Chemotherapy agent cisplatin induces 48 h Fos expression in the brain of a vomiting species, the house musk shrew (*Suncus murinus*)

Bart C. De Jonghe and Charles C. Horn*

Monell Chemical Senses Center, Philadelphia, PA 19104, USA.

**Running head**: Cisplatin-induced Fos expression in musk shrew brain

*Address correspondence to: Charles C. Horn, Ph.D.
Monell Chemical Senses Center
3500 Market St
Philadelphia, PA 19104
Telephone: 267-519-4865
Fax: 267-519-4865
Email: horn@monell.org
ABSTRACT

Cancer chemotherapy drugs, such as cisplatin, potently produce nausea and vomiting. Acute effects of these treatments are partly controlled by antiemetic drugs, but the delayed effects (>24 h), especially nausea, are more difficult to treat. It is unknown what brain pathways produce this delayed sickness. Our prior data show that brain Fos expression is increased for at least 48 h after cisplatin treatment in the rat, a non-vomiting species. Here, we extend these observations using house musk shrews (Suncus murinus), a species with an emetic response. Compared to saline injection, cisplatin treatment (30 mg/kg, i.p.) induced Fos expression in hindbrain areas known to play a role in the generation of emesis, the dorsal motor nucleus (DMN), area postrema (AP), and the nucleus of the solitary tract (NTS), for up to 48 h. Cisplatin also stimulated Fos expression in the parabrachial nucleus (PBN) of the midbrain and central nucleus of the amygdala (CeA) for at least 48 h after treatment. When animals were pretreated with the antiemetic palonosetron, a long-term 5-HT₃ receptor antagonist, cisplatin-induced Fos expression was significantly attenuated in the NTS, DMN, and CeA at 6 h but not at 48 h. These results indicate that cisplatin activates a neural system that includes the dorsal vagal complex and forebrain in the musk shrew, which is partially suppressed by a 5-HT₃ receptor antagonist. Our findings suggest the existence of an extensive neural system that could be targeted to reduce nausea, vomiting, and malaise in cancer patients receiving chemotherapy.

Key Words: Suncus, shrew, cisplatin, emesis, palonosetron, 5-HT₃
INTRODUCTION

Nausea and vomiting are among the most common and severe side effects of chronic disease states and pharmacological treatments. Some cancer chemotherapy drugs, such as cisplatin, are extremely potent agents for producing nausea and vomiting, as well as anorexia, body weight loss, and diarrhea (22, 33, 34, 47). Chemotherapy induces both acute (up to 24 h) and delayed (> 24 h) phases of nausea and emesis (57). Serotonin type 3 (5-HT₃) receptor antagonists largely control the severity of chemotherapy-induced acute-phase emesis, while neurokinin 1 (NK₁) receptor antagonists appear most useful for treatment of the delayed phase (16, 34, 72), although there is some overlap in their therapeutic time window. Despite the use of these first-line antiemetic drugs, many patients still experience nausea and emesis with chemotherapy, and although acute emesis is reasonably well controlled, nausea and delayed emesis are more difficult to treat (1, 57).

Several animal models have been used to study acute and delayed emesis and malaise induced by chemotherapy agents. Ferrets, pigs, dogs, and the house musk shrew (Suncus murinus) show delayed phases of emesis after injection with chemotherapy drugs (20, 42, 58, 63). Although rats, and likely all rodents, do not have a vomiting response, they do ingest kaolin clay (a form of pica), which has been used as a surrogate marker of emesis because this response appears to be a strategy to reduce toxicosis (toxins can bind to clay) and is inhibited by antiemetic drugs (5, 60, 68). Our laboratory has demonstrated that rats show acute and delayed phases of kaolin intake in response to cisplatin injection (13). We have also shown that, similar to emesis (4), cisplatin-induced kaolin ingestion is largely dependent on an intact vagus (13) and cisplatin activates the vagus (27). In general, however, the neural systems and possible
hindbrain-forebrain connections responsible for acute and delayed nausea and emesis are unknown.

The global activation of brain systems can be partly characterized by labeling Fos protein in neuronal cells after stimulation (61). Cisplatin treatment in vomiting species, such as the cat and ferret, produces acute Fos expression in the caudal hindbrain, including the nucleus of the solitary tract (NTS) and area postrema (AP), areas known to play a role in emesis (7, 44, 70). The rat also shows increased c-fos mRNA expression in the NTS and AP for up to 6 h after injection with cisplatin (18). Similarly, our laboratory has identified long-term increases in Fos expression (up to 48 h) in the NTS and AP and the amygdalar complex in rats treated with cisplatin (26). These data suggest a neural system that includes both the dorsal vagal complex (DVC) and forebrain areas that might be linked to the delayed phase of sickness produced by cisplatin treatment. Although our prior study defined the activation of neural pathways associated with chemotherapy-induced malaise in the rat over a 48-h period, it would be advantageous to assess activation of these neural connections using an animal model capable of vomiting.

