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Calcium-Sensing Receptor and Transient Receptor Ankyrin-1 Mediate Emesis Induction by Deoxynivalenol (Vomitoxin)

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ABSTRACT

The common foodborne mycotoxin deoxynivalenol (DON, vomitoxin) can negatively impact animal and human health by causing food refusal and vomiting. Gut enteroendocrine cells (EECs) secrete hormones that mediate DON's anorectic and emetic effects. In prior work utilizing a cloned EEC model, our laboratory discovered that DON-induced activation of calcium-sensing receptor (CaSR), a G-coupled protein receptor (GPCR), and transient receptor ankyrin-1 (TRPA1), a transient receptor potential (TRP) channel, drives Ca^{2+}-mediated hormone secretion. Consistent with these *in vitro* findings, CaSR and TRPA1 mediate DON-induced satiety hormone release and food refusal in the mouse, an animal model incapable of vomiting. However, the roles of this GPCR and TRP in DON's emetic effects remain to be determined. To address this, we tested the hypothesis that DON triggers emesis in mink by activating CaSR and TRPA1. Oral gavage with selective agonists for CaSR (R-568) or TRPA1 (allyl isothiocyanate; AITC) rapidly elicited emesis in the mink in dose-dependent fashion. Oral pretreatment of the animals with the CaSR antagonist NPS-2143 or the TRP antagonist ruthenium red (RR), respectively, inhibited these responses. Importantly, DON-induced emesis in mink was similarly inhibited by oral pretreatment with NPS-2143 or RR. In addition, these antagonists suppressed concurrent DON-induced elevations in plasma peptide YY_{3-36} and 5-hydroxytryptamine—hormones previously demonstrated to mediate the toxin's emetic effects in mink. Furthermore, antagonist co-treatment additively suppressed DON-induced emesis and peptide YY_{3-36} release. To summarize, the observations here strongly suggest that activation of CaSR and TRPA1 might have critical roles in DON-induced emesis.

Key words: mycotoxin; deoxynivalenol; calcium-sensing receptor; transient receptor potential channel; emesis; peptide YY3-36; PYY3-36; serotonin; 5-HT.

Deoxynivalenol (DON, vomitoxin), a trichothecene mycotoxin produced by *Fusarium graminearum*, occurs frequently in wheat, barley and corn as well as grain-based human food and animal feed (Pestka, 2010a). DON's capacity to elicit emetic responses in pigs resulted in it be given the colloquial name "vomitoxin" (Vesonder *et al.*, 1973). Ingestion of grains contaminated with DON have been etiologically associated with large outbreaks of noninfectious gastroenteritis in several countries including Russia, Japan, Korea, and India over the past half century (Pestka, 2010b). Between 1961 and 1991, 53 gastroenteritis outbreaks in China were linked to consumption of cereals containing *Fusarium* and/or DON, with the largest outbreak affecting

over 130,000 people (Luo, 1994). In United States, DON was found in the absence of other putative food poisoning agents in burritos associated with 16 large outbreaks of noninfectious gastroenteritis with a characteristic rapid onset of vomiting in over 1900 schoolchildren in 7 states (Steinberg *et al.*, 2006).

Emesis usually serves as a protective reflex against food poisoning by forcefully driving out contents of the upper gastrointestinal (GI) tract through the oral cavity (Andrews and Hawthorn, 1988; Stern *et al.*, 2011). However, severe emetic responses have the potential to affect health adversely by disrupting normal nutrition, hydration, and electrolyte balance (Andrews and Sanger, 2014). The mechanisms for emesis are highly complex and involve hormones, neurotransmitters, and visceral afferent neurons that are coordinated by a neuronal network known as the central pattern generator (CPG), also called the "vomiting center" (Andrews and Horn, 2006; Hornby, 2001). Emetic stimuli trigger emesis in at least 2 ways. One involves direct stimulation of the area postrema of the medulla by blood-borne and cerebrospinal fluid (CSF)-borne emetic mediators (eg, hormones and neurotransmitters), leading to activation of the CPG and subsequent emesis (Borison, 1989; Carpenter, 1990; Hornby, 2001). Another mechanism involves binding of emetic mediators to the corresponding receptors located on vagal afferent neurons that transfer signals to the nucleus tractus solitarius (NTS), ultimately activating the CPG (Andrews and Horn, 2006; Hornby, 2001). Using a mink model of emesis (Wu *et al.*, 2013a), our lab found that the DON-induced emetic response involves at least 2 hormonal mediators: the satiety peptide PYY_{3-36} and the monoamine neurotransmitter 5-hydroxytryptamine (5-HT; serotonin) (Wu *et al.*, 2013b).

PYY_{3-36} is secreted by L cells, a type of enteroendocrine cell (EEC) located in the distal ileum and colon (Hörsten *et al.*, 2004). As an important satiety hormone, PYY_{3-36} can induce anorectic effects via both upregulation of anorexigenic and downregulation of orexigenic signaling molecules within the brain in many species including humans, nonhuman primates, and rodents (Karra and Batterham, 2010; le Roux *et al.*, 2006; Challis *et al.*, 2003). Besides anorectic effects, exogenously administered PYY_{3-36} causes emesis in species capable of vomiting including human, dogs, cats, and mink (Gantz *et al.*, 2007; Harding and McDonald, 1989; Perry *et al.*, 1994; Sloth *et al.*, 2007; Wu *et al.*, 2013a,b, 2014, 2016a). Produced by enterochromaffin cells (EC), a type of EEC found widely throughout the gut, or by neurons of the CNS (Kim and Camilleri, 2000), 5-HT regulates appetite suppression, nausea, emesis, and various other GI functions (Endo *et al.*, 2000; Kucharczyk and Harding, 1990; Stables *et al.*, 1987). Pharmacologic antagonists to neuropeptide Y2 receptor and 5-HT3 receptor attenuate or abolish DON's emetic effects in the mink(Wu et al., 2013b). The mechanisms for DON-induced exocytosis of PYY_{3-36} and 5-HT *in vivo* are presently unknown.

G-protein-coupled receptors (GPCRs) and transient receptor potential channels (TRPs) in EEC are known to function as chemosensors of the GI lumenal content (Liou, 2013; Riemann et al., 2012). Employing the widely used EEC model, a murine neuroendocrine tumor STC-1 cell, our laboratory discovered that DON evokes release of the satiety hormones cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1) via activation of GPCR calcium-sensing receptor (CaSR)- and transient receptor potential ankyrin-1 (TRPA1)-mediated Ca^{2+} signaling (Zhou and Pestka, 2015). Relatedly, we recently reported that in the mouse, an animal incapable of vomiting (Horn *et al.*, 2013), DON elicits satiety hormone-driven anorexia by activating CaSR and TRPA1 (Wu *et al.*, 2016b). However, the roles of this receptor and channel in DON's emetic effects remain to be determined. Here we tested the hypothesis that DON triggers emesis in mink by activating CaSR and TRPA1. The results indicate that DON elicited both PYY_{3-36} elevation and emetic responses in a manner consistent with CaSR and TRPA1 activation.

