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Cisplatin induces neuronal activation and increases central AMPA and NMDA receptor subunit gene expression in mice

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HIGHLIGHTS

- Cisplatin reduces food intake and body weight in mice dose-dependently.
- 1st study examining cisplatin-induced c-Fos expression in mice.
- 1st study to examine cisplatin-induced AMPAR and NMDAR subunit gene expression.
- We present evidence for a novel central circuit of cisplatin-induced nausea.

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ABSTRACT

Although rats and mice do not vomit, these species are widely studied as models of energy balance and sickness behavior. Previous work has shown that rats exhibit similar neuroanatomical activation of brain and visceral afferent pathways following cisplatin chemotherapy compared to vomiting species. However, the neural response to cisplatin in mice is understudied. Here, food intake, body weight, and central c-Fos immunofluorescence were analyzed in the hindbrains of male C57BL/6 mice following IP saline or cisplatin (5 mg/kg, and 20 mg/kg doses). As glutamate receptor signaling is classically linked to inhibitory feeding pathways in the rodent, gene expression of selected α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartic acid (NMDA) receptor subunits were assessed in the dorsal vagal complex (DVC), parabrachial nucleus (PBN), amygdala, and bed nucleus of the stria terminalis (BNST). Our results show dose-dependent reductions in food intake and body weight following cisplatin treatment, as well as increases in cisplatin-induced c-Fos in the PBN and throughout the DVC. Quantitative PCR analysis shows cisplatin-induced increases in NMDA receptor subunit expression, particularly NR2B, in the DVC, PBN, BNST, and amygdala. In addition, upregulation of AMPA receptor subunits (GluA1 and/or GluA2) were observed in all regions examined except the amygdala. Taken together, these results suggest similar neural pathways mediating cisplatin effects in mice compared to other well-studied species, which are likely mediated by central upregulation of AMPA and NMDA receptors.

1. Introduction

Cisplatin chemotherapy is perhaps the most potent and widely studied emetogenic antineoplastic drug [1,2], having been used in the treatment of solid tumors for nearly 35 years. Without anti-emetic medication, cisplatin produces severe nausea, vomiting, anorexia and cachexia in a variety of vomiting species including ferrets, pigs, dogs, cats, shrews, and humans [3–7]. Although rats and mice do not vomit, these species are widely studied as models for food intake regulation, energy balance, and cisplatin-induced anorexia/sickness behavior [8–10]. Previous reports have shown that rats exhibit similar neuroanatomical activation in brain areas and visceral afferent pathways compared to vomiting species [11–14]. On the other hand, mice have been relatively understudied for neuronal pathways of sickness behavior caused by emetic stimuli such as cisplatin [15,16]. As the overwhelming majority of animal models used in cancer research involve mouse models, it is essential to study the neural correlates of cancer- and/or chemotherapy-induced anorexia, nausea, and sickness in mice.

Cisplatin robustly stimulates the vagus nerve via serotonin (5-HT) release from enteroendocrine cells and subsequent binding to 5-HT_3 receptors on afferent terminals innervating the gut (for more detailed discussion, see Ref. [17]). In rats, these chemotherapy-induced vagal signals are then transmitted to the brain; first synapsing in the NTS in the caudal hindbrain [17]. Rats exhibit similar distributions and magnitude of hindbrain c-Fos activation following cisplatin injection when compared to vomiting species [18,19]. Here, we are the first to examine c-Fos immunofluorescence in mice to test the hypothesis that cisplatin

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produces similar hindbrain neuronal activation in mice compared to other, more well-studied animal models. Although c-Fos quantification is a useful tool to identify relevant nuclei activated by cisplatin, it is also essential to ascertain which signaling pathways may be mediating anorectic and noxious behavioral effects of cisplatin.

It is well-established that vagal afferent projections to the nucleus of the solitary tract (NTS) are primarily glutamatergic [20,21]. Furthermore, presynaptic 5-HT_3 receptor activation in the gut, which occurs following cisplatin treatment, can facilitate glutamate release and synaptic input to the AP, NTS, and dorsal motor nucleus (DMN) neurons, likely leading to activation of AMPA or NMDA receptors within the hindbrain [22–24]. These studies suggest that cisplatin-induced vagal stimulation may trigger central glutamate receptor signaling which could contribute to anorexia and emetic-like behaviors in mice.

Therefore, the aims of the following studies are 1) to examine whether cisplatin treatment in mice produces anorexia, body weight suppression, and hindbrain neuronal activation similar to more widely studied animal models of nausea and vomiting, and 2) to assess whether glutamate receptor gene expression (i.e., AMPA and NMDA receptor subunits) in areas of the hindbrain and forebrain implicated in the control of feeding and emetic behaviors is altered following cisplatin treatment in mice.

2. Materials and methods

2.1. Animals

Thirty, 3-month old male C57BL/6J mice (The Jackson Laboratory) were used for experiments. Mice were individually housed in plastic bins in a temperature and humidity controlled room maintained on a 12-h light/12-h dark cycle (lights on at 0900 h). Animals were maintained ad libitum on pelleted chow (Lab Diet 5001) and water. All protocols and procedures were approved by the institutional care and use committee (IACUC, University of Pennsylvania).