Therefore, in the present study, we assessed the effects of cisplatin on brain Fos expression in the house musk shrew, *Suncus murinus*. The musk shrew is a well-defined model for emesis, including cisplatin-induced emesis (40, 59, 69), and shows a prominent Fos response in hindbrain emetic circuitry (6, 28, 29). We evaluated Fos expression at specific locations of the hind-, mid-, and forebrain at both acute (6 and 24 h) and long-term (48 h) time points. We chose these times because they align with sample times used in our study on cisplatin-induced Fos expression in the rat and also reflect times for acute and delayed emesis in the musk shrew.
(26, 62). Although we did not measure the emetic responses from animals used in this study, musk shrews from our colony do show a significant amount of emesis when injected with cisplatin. Furthermore, to address the issue of how Fos expression induced by cisplatin might relate to known emetic inputs, we used pretreatment with the antiemetic drug palonosetron to antagonize 5-HT₃ receptors. We chose this specific antagonist based on evidence that, unlike other clinically available 5-HT₃ antagonists, palonosetron might show usefulness for the delayed phase of emesis, as well as a potent effect on the acute phase, and has a very long action to inhibit 5-HT₃ receptors (2, 12, 16, 21).

**METHODS**

*Subjects*

We used 59 female house musk shrews (*Suncus murinus*) (34-75 days of age, 20-28 g) in this study. The musk shrews were born at the Monell Chemical Senses Center animal facility, in a colony created from stock animals obtained from Dr. Emilie Rissman at the University of Virginia, with animals originally captured from Guam (9, 14). We individually housed the musk shrews in plastic cages in a temperature-controlled vivarium on a 12/12-h light/dark cycle (lights on at 0600 h). All musk shrews had *ad libitum* access to tap water and food (75% Purina Cat Chow Complete Formula and 25% Complete Gro-Fur mink food pellets: Milk Specialty Co., New Holstein, WI, USA), except as indicated below. The protocol was approved by the Monell Chemical Senses Center Institutional Animal Care and Use Committee.

*Drug injections and tissue collection*
Musk shrews received two intraperitoneal injections, 15 min apart. All injections were completed between 1000 and 1145 h. The first injection was either saline (0.15 M NaCl) or palonosetron (0.5 mg/kg), and the second injection was either saline or cisplatin (30 mg/kg). Drugs were injected in a volume of 10 ml/kg body weight. Cisplatin (Sigma-Aldrich; cis-diamineplatinum dichloride, #P4394) and palonosetron (Haorui Pharma-Chem, Edison, NJ, USA) were dissolved in saline (0.15 M NaCl), sonicated until clear, and then vortexed immediately before injection. Animals were randomly assigned to receive one of three drug injection combinations: saline-saline, saline-cisplatin, palonosetron-cisplatin, or palonosetron-saline. Animals receiving saline-saline were euthanized at 6 h (n = 5), 24 h (n = 6), or 48 h (n = 5) post-injection. Animals receiving saline-cisplatin were euthanized at 6 h (n = 7), 24 h (n = 8), or 48 h (n = 6) post-injection. Animals receiving palonosetron-cisplatin were euthanized at 6 h (n = 8) or 48 h (n = 7) post-injection. An additional group receiving palonosetron-saline were euthanized at 6 h (n = 4). We removed water bottles and food jars 2 h before euthanization to minimize contributions of ingestive behavior to Fos expression.

We chose the dose of cisplatin based on previous work (62) and our own data showing reliable short-term induction of emesis (unpublished data). Doses of 20-40 mg/kg cisplatin produce acute and delayed emesis in musk shrews (62). We chose the dose of palonosetron (0.5 mg/kg) based on our experiments in the rat showing that this concentration maximally suppressed 5-HT-induced electrophysiological activation of afferent vagal fibers (unpublished data) and that lower doses suppress cisplatin-induced acute emesis in the dog and ferret (15). Single-bolus injections of palonosetron have inhibitory effects on chemotherapy-induced acute and delayed emesis in humans, with a half-life in plasma of 44-128 h (17).
At the time of euthanasia, we deeply anesthetized the animals by intraperitoneal injection of sodium pentobarbital (25 mg; 0.5 ml). We opened the thoracic cavity with scissors and inserted a blunt-edged syringe needle into the apex of the heart. Animals were then transcardially perfused with ~15 ml of 0.2 M phosphate-buffered saline (PBS; pH 7.4), followed by ~20 ml of 2% acrolein/4% paraformaldehyde in 0.1 M phosphate buffer (7.4). Whole brains were removed and placed in 10% sucrose/PBS followed by 20% and 30% sucrose/PBS, each overnight. After sucrose cryoprotection, brains were frozen using dry ice and subsequently cut at 50 µm using a cryostat.