MATERIALS AND METHODS

Chemicals. DON was obtained from Dr. Tony Durst (University of Ottawa) and purity (>98%) was verified by elemental analysis. R-568, NPS-2143, allyl isothiocyanate (AITC) and ruthenium red (RR) were purchased from Tocris Biosciences (Ellisville, Missouri). DON and RR were dissolved in filter-sterilized phosphate buffered saline. R-568 and NPS-2143 were dissolved in a vehicle (VEH) comprised of Pharmasolve (ISP Technologies, Wayne, New Jersey) plus 20% 2-hydroxyproply-β-cyclodextrin (Sigma-Aldrich, St. Louis). AITC was dissolved in 1% dimethyl sulfoxide (DMSO) in filter-sterilized phosphate buffered saline. Doses of various agonists and antagonists were selected based on supplier recommendations, prior animal investigations (Doihara *et al.*, 2009a; Nemeth *et al.*, 2001; Wada *et al.*, 1997) and preliminary studies in the mink. Delivery volumes of DON and pharmacologic agents were 1 ml/kg bw using a sterile 16-G, 5-cm stainless steel gavage tube.

Animals. Animal treatment followed National Institutes of Health guidelines and was approved by the Michigan State University Institutional Animal Care and Use Committee (AUF 02/11-017-00). Standard dark, female adult mink (Neovison vison) of 1–2 years of age (average weight = 1.25 ± 0.3 kg) ($n = 144$) were obtained from the Michigan State University (MSU) Experimental Fur Farm. Housing conditions and diet formulation (Supplementary Table 1) met standards of the American Association for Accreditation of Laboratory Animal Care (http://aaalac.org). Animals were housed singly in wire cages (62 cm long × 25 cm wide × 38 cm high) and provided with a nest box (24 cm long × 24 cm wide × 29 cm high) with aspen shavings or excelsior (wood wool) within an open-sided pole barn. Food and water were provided ad libidum until start of the experiment. Studies were conducted from April to June of 2013. These months are outside the mink's estrus cycle (February and March). Temperature (daily average = 13–21 °C), humidity, and photoperiod were dependent on the ambient environment. The mink used in the study were naive to all treatments. Animals were randomly allocated to experimental cohorts based on weight. Based on our preliminary experiments and prior trichothecene-induced emesis studies (Wu et al., 2013a, 2014, 2016a), group sizes (3–6) were selected to minimize the number of animals used but still be sufficient for statistical analyses. With the exception of transient emesis, no other signs of toxicity including fatigue, irritability, seizure, and death were evident in the treated mink for any of these agonists or antagonists used in this study.

Emesis studies. The general design of emesis experiments was the same as prior mink studies (Wu *et al.*, 2013a, b). To determine how CaSR agonist R-568 or the TRPA1 channel agonist AITC affected emesis (Figure 1A), groups of fasted mink ($n = 3$) were provided 50 g of feed at 8:30 h and then at 9:00 h were orally gavaged with R-568 (0, 2.5, 5, and 10 mg/kg bw) or AITC (0, 5, 10, and 15 mg/kg bw). Mink were then returned to their cages and monitored for emetic responses over the next 3 h. Each vomit or retch was counted and the counts combined to yield total emetic events (Wu *et al.*, 2013a). Vomiting was defined as rhythmic abdominal contraction with oral expulsion of either

