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Characterizing a new tool to manipulate area postrema GLP1R+ neurons across species

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Abstract

Nausea is an uncomfortable sensation that accompanies many therapeutics, especially diabetes treatments involving glucagon-like peptide-1 receptor (GLP1R) agonists. Recent studies in mice have revealed that GLP1R-expressing neurons in the area postrema play critical roles in nausea. Here, we characterized a ligand-conjugated saporin that can efficiently ablate GLP1R$^+$ cells from humans, mice, and the *Suncus murinus*, a small animal model capable of emesis. This new tool provides a strategy to manipulate specific neural pathways in the area postrema in the *Suncus murinus* and may help elucidate roles of area postrema GLP1R$^+$ neurons in emesis during therapeutics involving GLP1R agonists.

Keywords

nausea; GLP1R; emesis; area postrema; shrew

1. Introduction

Nausea is an unpleasant and distressing sensation that can significantly impact a person's quality of life. It is a common side effect of many medications, including chemotherapy drugs, opioids, and certain chronic disease treatments (Andrews 1992, Miller & Leslie 1994). Notably, nausea and emesis are among the top adverse side effects of glucagon-like peptide-1 (GLP1) receptor (GLP1R) agonists-based medications to treat type 2 diabetes and obesity (Wadden et al 2021, Wilding et al 2021). These medications act by mimicking the action of GLP1, a hormone that regulates blood glucose levels and can also influence appetite. Despite the impressive body weight loss in clinical trials, nausea and vomiting

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Author contributions

CZ designed the study, analyzed data, and wrote the manuscript; SF performed shrew behavioral experiments with the guidance of CCH; CZ performed other experiments.

Declaration of interests

None.

are prevalent, with up to 60% of participants reporting this side effect (Wadden et al 2021, Wilding et al 2021). Understanding and managing nausea is crucial to improving patient medication adherence and overall treatment outcomes.

The area postrema is a brain structure that mediates nausea effects to several visceral threats (Borison 1989, Miller & Leslie 1994, Price, Hoyda & Ferguson 2008, Ritter, McGlone & Kelley 1980). Neurons in the area postrema are anatomically privileged to sample circulating hormones and other chemicals in the periphery, as the area postrema has a reduced blood-brain-barrier (Borison 1989). Single nucleus RNA sequencing approaches recently provided a cell atlas of the area postrema, revealing excitatory neuron types that express multiple receptors for nausea-inducing stimuli, including GLP1R, the growth/differentiation factor 15 (GDF15) receptor (GFRAL), and the calcium sensing receptor (CaSR) (Zhang et al 2021). In mice, which are incapable of vomiting, chemogenetic activation of area postrema GLP1R$^+$ neurons promotes a characteristic behavioral response termed conditioned flavor avoidance, in which a novel flavor paired with visceral malaise cues causes future avoidance of that flavor (Zhang et al 2021). Area postrema GLP1R$^+$ neuron ablation eliminates avoidance imposed by GLP1R agonists, as well as several visceral poisons (Andrews 1992, Patel et al 2019, Sabatini et al 2021, Zhang et al 2021). However, it is unclear whether the same neuron type modulates nausea and emesis behaviors.

Suncus murinus (house musk shrew), has been used as an animal model in nausea research due to its ability to show emesis behaviors (vomiting) in response to nausea stimuli. This small mammal exhibits sensitivity to emetic agents (Borner et al 2020, Chan et al 2013, Horn et al 2014, Horn et al 2012, Hu et al 2003, Sam et al 2003) and motion (Horn, Meyers & Oberlies 2014), and has gastrointestinal features similar to the human gastrointestinal system (Tsutsui et al 2009), making it a great tool for studying the physiological and neural mechanisms of nausea and vomiting. However, genetic manipulations and tools to specifically target neural pathways in the house musk shrew are lacking, making it challenging to investigate the roles of defined cell types in nausea and emesis. Hence, a major goal of this study is to develop and characterize new tools that allow specific manipulation of neural pathways in the house musk shrew for nausea investigations.