We collected sections from three locations based on both previous work in the rat and the musk shrew (6, 26, 28-30): (i) the caudal hindbrain (dorsal motor nucleus [DMN], NTS, and AP), (ii) midbrain sections containing the parabrachial nucleus (PBN), and (iii) forebrain sections containing the central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis, ventral (BNSTv). The PBN, CeA, and BNSTv were selected for analysis because tract tracing in the musk shrew and rat indicates that these areas have primary rostral projections from the NTS and AP (31, 32, 65). The part of the BNST chosen for analysis is not the one represented in our paper on the rat (26), but instead is a division located ventral to the anterior commissure and shows a dense projection from the NTS of the shrew [see Plate IV, section R in Ref. (31)]. The dorsal lateral BNST that was reported in our paper using the rat (26) was difficult to locate in the shrew brain. Sections were collected in duplicate and placed into culture plate wells containing cryoprotectant (73), and either stored at -20°C or immediately processed for immunohistochemistry.

_Fos immunohistochemistry_
We processed sections using a slightly modified procedure previously established in our laboratory (26). Briefly, we include here only the important modifications. Sections were incubated at room temperature in 1:10,000 polyclonal anti-Fos antibody (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA, Lot# E1606) containing 1% normal donkey serum for 20 h. Biotinylated rabbit anti-rat was used as a secondary antibody (1:400, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) for 2 h at room temperature. This was followed by incubation in avidin–biotin and then DAB-nickel for the chromogen reaction.

**Cell counting and analysis**

We determined Fos cell staining by the presence of a blue-black reaction product in the cell nuclei. Tissue sections were viewed with a Zeiss microscope (Axiostar Plus) equipped with a digital camera (Scion CFW-1312C). Brain regions and cells expressing Fos were imaged using ImageJ software (NIH; http://rsb.info.nih.gov/ij). These images were subsequently merged with outlines of areas of interest and templates of Fluoro-Gold staining using CorelDraw (Graphics Suite X4). Subsequently, JPEG image files were generated and counted manually using ImageJ with the Measure and Label plug-in. Fos cell staining was counted by two people working independently without knowledge of the experimental conditions and the average of these two assessments is reported here (these values had an inter-rater Pearson correlation of 0.90 to 0.97).

Based on previous examinations of brain sections from rats treated with cisplatin (26), we counted cells in areas that consistently showed Fos expression, with boundaries determined by reference to other brain sections containing cresyl violet or Fluoro-Gold staining (see below). To
standardize the analyses, we counted cells from each area in coronal brain sections from each animal at approximately the same level (see Figs. 1 and 2). The brain areas analyzed were i) hindbrain: NTS, AP, and DMN at +250 µm from the obex; ii) midbrain: PBN; and iii) forebrain: CeA and BNSTv. Although we show in Fig.1 that Fos labeling produced by cisplatin did extend to rostral levels of the DVC, +400 and +700 um rostral to the obex, we did not attempt to count these areas because it was difficult to separate the NTS from the DMN. We did not count the cells in the reticular formation since this staining was very diffuse (see Fig. 6). Fos expression was not lateralized in any of the bilateral structures examined, and cell counts reflect the totals for both sides. Brains were processed in batches, and each batch also included a positive control for Fos expression, consisting of brain sections from rats treated with cholecystokinin (100 ug/kg, i.p.) 1 h before euthanasia [e.g., (49)]. These positive controls consistently showed high levels of Fos expression in the hindbrain and forebrain. We were also able to completely block Fos staining in samples of shrew and rat tissue by pre-incubation of the Fos antibody with Fos protein (sc-52P, Santa Cruz, Lot# J1805).

Hindbrain, CeA, and BNSTv areas were easily identifiable in almost all of the animals, however, we could not locate appropriate levels of the DVC in 2, CeA in 6, and BNSTv in 10 animals. The PBN proved to be particularly problematic because we typically observed only one section at the appropriate location in each animal (see Fig. 4). Furthermore, these sections often contained only one side, left or right, because even a slight difference in the leveling of the brain during cutting would completely change the position within the PBN. Therefore, for most cases we only used one side (47% of the animals) for analysis. We doubled this value to account for the missing side to produce a more accurate Fos cell count when compared to other brain areas.
In twelve animals we were unable to locate an appropriate PBN section. Missing sections were distributed across the different conditions.