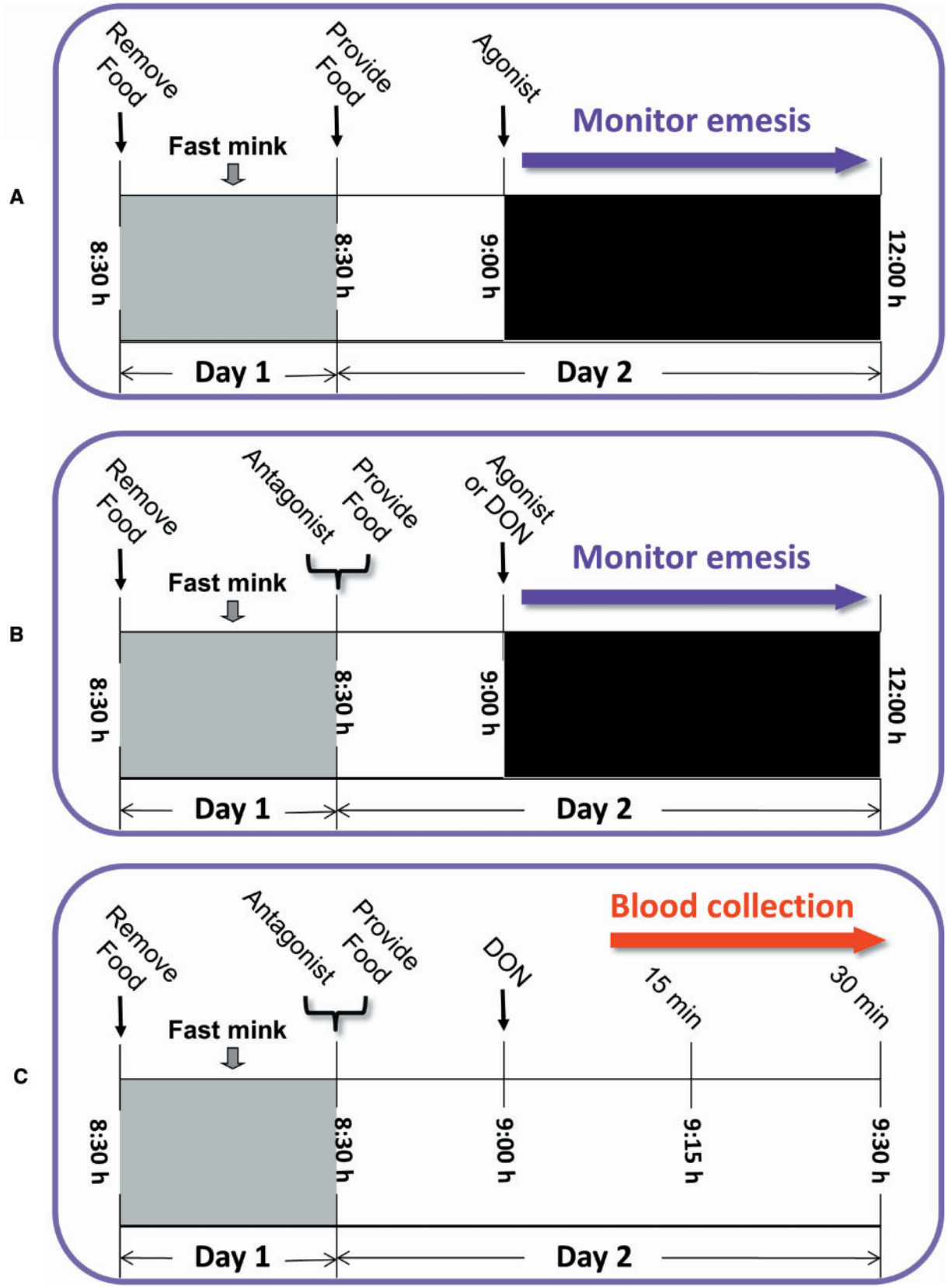

FIG. 1. Experimental design. A, Effect of CaSR or TRP channel agonists on emesis in mink. B, Effect of CaSR or TRP channel antagonists on CaSR or TRP channel agonist- and DON-induced emesis. C, Effect of CaSR and TRP channel antagonists on DON-induced plasma PYY_{3-36} and 5-HT elevation.

solid or liquid material, whereas, retching was defined as responses that mimicked vomiting but without any material being expelled.

To learn the impact of the CaSR antagonist NPS-2143 and the TRP channel antagonist RR on R-568- and AITC-induced emetic responses (Figure 1B), respectively, groups of fasted mink ($n = 3$) were orally treated with either NPS-2143 (2.5 mg/kg bw) or RR (0.5 mg/kg bw) in VEH or VEH alone at 8:30 h and provided 50 g of food. After 30 min, mink were gavaged with either 10 mg/kg bw R-568 or 15 mg/kg bw AITC in VEH, respectively. Afterwards, the mink were returned to their cages and emesis monitored over the next 3 h.

To gauge the effects of NPS-2143 or RR on DON-induced emetic responses (Figure 1B), groups of fasted mink ($n = 4$) were first gavaged with either NPS-2143 (0, 0.5, 1, and 2.5 mg/kg bw) or RR (0, 0.25, 0.5, and 1 mg/kg bw) in 1 ml VEH at 8:30 h and then provided 50 g of food. At 9:00 h, these groups were gavaged with 1.0 ml 0.5 mg/kg bw DON or VEH, respectively. Mink were monitored for emesis over the next 3 h. To assess the combined effects of NPS-2143 and RR on DON-induced emetic responses, groups of fasted mink ($n = 6$) were first gavaged with 2.5 mg/kg bw NPS-2143 or 0.5 mg/kg bw RR or both in 1 ml VEH or with VEH alone at 8:30 h, respectively, and then provided 50 g of food. At 9:00 h, animals were gavaged with 0.5 mg/kg bw DON or VEH and then monitored for emesis over the next 3 h.

PYY3-36 and 5-HT studies. The influence of CaSR and TRP channel antagonists on DON-induced PYY_{3-36} and 5-HT elevations in plasma was assessed using a previously described protocol (Wu *et al.*, 2013b) (Figure 1C). Briefly, fasted mink ($n = 5$) were first orally gavaged with 2.5 mg/kg bw NPS-2143 and/or 0.5 mg/kg bw RR in 1 ml VEH or VEH alone at 8:30 h, respectively, and provided 50 g feed immediately. After 30 min, mink were gavaged with 0.5 mg/kg bw DON or VEH. At experiment termination 30 min later, mink were anesthetized by intramuscular injection of ketamine (100 mg/kg bw). Blood was collected by heart puncture into vacutainers containing EDTA as anticoagulant, and mink were immediately euthanized by CO_2 exposure. Blood was centrifuged at 1000 × *g* for 10 min and resultant plasma stored at −80 °C until analysis. Plasma PYY_{3-36} and 5-HT were analyzed using enzyme immunoassay kits for PYY (PYY_{3-36}; mouse-, rat-, porcine-, and canine-specific) (Phoenix Pharmaceuticals, Burlingame, California) and 5-HT (Enzo Life Sciences, Plymouth Meeting, Pennsylvania), respectively.

Statistics. Data were analyzed statistically using Sigma Plot 11 for Windows (Jandel Scientific; San Rafael, California). Means were considered significantly different at $P < .05$. Fisher's Exact Test was used for incidence, and 1-way ANOVA using the Holm–Sidak method or *t*-test was used for latency, duration, retching, vomiting, total emetic events, and hormone concentrations. If the normality test failed, Kruskal–Wallis ANOVA on Ranks was used in conjunction with Student–Newman–Keuls test. Statistical comparisons between 2 groups were analyzed using a *t*-test unless normality failed and a Mann–Whitney Rank Sum Test was executed.

RESULTS

Selective CaSR Activation Induces Emesis in the Mink

When the effects of oral exposure to the selective CaSR agonist R-568 were determined, 5 and 10 mg/kg bw of the drug induced 3 ± 3 and 29 ± 6 total emetic events ($P < .05$), respectively (Figure 2A). Emesis occurred in 33 and 100% of the mink, respectively within 7–11 min and ended by 15 min (Table 1). Emetic responses were not evident in mink treated with 2.5 mg/kg bw R-568 or the VEH. The effects of NPS-2143, a selective antagonist to CaSR on R-568-induced emesis were assessed. Mink pretreated with VEH and then with 10 mg/kg bw R-568 responded with 22 ± 8 total emetic events (Figure 2B). Again, emesis began within 10 min and ended by 15 min (Table 2). In animals receiving the NPS-2143 prior to exposure, however, R-568-induced emesis was completely ablated ($P < .05$). Accordingly, CaSR activation appeared to induce emesis in the mink.