Here, we characterize a GLP1R agonist conjugated saporin toxin that can be used to specifically ablate GLP1R-expressing human-derived cells *in vitro*, and GLP1R-expressing area postrema neurons in mice and house musk shrews *in vivo*. Neuron-ablated house musk shrews recovered fully and could undergo behavioral experiments involving feeding and emesis measurements. This study examines a new tool to manipulate defined neural pathways in the house musk shrew and provides proof of principle characterization of area postrema GLP1R$^+$ neurons in nausea responses to GLP1R agonists.

2. Material and Methods

2.1. Animals

All animal husbandry and procedures were performed in compliance with institutional animal care and use committee guidelines. All animal husbandry and procedures

followed the ethical guidelines outlined in the NIH Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf), and all protocols were approved by the institutional animal care and use committee (IACUC) at Harvard Medical School (mice) and University of Pittsburgh (shrews). Wild-type C57BL/6J (000664) mice were purchased (Jackson Laboratory). In total, 9 experimental naive C57BL/6J were used (5 virgin male and 4 virgin female mice between 8–24 weeks old, 20–30g body weight), and no differences based on sex were observed. Mice of the same sex were housed in groups and fed a 5053 LabDiet chow, with free access to food and water. Mice were maintained on a 12-h light/12-h dark cycle (lights on at 0700 h). In total, 19 experimentally naive male musk shrews were used (2–17 month of age, 43–72 g body weight); shrews were offspring from breeding stock obtained from the Chinese University of Hong Kong (a strain originating from Taiwan; (Wang 1994)). Animals were housed individually and fed a mixture of 75% Purina Cat Chow Complete Formula and 25% Complete Gro-Fur mink food pellets (Milk Specialty, New Holstein, WI; (Temple 2004)). Musk shrews were maintained on a 12-h light/12-h dark cycle (lights on at 0700 h), with free access to food and water.

2.2. Cell culture and cytotoxicity assay

HEK293T cells were cultured in medium [DMEM (ThermoFisher 11995073) with 10% fetal bovine serum (VWR, FBS-01–0500), and Penicillin-Streptomycin (ThermoFisher 15140122)]. Cells were plated on 96-well plates and transfected (overnight, 37°C) with a viral vector encoding human GLP1R (Montana Molecular Z0600N). Cells were also tested for transfection efficiency in separate plates with identical construct as human GLP1R, but encoding GFP instead (mNeonGreen CMV kit, F0500G, Montana Molecular), which showed over 95% transfection efficiency.

Cytotoxicity assay was conducted as described in the sample protocol from Advanced Targeting Systems. Briefly, Ex4-SAP and control SAP (Advanced Targeting Systems, IT-90 and IT-27B, respectively) series dilutions were prepared in HEK293T culture medium in four replicates and added in the morning after transfection. The plate was incubated for 72 hours in normal cell culture conditions. XTT cell proliferation assay (ATCC, 30–1011K) was used according to manufacturer instructions to test for viable cells remaining on the day of development.

2.3. Stereotaxic brain injections

Mice were not fasted before surgery. Mice were anesthetized with avertin. The dorsal neck surface was shaved, and the skin sterilized with betadine surgical scrub and 70% isopropyl alcohol and placed on a stereotaxic frame (David Kopf Instruments) with heads facing down ~45°. The fourth ventricle and area postrema were surgically exposed after removal of the meninges, and a Nanoject III Injector (Drummond) was positioned directly into the area postrema for injections of Ex4-SAP or SAP (200 ng/μl, 400 nl, 2 nl/second). All injection mixtures contained 10% cholera toxin subunit B, Alexa Fluor 647 conjugate (ThermoFisher, C34778) to mark injection sites. Animals recovered for 2 weeks for brain tissue collection and *in situ* hybridization. We injected SAP in a total of 3 mice (2 males, 1 female) and Ex4-SAP in 6 (3 males, 3 females) mice.