*Cresyl violet and Fluoro-Gold staining to determine nuclear boundaries*

No detailed brain atlases are available for *Suncus murinus*, and initial observations indicated that it was difficult to determine boundaries of nuclear areas in this species. Therefore, we used cresyl violet staining to determine gross anatomical landmarks with reference to homologous areas in the rat brain, and retrograde tracing to define distinctly the DMN relative to the NTS.

We used one musk shrew to stain the entire brain with cresyl violet to more accurately depict the cytoarchitectural boundaries. Animal euthanization and brain tissue collection were the same as described above, except that sections (50 µm) were placed directly on slides as they were cut. Fig. 1 (right column) shows representative images collected from the hindbrain.

In a separate experiment, two animals were injected with 0.2 ml (i.p., 1% solution) of the retrograde tracer Fluoro-Gold (Fluorochrome, LLC, Denver, CO, USA) to specifically identify the location of DMN neurons. This procedure has been used previously in our laboratory and others to define the DMN in the rat (13, 51). Four to five days after injection with Fluoro-Gold, animals were euthanized as described above, and 50-µm sections were processed for Fluoro-Gold immunoreactivity as described previously (13). Fig. 1 (middle column) shows representative images collected from the hindbrain.

*Statistical analysis*
Fos cell counts were analyzed using two-way analysis of variance (ANOVA) for each brain area. To assess the time effects of cisplatin, 2 (saline or cisplatin) x 3 (6, 24, or 48 h) ANOVAs were used. For the analysis of palonosetron effects, 3 (saline, cisplatin, or cisplatin plus palonosetron) x 2 (6 or 48 h) ANOVAs were used. Planned comparisons were conducted using least significant difference tests (LSD-tests). Although it was not possible to do a full three-way ANOVA using palonosetron as a factor (i.e., time by cisplatin by palonosetron), saline-saline and palonosetron-saline groups, at 6 h, were compared using independent two sample Student’s t-tests (two-tailed). The palonosetron-saline data are included in Fig. 7 and 8 for reference. For all analyses, a P-value of 0.05 was used to determine statistical significance. Group values are reported as the mean ± SEM. Statistical analyses were conducted using Statistica (Version 8, StatSoft, Inc.).

RESULTS

**Hindbrain Fos expression after cisplatin treatment**

Cisplatin produced both short- and long-term increases in Fos expression in the hindbrain at several levels rostral to the obex (Fig. 1). There was also a scattering of Fos expression in the reticular formation, which likely included nucleus ambiguous and C1/A1 regions, produced by cisplatin (Fig. 6). Using Fluoro-Gold and cresyl violet staining (Fig. 1) to delineate nuclear boundaries, it was clear that Fos expression was elevated in the DMN, NTS, and AP at 6, 24, and 48 h after cisplatin treatment compared to saline injection (Fig. 2).

Fos cell counts (250 µm rostral to the obex) were increased in the DMN, NTS, AP, and DVC by cisplatin treatment [all F(1,30) ≥ 34.3, all P ≤ 0.00001, cisplatin main effect for each
brain region; Fig. 3]. Planned comparisons showed that in all cases cisplatin treatment produced greater Fos cell counts than saline treatment (LSD-tests; Fig. 3). There were no significant interaction effects for treatment by time, however, there was a significant decrease in Fos cell counts over time in the NTS [F(2,30) = 3.3, P < 0.05, main effect of time].

**Mid- and forebrain Fos expression after cisplatin treatment**

We observed scattered levels of Fos staining in the mid- and forebrain, but the PBN and CeA showed clear effects of cisplatin treatment (Fig. 4). The PBN and the CeA showed significantly increased Fos expression after cisplatin treatment [PBN, F(1,24) = 14.3, P < 0.001; CeA, F(1,27) = 32.0, P = 0.000005; main effects of cisplatin, Fig. 5]. Planned comparisons showed that at all time points in the CeA and PBN, except at 6 h for the PBN, cisplatin treatment produced greater Fos cell counts than saline treatment (LSD-tests; Fig. 5). There were no significant interaction effects or main effects of time. Furthermore, there were no significant effects of cisplatin at any time point on Fos expression in the BNSTv (ANOVA and planned comparisons, Ps > 0.05).