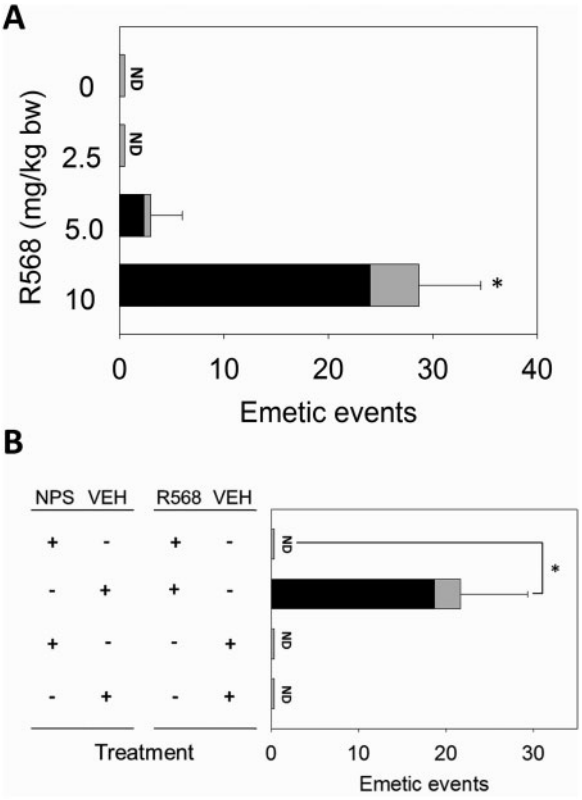

FIG. 2. CaSR mediates emesis in the mink. A, CaSR agonist R-568 induces emetic response in mink. Fasted mink were provided 50 g of feed and 30 min later were orally gavaged with 0, 2.5, 5, and 10 mg/kg bw R-568. Emetic events include vomiting (gray) and retching (black) were monitored over a 3 h period. ND = not detected. Data are mean ± SEM ($n = 3$/group). Asterisk indicates statistically significant differences in emetic events between treatment and control group ($P < .05$). B, CaSR antagonist NPS-2143 inhibits R-568-induced emetic responses in mink. Fasted mink were orally treated with either 2.5 mg/kg bw NPS-2143 or VEH alone and then provided 50 g of food. After 30 min, mink were gavaged with either 10 mg/kg bw R-568 or VEH, respectively. Following these treatments, mink were returned to their cages and monitored for emesis over a 3 h period. Data are mean ± SEM ($n = 3$/group). Asterisk indicates statistically significant difference in R-568-induced emesis between animals pretreated with NPS-2143 or VEH ($P < .05$).

Selective TRPA1 Activation Induces Emesis in the Mink

The impact of oral exposure to the selective TRPA1 agonist AITC on emesis was evaluated. The minimum emetic dose for the compound was 10 mg/kg bw (Figure 3A), with two-thirds of the treated mink showing emesis (Table 3). When the dose was increased to 15 mg/kg bw, all mink exhibited emetic responses. Emesis began within 4–8 min and ended by 15 min with 11 ± 8 and 36 ± 12 total emetic events being observed after treatment with AITC at 10 and 15 mg/kg bw ($P < .05$), respectively.

TABLE 1. Effects of CaSR Agonist R-568 on Emesis in Mink

Treatment	Incidence (Responding/Tested)	Latency to Emesis (min) [a,b]	Duration of Emesis (min) [a,b]
VEH	0/3	–	–
2.5 mg/kg R-568	0/3	–	–
5 mg/kg R-568	1/3	11 ± 0	1 ± 0
10 mg/kg R-568	3/3	8 ± 1	4 ± 1

[a]Average of positive responders only.
[b]If animals failed to retch or vomit, the latency and duration of emesis are shown as "–". Data are presented as the mean ± SEM.

Pretreatment with VEH prior to AITC dosing at 15 mg/kg bw resulted in animals having 39 ± 14 total emetic events within 6 min and ending by 15 min (Figure 3B and Table 4). Pre-treatment with the TRP antagonist RR at 30 min prior to AITC exposure totally abrogated emesis ($P < .05$). These data suggest that TRPA1 activation can trigger emesis in the mink.

CaSR and TRP Channel Antagonism Attenuates DON-Induced Emesis

The effect of the selective CaSR antagonist NPS-2143 on DON-induced emesis was assessed in the mink. Oral administration of DON at 0.5 mg/kg bw caused emesis in all animals within 10 min, which ceased by 30 min (Figure 4 and Table 5). DON-induced emetic events in mink pre-treated with NPS-2143 were reduced by 30, 41, and 53% at 0.5, 1, and 2.5 mg/kg bw ($P < .05$), respectively. In similar fashion, pretreatment with RR dose-dependently attenuated DON-induced emesis by 23, 50, and 54% at 0.25, 0.5 ($P < .05$), and 1 mg/kg bw ($P < .05$), respectively (Figure 5 and Table 6). There was also a general trend toward increased latency and/or decreased duration of DON-induced emesis in animals pretreated with NPS-2143 or RR.

The combined effects of NPS-2143 and RR on DON-induced emetic response was determined. Consistent with the prior experiment, mink treated with 0.5 mg/kg bw DON exhibited 79 ± 11 total emetic events within 10 min that lasted up to 30 min (Figure 6 and Table 7). Upon pretreatment with 2.5 mg/kg bw NPS-2143 or 0.5 mg/kg bw RR, DON-induced emetic events were attenuated by 44 and 52%, respectively ($P < .05$). However, in mink that were treated with both antagonists, total emetic events and duration of emesis were reduced by 80 and 84%, respectively ($P < .05$). There was a general trend toward increased latency in animals pretreated with both antagonists as compared with animals pretreated with VEH or each antagonist.

DON-Induced PYY_{3-36} and 5-HT Plasma Elevation Is Attenuated by CaSR and TRP Antagonist

Effects of CaSR and TRP antagonism on DON-induced hormone elevation were gauged. At 30 min after oral gavage with the toxin, plasma PYY_{3-36} concentration was significantly increased ($P < .05$) (Figure 7A). DON-treated mink pretreated with 2.5 mg/kg bw NPS-2143 or 0.5 mg/kg bw RR alone displayed modest trends toward reduced plasma PYY_{3-36} concentrations ($P = .895$ and .066, respectively). However, pretreatment with both NPS-2143 and RR caused a 48% reduction in plasma PYY_{3-36} concentrations compared with DON treatment alone ($P < .05$).