Shrews were fasted for 2 hours before being anesthetized. Shrew were anesthetized with isoflurane (2 to 3%) using an induction chamber followed by a nose cone during surgery. The dorsal neck surface was shaved, and the skin sterilized with betadine surgical scrub and 70% isopropyl alcohol, and then placed on a stereotaxic frame (David Kopf Instruments) with heads facing down ~45°. The fourth ventricle and area postrema were surgically exposed after removal of the meninges, and a Nanoject III Injector (Drummond) was positioned directly into the area postrema for injections of Ex4-SAP or SAP (500 ng/μl, 200 nl, 2 nl/second). All injection mixtures contained 10% cholera toxin subunit B, Alexa Fluor 647 conjugate (ThermoFisher, C34778) to mark injection sites. The dorsal neck was sutured (4–0 silk; Ethicon), and the skin was closed with surgical staples (7.5 × 1.75 mm, Michel). Animals were weighed daily after surgery to assess body weight changes and were allowed to recover for 10 days before behavioral testing. For 2 days after surgery, animals received the analgesic ketoprofen (Sigma-Aldrich; 2 mg/kg sc, twice daily). We injected SAP in a total of 5 shrews and Ex4-SAP in 14 shrews. Of the 14 Ex4-SAP injected shrews, 3 were discarded due to damaged brainstem tissues for *post hoc* analysis. Of the remaining 11 shrews, 8 showed Alexa Fluor 647 cholera toxin conjugate expressions in either the cerebellum or hypoglossal nucleus (GLP1R has little expression in the cerebellum and hypoglossal nucleus), while 3 showed expressions in at least the area postrema with possible nearby NTS. These 8 off-targeted shrews showed wild-type-like GLP1R expression in the area postrema. Hence, the 8 off-targeted shrews were combined with the 5 SAP-injected shrews as control animals for data analysis.

2.4. Analysis of RNA expression

For detecting mouse *Glp1r* and *Calcr*, hybridization chain reaction (HCR) RNA *in situ* hybridization was performed on cryosections of unfixed brain (25 μm) following the HCR-FISH 3.0 protocol (Choi et al 2018, Choi, Schwarzkopf & Pierce 2020). *Glp1r* probe, *Calcr* probe, probe hybridization buffer, probe wash buffer, amplification buffer, and fluorescent HCR hairpins were purchased from Molecular Instruments (Los Angeles, USA). *Glp1r* probe was designed to be associated with the B1 initiator sequence and detected by hairpins labeled with amplifier 488. *Calcr* probe was designed to be associated with the B3 initiator sequence and detected by hairpins labeled with amplifier 594. Fluorescent images were analyzed with a Leica SP5 II confocal microscope.

For detecting shrew *Glp1r*, colorimetric *in situ* hybridization was performed on coronal brain (25 μm) cryosections of unfixed tissue involving digoxigenin-conjugated cRNA riboprobes, alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche 11093274910) and staining with NBT/BCIP (Thermo Scientific 34042), as described previously with minor modifications. High-stringency washes used 5X SSC (2×5 minutes, 70°C, VWR 45001–046) and 0.2X SSC (2×30 minutes, 70°C) and subsequent washes and antibody incubations used PBS buffer with 0.05% Tween-20. Phosphatase and peroxidase reporter reactions were performed according to manufacturer's protocols. Colorimetric images were analyzed with a Nikon Ti2 Inverted microscope. cRNA probes were synthesized with digoxigenin-conjugated (Roche 11277073910) dNTPs and transcribed *in vitro* following manufacturer's protocols (MegaScript T7 or T3 Kits, Invitrogen AM1334 or AM1338). Probes (listed 5' to 3') were amplified using the following primer pairs: shrew *Glp1r* (1392 bp)

TGACGACTATGCTTGCTGGC and CGTCCATCACGAAAGCGAAG. The design of the probe was based on *Sorex Araneus Glp1r* gene.