**Effect of palonosetron on cisplatin-induced hindbrain Fos expression**

Cisplatin treatment induced Fos expression in the DMN, NTS, AP, and DVC [all F(2,30) ≥ 10.7, all P ≤ 0.001, main effects of treatment, Fig. 7]. Treatment condition and time significantly affected Fos expression in the DMN [F(2,30) = 3.3, P < 0.05, treatment by time interaction, Fig. 7]. There were no other significant interaction effects or main effects of time. Mean comparisons revealed that cisplatin-induced Fos expression in the DMN and NTS was
attenuated by pretreatment with palonosetron (LSD-tests, Ps < 0.05, cisplatin vs. cisplatin plus palonosetron, Fig. 7). The scattering of Fos expression in the reticular formation produced by cisplatin appeared to be attenuated by palonosetron (Fig. 6).

To determine if palonosetron might reduce basal levels of Fos expression in the hindbrain we compared saline-saline with palonosetron-saline treated shrews. There was no significant difference at 6 h between shrews treated with saline-saline or palonosetron-saline in the DMN, NTS, AP, or DVC [all t(7) ≤ 0.8, all P ≥ 0.5; Fig. 7].

**Effect of palonosetron on cisplatin-induced mid- and forebrain Fos expression**

Cisplatin treatment produced Fos expression in the PBN and CeA [PBN, F(2,23) = 4.6, P = 0.02; CeA, F(2,28) = 7.9, P = 0.002, main effects of treatment, Fig. 8]. Treatment condition and time significantly affected Fos expression in the CeA [F(2,28) = 5.9, P = 0.007, treatment by time interaction, Fig. 8]. There were no other significant interaction effects or main effects of time. In the PBN, only cisplatin at 48 produced an increase in Fos expression (LSD-test, Fig. 8). In the CeA, planned comparisons revealed that cisplatin-induced Fos expression at 48 h was attenuated by pretreatment with palonosetron (LSD-test; P < 0.05, cisplatin vs. cisplatin plus palonosetron, Fig. 8). Both cisplatin and cisplatin plus palonosetron conditions produced significant increases in Fos expression in the CeA at 48 h (LSD-tests; Fig. 8). In the BNSTv, there were no significant effects of cisplatin or cisplatin plus palonosetron at any time point on the level of Fos expression (ANOVA and planned comparisons, Ps > 0.05).

To determine if palonosetron might reduce basal levels of Fos expression in the PBN, CeA, and BNSTv we compared saline-saline and palonosetron-saline treated animals. There
were no significant differences at 6 h between shrews treated with saline-saline or palonosetron-saline in PBN, CeA, or bed nucleus [all t(5 or 6) ≤ 1.5, all P ≥ 0.2; Fig. 8].

**DISCUSSION**

Cisplatin treatment induced Fos expression for at least 48 h in the DVC, PBN, and CeA of the house musk shrew, *Suncus murinus*, a species with a vomiting response. Furthermore, cisplatin-induced Fos expression at 6 h postinjection in the DMN, NTS, and CeA was potently inhibited by pretreatment with the antiemetic palonosetron, indicating the involvement of 5-HT₃ receptors. This is the first study using a vomiting species to show long-term activation of the hindbrain with cisplatin treatment, up to 48 h posttreatment, and also in brain regions rostral to hindbrain emetic circuitry. These results indicate that cisplatin activates a neural system that includes several levels of the brain’s neuroaxis and suggest potential brain areas that might play a role in the longer-term sickness produced by this chemotherapy agent.

*Cisplatin-induced Fos expression indicates activation of emetic circuitry*

The anti-neoplastic agent cisplatin, a common chemotherapy agent, exerts its cytotoxic effects primarily through direct interference with DNA synthesis, replication, or repair (11). Cisplatin has many deleterious side effects, including the stimulation of nausea, vomiting, anorexia, and behaviors indicative of malaise (10, 13, 37, 38, 57). Potentially, any of these physiological effects might affect brain Fos expression. However, cisplatin’s effects on Fos
expression in specific brain areas known to play a role in emesis, and the inhibition of this response by an antiemetic agent, suggest that at least part of the Fos expression induced by cisplatin treatment in musk shrews is related to activation of emetic pathways.