Plasma 5-HT was significantly increased at 30 min (Figure 7B) after oral gavage with DON. Mink pretreated with 2.5 mg/kg bw NPS-2143 alone had markedly decreased DON-

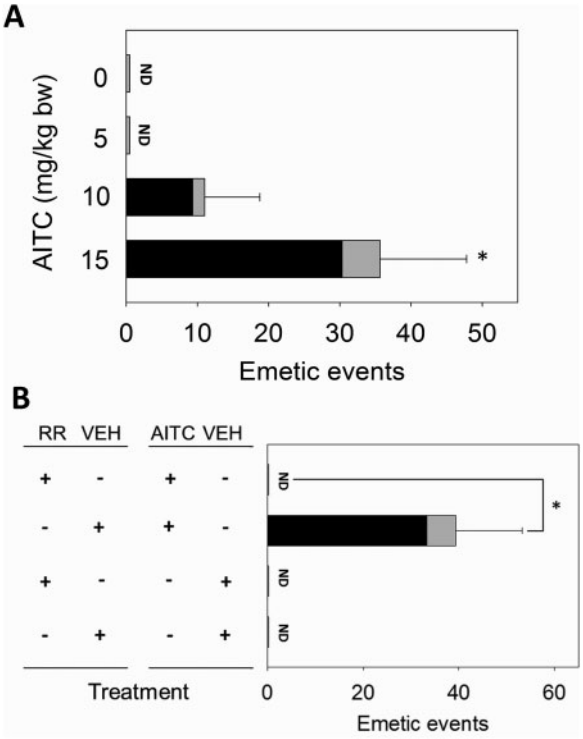

FIG. 3. TRPA1 mediates emesis in the mink. A, TRPA1 agonist AITC induces emetic response in mink. Fasted mink were provided 50 g of feed and after 30 min were orally gavaged with 0, 5, 10, and 15 mg/kg bw AITC. Mink were returned to their cages and monitored for emesis including vomiting (gray) and retching (black) over a 3 h as descrined in Figure 2 legend. Emetic events episodes. ND = not detected. Data are mean ± SEM ($n = 3$/group). Asterisk indicates statistically significant differences in emetic events between treatment and control group ($P < .05$). B, TRP channel antagonist ruthenium red (RR) inhibits AITC-induced emetic response in mink. Fasted mink were orally treated with 0.5 mg/kg bw RR or VEH alone and provided 50 g of food. After 30 min, mink were gavaged with 15 mg/kg bw AITC or VEH, and then emesis monitored as described above. ND = not detected. Data are mean ± SEM ($n = 3$/group). Asterisk indicates statistically significant difference AITC-induced emesis between animals pretreated with RR or VEH ($P < .05$).

TABLE 2. Effects of CaSR Antagonist NPS-2143 on R-568-Induced Emesis in Mink

Treatment	Incidence (Responding/Tested)	Latency to Emesis (min)[a,b]	Duration of Emesis (min)[a,b]
VEH + VEH	0/3	–	–
2.5 mg/kg NPS + VEH	0/3	–	–
VEH + 10 mg/kg R-568	3/3	9 ± 1	3 ± 1
2.5 mg/kg NPS + 10 mg/kg 568	0/3	–	–

[a]Average of positive responders only.
[b]If animals failed to retch or vomit, the latency and duration of emesis are shown as "–". Data are presented as the mean ± SEM.

TABLE 3. Effects of TRPA1 Agonist AITC on Emesis in Mink

Treatment	Incidence (Responding/Tested)	Latency to Emesis (min) [a,b]	Duration of Emesis (min) [a,b]
DMSO	0/3	–	–
5 mg/kg AITC	0/3	–	–
10 mg/kg AITC	2/3	7 ± 1	1 ± 0.3
15 mg/kg AITC	3/3	5 ± 1	5 ± 1

[a]Average of positive responders only.
[b]If animals failed to retch or vomit, the latency and duration of emesis are shown as "–". Data are presented as the mean ± SEM.

TABLE 4. Effects of TRP Channel Antagonist Ruthenium Red (RR) on AITC-Induced Emesis in Mink

Treatment	Incidence (Responding/Tested)	Latency to Emesis (min) [a,b]	Duration of Emesis (min) [a,b]
PBS + DMSO	0/3	–	–
0.5 mg/kg RR + DMSO	0/3	–	–
PBS + 15 mg/kg AITC	3/3	7 ± 1	4 ± 1
0.5 mg/kg RR + 15 mg/kg AITC	0/3	–	–

[a]Average of positive responders only.
[b]If animals failed to retch or vomit, the latency and duration of emesis are shown as "–". Data are presented as the mean ± SEM. Values for each compound with different superscript within a column indicate significant differences at $P < .05$.

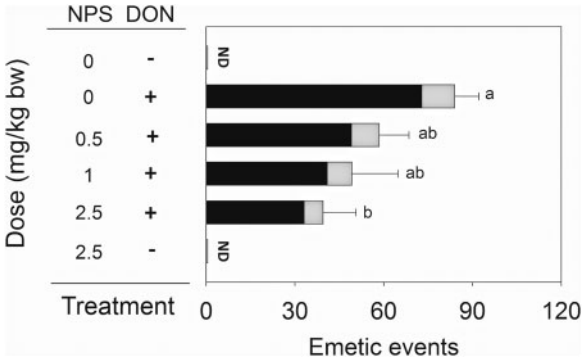

FIG. 4. CaSR antagonist NPS-2143 inhibits DON-induced emetic response in mink. Fasted mink were first gavaged with 0, 0.5, 1, and 2.5 mg/kg bw NPS-2143 and then provided 50 g of food. After 30 min, they were orally gavaged with 0.5 mg/kg bw DON or VEH and then monitored for emesis over a 3 h period. Emetic events include vomiting (gray) and retching (black) episodes. ND = not detected. Data are mean ± SEM ($n = 4$/group). Bars without the same letter are significantly different ($P < .05$).

induced plasma 5-HT at 30 min (59%) ($P < .05$). Pretreatment with 0.5 mg/kg bw RR alone also caused a significant reduction in DON-induced 5-HT concentrations (60%) ($P < .05$). DON-treated mink receiving both NPS-2143 and RR also exhibited a significant reduction in plasma 5-HT compared with DON alone (67%) ($P < .05$), but were not different from those treated with NPS-2143 plus DON or RR plus DON treatment alone ($P = .995$ and .973, respectively). Thus, each antagonist alone could abolish the plasma 5-HT response.

DISCUSSION

Emesis is a hallmark effect of trichothecene mycotoxin food poisoning in humans and animals such as pigs, dogs, cats, and mink. Prior investigations in this species have revealed that PYY_{3-36} and 5-HT, likely originating from EECs, contribute to emesis induction by DON and other trichothecenes (Wu *et al.*, 2013b, 2014, 2015). In the STC-1EEC model, DON elicits hormone exocytosis by activating both CaSR and TRPA1-mediated Ca^{2+} signaling (Zhou and Pestka, 2015). In that paper, it was proposed that DON-triggered hormone excytosis is mediated by the following pathway: CaSR-mediated activation of phospholipase C → phospholipase C-triggered activation of the IP3 receptor and mobilization of intracellular Ca^{2+} stores → TRPM5 activation and resultant L-type voltage-sensitive Ca^{2+} channel-facilitated extracellular Ca^{2+} entry → amplification of extracellular Ca^{2+} entry by TRPA1 activation → intracellular Ca^{2+}-driven hormone exocytosis. Hence, in the STC-1 model, CaSR activation was upstream of TRPA1 activation.