2.2. Experiment 1: Food intake and body weight measurements following intraperitoneal (IP) cisplatin in mice

Mice were randomly assigned to one of three conditions and received IP injections of saline (0.15 M NaCl: 4 ml/kg, n = 10, BW = 23.5 g ± 1.3) or cisplatin (5 mg/kg, n = 10, BW = 22.9 g ± 1.0; 20 mg/kg, n = 10, BW = 23.1 g ± 1.1) in a weight-matched, between subjects design. All animals were injected between 0900 h–1030 h. Food intake and body weight measurements were taken as previously described [25] immediately prior to injections and 24 h post-injection. Doses of cisplatin were chosen based on previously published cisplatin studies in mice as well as our experiments in the house musk shrew *Suncus murinus* (i.e., vomiting animals of similar size to laboratory mice) [15,26]. Cisplatin (Sigma-Aldrich; *cis*-diamineplatium dichloride, no. P4394) was dissolved in saline (0.15 M NaCl), sonicated until clear, and vortexed prior to injection. Following 24 h measurements, all mice were euthanized as described below to facilitate Experiments 2 and 3.

2.3. Experiment 2: Cisplatin-induced hindbrain c-Fos immunofluorescence in mice

Twenty-four hours after cisplatin injection, half of the mice from experiment 1 (n = 5/condition) were deeply anesthetized via intraperitoneal ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg), and transcardially perfused with 10 ml of 0.2 M phosphate-buffered saline (PBS; pH 7.4), followed by 10 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (7.4) [11]. The 24 hour time point was chosen based on near maximal c-Fos expression observed in the rat and musk shrew in previous published data [12,18]. Whole brains were removed and placed in 10% sucrose-PBS, followed by 20% and 30% sucrose-PBS, each overnight. After sucrose incubation, brains were cut at 30 μm with a cryostat (Leica 3050 s). We collected sections from four locations based previous work in both the rat and the musk shrew [12,18]: 1) the caudal hindbrain [dorsal vagal complex (DVC), including NTS, and AP], 2) PBN.

Sections were processed for immunofluorescence using a modified procedure [18] (n = 5/condition). We include here only the important modifications. Sections were incubated at room temperature with a polyclonal goat anti-Fos primary antibody (1:2000, sc-52G, Santa Cruz Biotechnology, Santa Cruz, CA; lot no. B2856) containing 2% normal donkey serum for 20 h. Sections were then incubated in donkey Anti-Goat AlexaFluor 594 secondary antibody (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. Sections were mounted onto microscope slides and cover slipped with Vectastain Hard Set Mounting Medium and viewed with a fluorescence microscope (Olympus BX60). Images were captured (Nikon DS-U3) and quantified by two authors working independently and blind to experimental conditions; the average of these two assessments is reported here. Counts for three coronal brain sections per animal corresponding to each coordinate examined were averaged for inclusion in statistical analysis.

2.4. Experiment 3: AMPA and NMDA receptor subunit expression in the amygdala, BNST, PBN, and NTS-enriched DVC regions of cisplatin-treated mice

Half of the mice from experiment 1 (n = 5/condition) were euthanized immediately following 24 h food intake and body weight measurements via CO_2 asphyxiation. Whole brains were collected and flash frozen in liquid nitrogen according to procedures previously published [27]. Bilateral micropunches of BNST, amygdala, NTS-enriched DVC and PBN were collected according to previously published methods [28]. The targeted micropunches for each tissue corresponded to the following starting coordinates, with punches extending rostrally, based on [29] (also see Figs. 4A and C, 5A and C): DVC-enriched hindbrain (B: −7.76 mm, punch depth 1.0 mm), PBN (B: −5.52 mm, punch depth 0.5 mm) and BNST (B: −0.10 mm, punch depth 0.5 mm) and amygdala (B: −1.58 mm, punch depth 0.75 mm). Regions were chosen based on previous reports [12,18] and our a priori hypothesis implicating these areas in cisplatin-induced neuronal activation.

Briefly, total RNA was extracted from micropunches using TRIzol (Invitrogen) and the RNeasy kit (Qiagen). cDNA was synthesized from 0.5 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan gene expression kits and PCR reagents from Applied Biosystems were used to quantify relative mRNA levels of AMPA receptor subunits GluA1 (Gria1; Mm00433753_m1), GluA2 (Gria2; Mm00442822_m1), and NMDA receptor subunits NR1 (Grin1; Mm00626390_m1), NR2A (Grin2A; Mm00433802_m1), NR2B (Grin2b-Mm00433820_m1) by quantitative real-time PCR. Mouse β-actin (VIC-MGB, #4352341E Applied Biosystems) was used as an internal control. Samples were analyzed using the Eppendorf Mastercycler® ep realplex2. Relative mRNA expression was calculated using the comparative Ct method as described previously [30].

2.5. Statistics

All data are expressed as means ± SEM. For all experiments, one way ANOVAs were performed to evaluate group differences using drug treatment (cisplatin) as a main effect for food intake, body weight change, c-Fos immunofluorescence, and real time PCR. Planned comparisons were conducted with least significant difference (LSD) tests. Statistical differences between mean values were calculated using SAS 9.2 (SAS Institute Inc., Cary, NC).