Previous evidence indicates that cisplatin stimulates a vagal pathway to the hindbrain to produce emesis, and the present data showing cisplatin-induced Fos expression in the NTS and AP are consistent with this action. Although little neuroanatomical information is available for musk shrews, in the rat, vagal afferents from the gastrointestinal tract project to the NTS and AP (48). The initial stimulus for emesis induced by cisplatin appears to stem from the release of 5-HT from enterochromaffin cells, which activates vagal afferent fibers in the gut containing 5-HT\textsubscript{3} receptors that project to the DVC (4, 45, 57). The essential neural circuitry for vomiting is contained in the hindbrain. A hindbrain that has been surgically disconnected from mid- and forebrain regions can still produce emetic-like responses, for example, in the cat (43). The DVC and regions of the reticular formation play an integral role in producing emesis (8, 19). In the present study, cisplatin treatment increased Fos expression in the NTS, AP, DMN, and reticular formation, which likely included activation of the nucleus ambiguous and C1/A1 regions. In our previous study using the rat, a non-vomiting species, cisplatin injection induced Fos expression in the NTS and AP but not in the DMN (26). DMN neurons make extensive projections to the upper gastrointestinal tract [e.g., in musk shrew, Ref. (74)] and possibly initiate the giant retrograde contraction that moves intestinal contents back to the stomach before emesis, and the nucleus ambiguous likely participates in the esophageal shortening that occurs during vomiting (66). Therefore, cisplatin-induced Fos expression in the DMN might reflect the activation of motor outputs related to emesis.
Inhibition of cisplatin-induced hindbrain Fos expression by pretreatment with the antiemetic palonosetron, a 5-HT$_3$ receptor antagonist, also indicates the activation of emetic circuits. In vomiting species, for example, humans, dogs, and ferrets, palonosetron is an effective antiemetic against chemotherapy-induced emesis, especially during the acute phase (15, 39). In the ferret, Fos expression in the NTS at ~3 h after cisplatin injection was also reduced by pretreatment with granisetron, another 5-HT$_3$ receptor antagonist (55). Cisplatin-induced Fos expression in the NTS of musk shrews, at least at 6 h post-treatment, likely depends on vagal input, given that in the ferret vagotomy blocks cisplatin-induced Fos expression in the NTS (55). The NTS has a dense projection to the DMN in musk shrews and other animals (31, 56), and inhibition of DMN Fos expression by palonosetron is possibly due to suppression of activity in the NTS. Fos expression in the AP could reflect nonvagal action of cisplatin, because in the present study palonosetron did not greatly alter cisplatin-induced Fos expression in the AP, and because in the ferret vagotomy does not affect cisplatin-induced Fos expression in the AP (55). Cisplatin or humoral factors might activate the AP directly. For example, systemic injection of cisplatin increases plasma levels of substance P and 5-HT, possibly derived from release of these factors from enteroendocrine cells of the gut. Direct application of serotonin and substance P can activate neurons in the AP and intracerebroventricular injection of cisplatin in the cat can produce emesis (23, 41, 67).

Relationship between Fos expression and emesis 48 h after cisplatin treatment

Although cisplatin treatment increased brain Fos expression for at least 48 h, Fos cell counts did not decline significantly over this duration. In a similar study in the rat, we also found
that cisplatin treatment elevated Fos expression for at least 48 h post-treatment (26). In addition, we have shown that rats continue to ingest clay, a behavioral marker of sickness, for up to 2 weeks after a single injection of cisplatin (13). The relationship in musk shrews between Fos expression and emesis produced by cisplatin treatment is not straightforward. In a study by Sam et al. (62), examining emetic episodes over 72 h, musk shrews vomited primarily within the first few hours of cisplatin treatment, followed by a prolonged (>24 h) period of little emesis, with a recurrence of emesis near 48 h after cisplatin injection. Long-term Fos expression in the emetic circuitry induced by cisplatin treatment might indicate a sustained level of activation of these nuclei that could contribute to nausea, malaise, and a reduced threshold for emesis, despite a period of little to no direct emetic behavior. In dogs, by 48 h after a bolus injection of cisplatin (3 mg/kg, ip) only about 40% of this chemical is recovered in urine, the primary method of elimination (35), therefore, the direct effects of cisplatin on physiology might persist for a significant period of time.

The present results also suggest that 5-HT\textsubscript{3} receptors play little role in activation of Fos expression 48 h after cisplatin treatment, because palonosetron did not affect emesis at this time point. This is in agreement with results of another study on delayed emesis in musk shrews in which injection of granisetron, another 5-HT\textsubscript{3} receptor antagonist, every 12 h also had no effects on emesis occurring from 24 to 72 h after cisplatin treatment (62). However, in the present study we administered palonosetron only once, 15 min before cisplatin injection, and it is unknown how long this agent remains active in musk shrews. An injection of palonosetron in humans has a half-life in plasma of 44 to 128 h (17).

The relative role of the GI vagal afferent fibers and the area postrema in acute and delayed cisplatin-induced emesis is not well defined. Palonosetron significantly reduced
cisplatin-induced Fos expression at 6 h in the NTS and there was a trend for the AP (Fig. 7), but had no effect on 48 h Fos expression after cisplatin treatment. It is not clear where palonosetron might act to produce this effect because 5-HT3 receptors are present in the NTS, AP, and on vagal afferents (peripherally and centrally) (36, 54). 5-HT3 receptor antagonism compared to vagotomy produces a greater reduction in cisplatin-induced emesis (4, 62). Vagotomy in musk shrews appears to only delay cisplatin-induced emesis for the first few hours but with no actual reduction in the total amount of emesis over 72 h (62). There appear to be no reports for the effects of AP ablation on cisplatin-induced delayed emesis, however, this method of study could be difficult to interpret because vagal afferent fibers also terminate in the AP (48, 53).