Using the mink, an animal capable of emesis, 3 novel observations were made here. First, directly activating either CaSR or TRPA1 by oral administration of selective agonists caused emesis in the mink. These effects could be blocked by oral pretreatment with their respective antagonists. Second, antagonists for either CaSR or TRPA1 attenuated DON-induced emetic effects; antagonism by both additively inhibited emesis induction by DON. Finally, inhibition of CaSR and TRPA1 impaired DON-induced plasma elevation of PYY_{3-36} and 5-HT, both of which have been previously shown to mediate DON-induced emesis. Accordingly, the dependency of DON-induced hormone elevation and emesis by mink on CaSR and TRPA1 as observed here harmonize with linkages to this GPCR and TRP channel previously proposed for DON-driven hormone excytosis in STC-1 cells (Zhou and Pestka, 2015) and for DON-triggered plasma hormone elevation and anorexia in the mouse (Wu *et al.*, 2016b).

Widely expressed in many organs including GI tract, kidney and parathyroid, CaSR has diverse functions that include regulation of serum calcium homeostasis (D'Souza-Li, 2006). Whereas CaSR is activated by allosterically binding extracellular Ca^{2+}, other inorganic cations and organic polycations (Chakravarti *et al.*, 2012; Ward *et al.*, 2012), this GPCR also functions as a chemosensor of intestinal lumenal content and can be activated by dietary peptides, amino acids, fatty acids, and polyamines. A number of drugs known as calcimimetics and calcilytics have been designed to induce (eg, R-568) or suppress

TABLE 5. Effect of CaSR Antagonist NPS-2143 on DON-Induced Emesis in Mink

Treatment	Incidence (Responding/Tested)	Latency to Emesis (min)[a,B]	Duration of Emesis (min)[A,b]
VEH + PBS	0/4	–	–
VEH + 0.5 mg/kg DON	4/4	8 ± 1	15 ± 4
0.5 mg/kg NPS + 0.5 mg/kg DON	4/4	10 ± 1	10 ± 3
1 mg/kg NPS + 0.5 mg/kg DON	4/4	9 ± 1	6 ± 2
2.5 mg/kg NPS + 0.5 mg/kg DON	4/4	13 ± 2	4 ± 1
2.5 mg/kg NPS + PBS	0/4	–	–

[a]Average of positive responders only.
[b]If animals failed to retch or vomit, the latency and duration of emesis are shown as "–". Data are presented as the mean ± SEM.

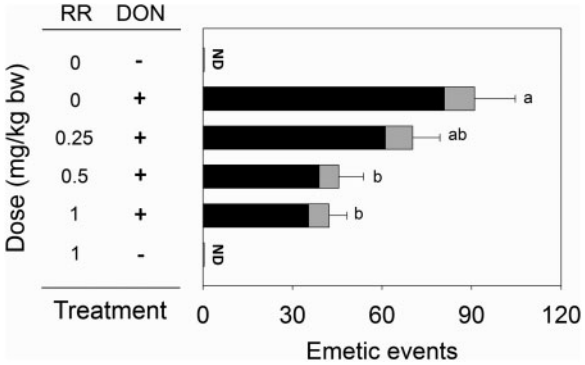

FIG. 5. TRP channel antagonist RR inhibits DON-induced emetic response in mink. Mink were first gavaged with 0, 0.25, 0.5, and 1 mg/kg bw RR and provided 50 g of food. After 30 min, they were gavaged with 0.5 mg/kg bw DON or VEH and emesis monitored for 3 h. Emetic events include vomiting (gray) and retching (black) episodes. ND = not detected. Data are presented as mean ± SEM ($n = 4$/group). Bars without the same letter are significantly different ($P < .05$).

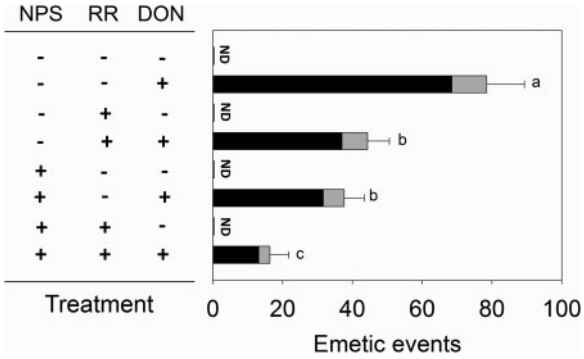

FIG. 6. CaSR antagonist NPS-2143 and TRP channel antagonist RR additively inhibit DON-induced emetic response in mink. Fasted mink were first gavaged with 2.5 mg/kg bw NPS-2143, 0.5 mg/kg bw RR and/or VEH and then provided 50 g of food. After 30 min, they were gavaged with 0.5 mg/kg bw DON or VEH and then monitored for emesis over a 3 h period. Emetic events include vomiting (gray) and retching (black) episodes. ND = not detected. Data are presented as mean ± SEM ($n = 6$/group). Bars without the same letter are significantly different ($P < .05$).

TABLE 6. Effect of TRP Channel Antagonist Ruthenium Red on DON-Induced Emesis in Mink

Treatment	Incidence (Responding/Tested)	Latency to Emesis (min)[a,b]	Duration of Emesis (min)[a,b]
PBS + PBS	0/4	–	–
PBS + 0.5 mg/kg DON	4/4	9 ± 1	20 ± 6
0.25 mg/kg RR + 0.5 mg/kg DON	4/4	9 ± 1	12 ± 2
0.5 mg/kg RR + 0.5 mg/kg DON	4/4	10 ± 1	11 ± 2
1 mg/kg RR + 0.5 mg/kg DON	4/4	12 ± 2	7 ± 1
1 mg/kg RR+ PBS	0/4	–	–

[a]Average of positive responders only.
[b]If animals failed to retch or vomit, the latency and duration of emesis are shown as "–". Data are presented as the mean ± SEM.

