. Varying doses of netupitant or its vehicle (i.e. 0 mg/kg) were injected (i.p.) to different groups of shrews (n = 6). Following 30 min exposure, animals were challenged with FPL 64176 (10 mg/kg, i.p.) and were observed for the next 30 min. The vomiting frequency data were analyzed using the Kruskal-Wallis non-parametric one-way ANOVA followed by Dunn's post hoc *test* and expressed as mean ± S.E.M. The percentage of animals vomiting within groups at different doses was compared using Chi-square test. ***P* < 0.01, ****P* < 0.001 vs. 0 mg/kg. C–D) A combination of ineffective dose of serotonin type 3 receptor antagonist palonosetron with netupitant exerted additive antiemetic efficacy against FPL64176. Palonosetron (0.1 mg/kg, s.c.) plus netupitant (1 mg/kg, i.p.) or corresponding vehicles (i.e. 0 mg/kg) were administered to different groups (n = 8) of shrews 30 min prior to FPL 64176 (10 mg/kg, i.p.) challenge. **P* < 0.05, ***P* < 0.01 vs. Palonosetron 0.1 mg/kg + Netupitant 1 mg/kg.

Figure 7. Example of images (20×) of serotonin and substance P immunolabeling following injection of the emetic agent, FPL64176

Shrews ($n = 3$ per group) were euthanized at 15 min post vehicle control or FPL64176 (10 mg/kg, i.p.) injection, and immunohistochemistry were conducted as described in methods section “5-HT and SP Immunohistochemistry”. Coronal brain sections (20 μ m) containing the brainstem DVC emetic nuclei, area postrema (AP), nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMNX) were used. Sections were immunolabeled with rabbit anti-serotonin (5-HT) antibody and rat anti-substance P (SP) antibody overnight followed by FITC-conjugated donkey anti-rabbit secondary antibody incubation and Cy3TM-conjugated donkey anti-rat secondary antibody incubation. After counterstaining with DAPI, images were acquired using a confocal microscope. Left panels A, C, and E show representative images of sections from vehicle controls labelled with anti-5-HT (green), anti-SP antibodies (red) and DAPI (blue) respectively. Right panels B and D show representative images of sections from FPL64176-treated shrews labelled with anti-5-HT and anti-SP antibodies respectively. Image from DAPI-stained sections of FPL64176-treated shrews is not shown. Scale bar, 100 μ m. Boxed areas (the dorsomedial NTS subnucleus) in panels A and B were further magnified and displayed as Figure 8. Panel F. Average

Fluorescence Intensities of 5-HT in boxed area in A and B and SP staining in the field as boarded by the dashed lines in C and D. $**P < 0.01$. *n.s.*, non-significant. Unpaired t-test.

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Figure 8. Example of images (100×) of serotonin-immunoreactivity in the dorsomedial NTS related to administration of the emetic agent, FPL64176

Shrews were treated and immunohistochemistry were performed as described in Figure 7. Panel A and B, high magnification (100×) of boxed area (the dorsomedial NTS) of figures 7A and 7B, show more evidence of density of serotonin (5-HT)-immunoreactive varicosities (green) at 15 min post FPL64176 (10 mg/kg., i.p.) administration (panel B), in contrast with vehicle control (panel A). C–D) Cell nuclei counterstained with DAPI (blue). E–F) Merged version demonstrates co-labeled neurons. Scale bar, 10 μ m.