**Cisplatin-induced Fos expression in the PBN and the CeA**

Similar to the rat (65), in the musk shrew a substantial number of ascending fibers from the NTS and AP terminate in the PBN of the midbrain (31). Although there are no reports of PBN outputs in musk shrews, the PBN of the rat has projections to the CeA and other forebrain areas (32) and forms a critical relay for viscerosensory pathways from hindbrain to forebrain (65). Lesions of the PBN in the rat demonstrate a critical role for this brain area in the formation of conditioned taste aversion produced by toxins, including lithium chloride and cyclophosphamide (46). Cyclophosphamide, like cisplatin, is a chemotherapy agent that produces emesis by action on a 5-HT3 pathway from the gut (4). In the present study, cisplatin treatment in musk shrews induced Fos expression in the PBN at 24 and 48 h, indicating long-term activation of this nucleus.
The CeA is a highly differentiated nucleus that has afferent and efferent connections with cortices, PBN, hypothalamus, DVC, and other brain regions (3). In the present study, Fos expression in the CeA paralleled similar effects in the DVC after cisplatin treatment, and pretreatment with palonosetron inhibited Fos cell counts in both areas at 6 h after cisplatin treatment. Studies suggest that the CeA is an important integrator for feeding behavior, including conditioned flavor aversion (52), so is it difficult to say whether cisplatin-induced Fos expression in the CeA of musk shrews is a direct function of emetic behaviors or a generalized function of homeostatic and stress-related experience.

In summary, it is unknown why cisplatin induces Fos expression in the PBN and CeA. Presumably, areas rostral to the hindbrain emetic circuitry would not be needed for the emetic response to cisplatin treatment because cisplatin acts on a vagal pathway to produce emesis, at least acutely, and the caudal hindbrain is sufficient for generation of emetic output (4, 43). Activation of the PBN and CeA might be related to stress, disrupted gastrointestinal function, altered water intake, or decreased food intake that occurs with cisplatin treatment (10, 13, 71). Alternatively, these actions on PBN and CeA might represent a pathway for nausea, which likely involves a forebrain projection. Vomiting and nausea in humans can occur separately and likely engage neural systems that are at least partially separate (25, 64). Although it is very difficult to determine when laboratory animals are experiencing nausea, conditioned taste aversion has been used as a correlate of nausea in animal models (50). Importantly, lesions of the PBN or CeA disrupt conditioned taste aversion in rats (46, 52).

It would be highly informative to assess Fos expression in multiple forebrain regions of musk shrews in order to determine cisplatin responsiveness over time. However, a detailed
neuroanatomical atlas for *Suncus murinus* does not exist, making many regional and nucleus-specific analyses difficult. In the present study, we did observe Fos expression in the cortex and hypothalamus, but this labeling was not specific and also occurred in saline-treated control animals. As a treatment effect, amygdalar labeling was relatively easy to discern because it presented as a cluster of cells. We were also able to routinely see a clustering of Fos expressing cells in the BNSTv. Although this part of forebrain receives a dense projection from the NTS in the musk shrew (31) it did not show changes in Fos expression after cisplatin. This suggests that not all pathways from the NTS are activated by cisplatin treatment.

**Perspectives and significance**

This is the first study using a vomiting species to show activation of the hindbrain up to 48 h after cisplatin treatment, and also in brain regions rostral to hindbrain emetic circuitry. This time course parallels the acute and delayed phases of nausea and emesis in cancer patients and emesis in musk shrews after cisplatin treatment (34, 62). Cisplatin treatment stimulated Fos expression in brain areas known to play a role in emesis, which was inhibited by the antiemetic agent palonosetron, suggesting that at least part of the Fos expression induced by cisplatin treatment is related to activation of emetic pathways. It is unknown why cisplatin stimulated Fos expression in the PBN and CeA, because evidence indicates that cisplatin acts on a vagal pathway to induce emesis (4), at least acutely, and the hindbrain contains the circuitry to generate emesis (43). PBN and CeA activation by cisplatin might be related to nausea, stress, disrupted gastrointestinal function, or decreased food intake. The present findings suggest the existence of an integrated neural system that could be targeted to reduce chemotherapy-induced
nausea, malaise, and vomiting during acute and delayed phases in cancer patients. There is a host of current and investigational antiemetic drugs that should be evaluated to suppress the activity of this neural system (24).