(eg, NPS-2143) CaSR activation in an allosteric fashion, respectively, to treat human diseases related to aberrant regulation of Ca^{2+} (Nemeth *et al.*, 2001; Terranegra *et al.*, 2010). In CaSR-transfected HEK 293 cells, the EC50 for R-568 is 1.4 uM (Terranegra *et al.*, 2010) and the IC50 for NPS-2143 is 43 nM (Nemeth *et al.*, 2001). Although ADME data for these drugs following oral delivery have not been published, the doses employed here for R-568 (2.5, 5, and 10 mg/kg bw) and NPS-2143 (2.5 mg/kg bw) are consistent with those utilized *in vivo* in prior studies. When partially nephrectomized rats were treated orally with 3 and 30 mg/kg bw of R-568 to activate CaSR, parathyroid cell hyperplasia was suppressed (Wada *et al.*, 1997). A 45 mg/kg bw dose of NPS-2143 was used in rats to antagonize CaSR-mediated parathyroid hormone secretion (Nemeth *et al.*, 2001). Recently, we used 15 mg/kg bw R-568 to induce plasma satiety hormone increases and anorexia in the mouse; NPS-2143 at 20 mg/kg bw suppressed these responses (Wu *et al.*, 2016b). Overall, our data suggest that CaSR activation plays a critical role in DON-induced PYY_{3-36} and 5-HT release and furthermore, this GPCR contributes, in part, to emesis. Further clarification is needed on the specific mechanisms by which DON activates CaSR in EEC.

TABLE 7. Effect of CaSR and TRP Antagonists on DON-Induced Emesis in Mink

Treatment NPS RR DON	Incidence (Responding/Tested)	Latency to Emesis (min) [a,b]	Duration of Emesis (min) [a,b]
− − −	0/6	–	–
− − +	6/6	8 ± 1 [a]	18 ± 4 [a]
− + −	0/6	–	–
− ++	6/6	12 ± 2 [a]	8 ± 2 [b]
+ − −	0/6	–	–
+ − +	6/6	10 ± 1 [a]	9 ± 1 [b]
++ −	0/6	–	–
++ +	5/6	17 ± 5 [a]	3 ± 1 [b]

[a]Average of positive responders only.
[b]If animals failed to retch or vomit, the latency and duration of emesis are shown as "–". Data are presented as the mean ± SEM. Values for each compound with different superscript within a column indicate significant differences at $P < .05$. The doses for NPS, RR and DON were 2.5 , 1, and 0.5 mg/kg, respectively.

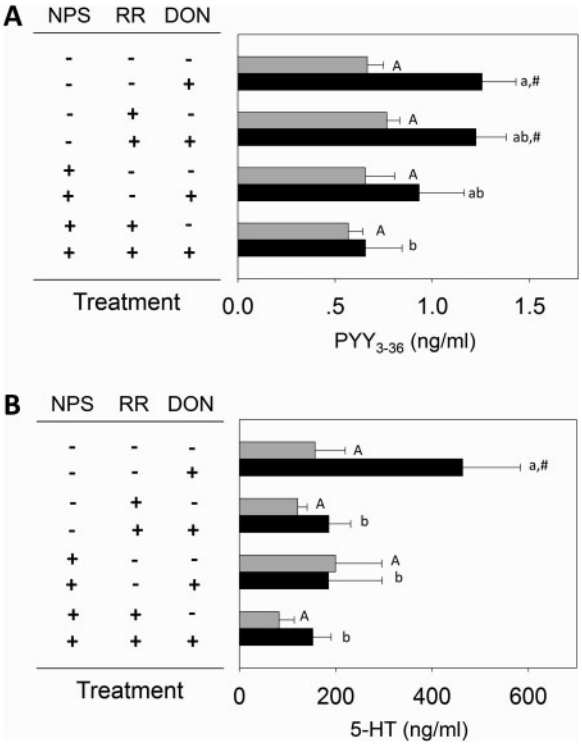

FIG. 7. CaSR antagonist NPS-2143 and TRP channel antagonist RR inhibit DON-induced plasma (A) PYY_{3-36} and (B) 5-HT elevation in mink. Fasted mink were first gavaged with 2.5 mg/kg bw NPS-2143, 0.5 mg/kg bw RR and/or VEH and then provided 50 g of food. Thirty minutes later, mink were gavaged with 0.5 mg/kg bw DON or VEH. After 30 min, blood was collected for PYY_{3-36} and 5-HT plasma concentration determined by ELISA. Grey bars indicate mink treated with DON; black bars indicate mink treated with vehicle only. Data are presented as mean ± SEM ($n = 5$/group). Bars without the same letter are significantly different ($P < .05$). The # sign indicates significant difference between VEH and corresponding DON-treated group ($P < .05$).

Another lumenal chemosensor, TRPA1 can be activated by noxious chemical agents such as AITC, formalin, acrolein, 4-hydroxynonenal, cinnamaldehyde, and bradykinin (Nilius *et al.*, 2012). In TRPA1-transfected HEK-293 cells, the selective TRPA1 agonist AITC EC50 is 2.2 μM (Nagata *et al.*, 2005) and the TRP antagonist RR IC50 is 3.4 μM (Liu *et al.*, 2010), respectively. Although ADME studies for these drugs following oral delivery have not been reported, the doses utilized here for AITC (1, 5, and 15 mg/kg bw) and RR (0.5 mg/kg bw) are in line with those employed in other *in vivo* studies. In rats, oral gavage with 10 mg/kg bw AITC induces TRPA1-mediated gastric emptying that was abrogated by oral pre-treatment with 1 mg/kg bw RR (Doihara *et al.*, 2009b). Similarly, oral delivery of 1 mg/kg bw AITC induced TRPA1-mediated gastric motility and vomiting in dogs that could be ablated by oral pretreatment with 3 mg/kg RR (Doihara *et al.*, 2009a). In addition, administration of TRPA1 agonist methyl syringate in mice elicited TRPA1-driven PYY secretion and anorexia in ICR mice, both of which were blocked by oral pretreatment with 0.33 mg/kg bw of RR (Kim *et al.*, 2013). Finally. in recent work, we similarly observed in mice that 15 mg/kg bw AITC evoked food refusal that could be blocked with 2.5 mg/kg bw RR (Wu *et al.*, 2016b).