ACKNOWLEDGMENTS

The authors thank Matthew Rosazza and Liz Still for expert assistance in collecting these data. The authors also thank Dr. Emilie Rissman at the University of Virginia for providing the initial stock animals to create a musk shrew colony. This work was supported by NIH grants DK065971 and DC000014.
Figure Legends

**Fig. 1.** Representative images from the *Suncus murinus* hindbrain showing the rostral extent of Fos expression induced by cisplatin treatment in the dorsal vagal complex. Areas analyzed for Fos expression included the NTS (nucleus of the solitary tract), AP (area postrema), and DMN (dorsal motor nucleus). Six hours after cisplatin (i.p., 30 mg/kg) treatment, Fos expression (left column) was increased at several levels (+250, +400, and +700 µm) rostral to the obex. The boundaries of hindbrain areas were determined by retrograde labeling of the DMN with Fluoro-Gold (center column) and cresyl violet staining of cytoarchitecture (right column).

**Fig. 2.** Representative images of hindbrain Fos expression at the level of the area postrema (AP; +250 µm rostral to the obex) 6, 24, and 48 h after intraperitoneal injection with saline or cisplatin (30 mg/kg). NTS = nucleus of the solitary tract, DMN = dorsal motor nucleus.

**Fig. 3.** Fos cell counts (mean ± SEM) in the hindbrain 6, 24, and 48 h after intraperitoneal injection with saline or cisplatin (30 mg/kg). DMN = dorsal motor nucleus, NTS = nucleus of the solitary tract, AP = area postrema, DVC (DMN + NTS + AP) = dorsal vagal complex. * P < 0.05, LSD test, cisplatin compared to saline injection.

**Fig. 4.** Representative images of midbrain and forebrain Fos expression at the parabrachial nucleus (PBN), central nucleus of the amygdala (CeA), and bed nucleus of the stria terminalis, ventral (BNSTv) 6 h after intraperitoneal injection with cisplatin (30 mg/kg). *ic = inferior
colliculus, 4th = fourth ventricle, 3rd = third ventricle, lv = lateral ventricle, ac = anterior commissure.

**Fig. 5.** Fos cell counts (mean ± SEM) in the parabrachial nucleus (PBN), central nucleus of the amygdala (CeA), and bed nucleus of the stria terminalis, ventral (BNSTv) 6, 24, and 48 h after intraperitoneal injection with saline or cisplatin (30 mg/kg). * P < 0.05, LSD test, cisplatin compared to saline injection.

**Fig. 6.** Representative images of hindbrain Fos expression in the reticular formation (oval areas) at the level of the area postrema (AP; +250 µm rostral to the obex, see Fig. 2) 6 h after intraperitoneal injection with saline, cisplatin (30 mg/kg), or cisplatin plus palonosetron (0.5 mg/kg). Palonosetron, a 5-HT3 receptor antagonist, was injected 15 min before injection with cisplatin. Images show only one half of the hindbrain and Fos expression in the reticular formation was present on both sides. The lower 1/3 of the region of interest likely includes the nucleus ambiguous and C1/A1.

**Fig. 7.** Fos cell counts (mean ± SEM) in the hindbrain 6 and 48 h after intraperitoneal injection with saline or cisplatin (30 mg/kg), with and without pretreatment with palonosetron (a 5-HT3 receptor antagonist). DMN = dorsal motor nucleus, NTS = nucleus of the solitary tract, AP = area postrema, DVC (DMN + NTS + AP) = dorsal vagal complex. * P < 0.05, LSD test, cisplatin or cisplatin plus palonosetron compared to saline injection. a P < 0.05, LSD test, cisplatin vs. cisplatin plus palonosetron.
Fig. 8. Fos cell counts (mean ± SEM) in the parabrachial nucleus (PBN), central nucleus of the amygdala (CeA), and bed nucleus of the stria terminalis, ventral (BNSTv) 6 and 48 h after intraperitoneal injection with saline or cisplatin (30 mg/kg), with or without palonosetron (a 5-HT3 receptor antagonist). * = p < 0.05, LSD test, cisplatin or cisplatin plus palonosetron compared to saline injection. a P < 0.05, LSD test, cisplatin vs. cisplatin plus palonosetron.
References


64. **Sanger GJ and Andrews PL.** Treatment of nausea and vomiting: gaps in our knowledge. *Auton Neurosci* 129: 3-16, 2006.


72. **Warr DG, Grunberg SM, Gralla RJ, Hesketh PJ, Roila F, Wit R, Carides AD, Taylor A, Evans JK, and Horgan KJ.** The oral NK(1) antagonist aprepitant for the