Based on the data presented here and previous in vitro (Zhou and Pestka, 2015) and mouse (Wu *et al.*, 2016b) studies, we speculate that TRPA1 is a critical calcium channel associated with mediating DON's toxic effects. In support of this contention, TRPA1 activation has been reported to be elicited by GPCR-mediated activation (Nilius *et al.*, 2012). It is thus possible that TRPA1 activation follows DON-induced CaSR activation. DON is nonreactive, because its epoxide group is shielded by the parent ring and prevents it from reacting with other compounds (Zhou and Pestka, 2015). Nevertheless, some other nonreactive chemicals including nicotine have been mentioned to directly evoke TRPA1 activation (Talavera *et al.*, 2009). Another limitation of this study is that we cannot yet exclude the possibility that other TRP channels might be involved in DON's emetic effects. Although AITC is widely used as a TRPA1 channel agonist (McNamara *et al.*, 2007), it can also activate TRPV1 (Everaerts *et al.*, 2011; Hsu and Lee, 2015). Furthermore, RR, the TRP channel antagonist used here is a nonspecific inhibitor of TRP channels as well as other calcium channels (Tapia and Velasco, 1997; Vriens *et al.*, 2009). Therefore, additional investigations employing other selective TRP agonists and inhibitors are needed to verify the role of TRPA1 and other TRP channels further in DON-induced emesis.

The mink data presented here are qualitatively in concert with prior findings in STC-1 cells (Zhou and Pestka, 2015) that showed DON induced intracellular Ca^{2+} elevation and hormone secretion. However, the DON concentrations required for STC-1 responses were 0.5–2.0 mM and unlikely to be encountered physiologically. Based on a prior study (Wu *et al.*, 2013b), we estimate that DON plasma concentrations would be 1–2 μM in mink during the first 60 min following orally gavage with the toxin at 0.5 mg/kg bw. Consequently, the mink model might be orders of magnitude more sensitive to DON than the STC-1 EEC model.

In summary, the results provided herein and previously suggest that DON elicits PYY_{3-36}- and 5-HT-driven emesis by

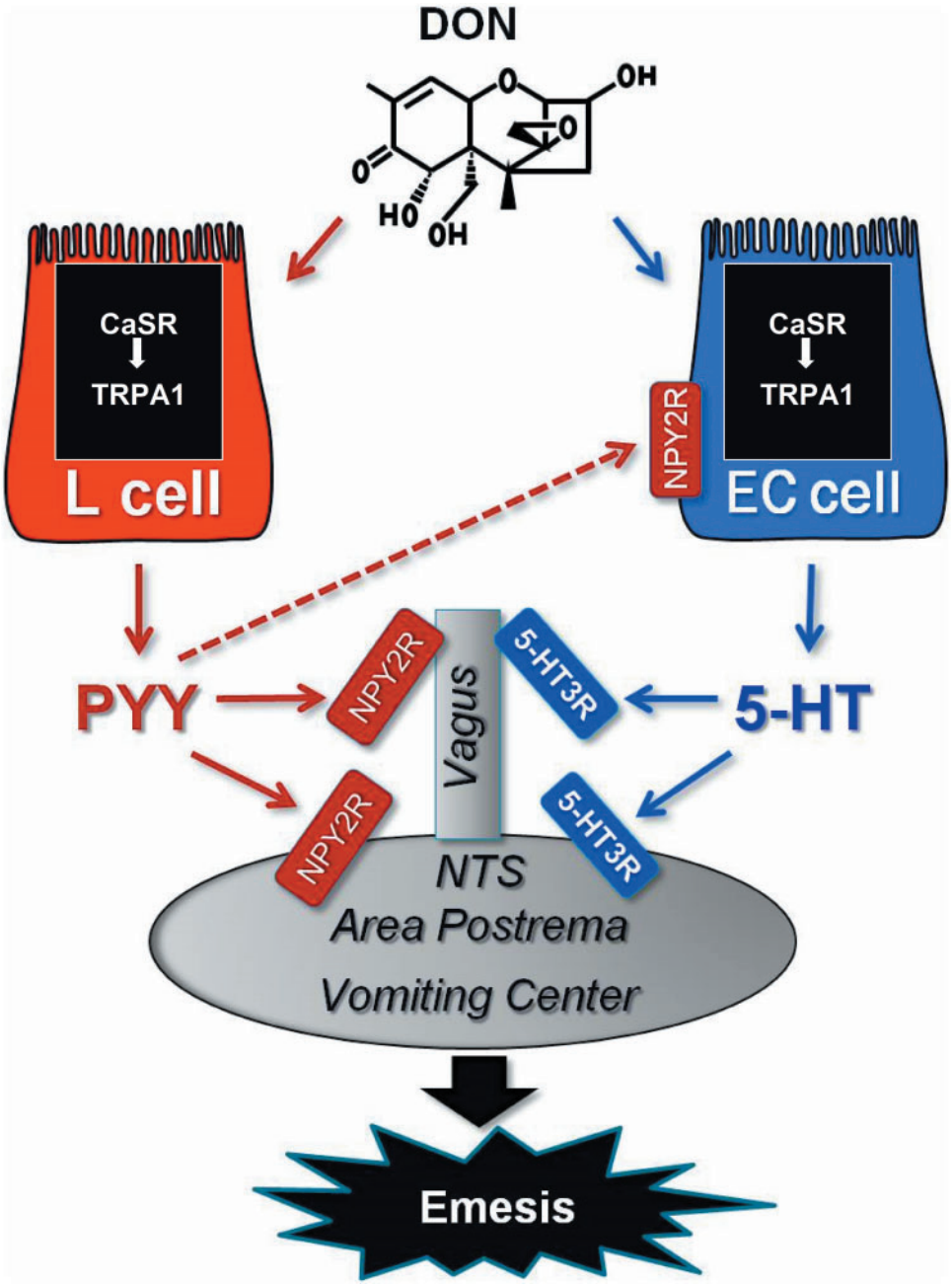

FIG. 8. Putative role of CaSR and TRP channel on enteroendocrine cell (EEC) in DON-induced emesis. The results presented here and previously (Wu *et al.*, 2016b) suggest that DON sequentially activates CaSR and TRP channel in EEC to induce release of PYY_{3-36} (L cells) and 5-HT (EC cells). These may activate neuropeptide Y2 receptor (NPY2R) and 5-HT_3 receptor (5-HT_3R), respectively, in the peripheral and central nervous systems, ultimately inducing emesis via the vomiting center. The potential exists for crosstalk (dotted line) whereby PYY_{3-36} activates neuropeptide Y2 receptor (NPY2R) on EC cells to release 5-HT at peripheral and central sites.

activating CaSR and TRP channels as depicted in Figure 8. Future investigations should focus on how DON and other trichothecenes act on primary EEC to elicit hormone exocytosis as well as their linkages to subsequent emetic responses. From a public health perspective, studies like this will improve our understanding of the cellular and molecular mechanisms for DON's emetic effects. This could lead to strategies to block this and other adverse effects of exposure to this or other trichothecenes in humans and animals. Over the long term, such research will serve as a template to determine GPCR- and TRP-mediated emesis induction by foodborne toxins, environmental toxicants, and chemotherapeutic drugs.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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