2.5. Behavioral assays

For the exendin-4 (Tocris #1933) dose test, four emetic tests (0 or saline, 0.06, 0.3, 3 mg/kg) were conducted with 1 week between each test. The order of exposure was randomly determined for each shrew. For the ablation test, only exendin-4 at 3 mg/kg was used for all shrews. All tests were conducted 2 hours after the light cycle started. Animals were not fasted before the experiments. Prior habituation to the testing chamber was not conducted before the first test. After exendin-4 intraperitoneal injections, shrews were transferred to the testing chamber (19 × 27 cm, width and length) for 120 min. Animal behavior was recorded using digital video cameras (Sony DCR-SR300 or HDR-XR550V, wide field lenses) positioned above the testing chambers. While in the chambers, animals were observed and recorded for emetic episodes using keystrokes on a laptop computer running JWatcher software (http://www.jwatcher.ucla.edu) (Blumstein & Daniel 2007, Horn et al 2013). Emetic episodes in musk shrews occur as a series of closely spaced retches, which end with an expulsion phase. A retch = an abdominal contraction with a rostral movement of the body while standing in place; an emetic episode = ~ 6 retches plus a larger expulsion movement with ~ 0.2 s between each retch or movement (Huang et al 2011). Emetic episodes with and without vomiting (expulsion) were recorded. For food consumption, a small bowl containing shrew chow (details above) was placed inside each testing chamber. For water consumption, a sipper tube containing 20 mL water was placed inside each testing chamber. Shrews could freely access both food and water. Total food and water (including containers) were weighed and measured in grams before shrews entered the testing chamber and after they left the testing chambers. After all behavioral tests, animals were sacrificed for brain tissue collection and *in situ* hybridization.

2.6. Data analysis

Sample sizes from left to right: Figure 1C (N=6, 11, 16, 19 sections from 3 (SAP) or 6 (Ex4-SAP) mice, Figure 2B (N=25, 11 sections from 13 (SAP) or 3 (Ex4-SAP) shrews, Figure 3A (N= 6, 3, 3, 3 shrews), Figure 3B (N=13, 3 shrews).

In cytotoxicity experiments, cytotoxicity data are analyzed by comparing well readings of the treated wells to those of the control wells, expressed as a percentage. The darkness of color in the untreated wells is considered to be 100% survival. Statistical significance was measured on Prism 9 software (Graphpad) using an ordinary one-way ANOVA test with Tukey's multiple comparisons test (Figure 1C), and an unpaired t-test (Figure 2B, 3B).

3. Results

3.1. Ex4-SAP as a tool to ablate GLP1R-expressing cells in a human cell line and in mice

First, we obtained exendin-4 conjugated saporin toxins (Ex4-SAP) and characterized its efficiency in ablating GLP1R-expressing cells. Exendin-4 binds to many mammalian GLP1Rs, including humans and rodents, making it possible to target $GLP1R^+$ cells across species. Saporin is a ribosome-inactivating protein that requires cell surface binding to

be internalized. As a result, Ex4-SAP can be specifically internalized by cells expressing GLP1R and induce cell death. The cytotoxicity of Ex4-SAP partly depends on receptor internalization, which has been reported for GLP1R. Saporin conjugated to a shuffled peptide that does not recognize cell surface receptors was used as a control for non-specific toxicity (termed SAP in the paper). We first used HEK293T cells expressing human GLP1R to test the cytotoxic effect of Ex4-SAP *in vitro*. In HEK293T cells that did not express GLP1R, both SAP and Ex4-SAP showed comparable levels of toxicity (EC_{50} = 9.4 nM and 11 nM, respectively), likely due to fluid-phase endocytosis. In HEK293T cells that expressed GLP1R, it required 100-fold less Ex4-SAP (EC_{50} = 0.068 nM) compared to the non-targeted control SAP (EC_{50} = 2.3 nM) to effectively kill cells (Figure 1A). So, Ex4-SAP demonstrates specific cytotoxic effects in cells expressing GLP1R.

To test the functions of Ex4-SAP *in vivo*, we first injected Ex4-SAP and control SAP into the mouse area postrema using a previously modified stereotaxic injection approach (Zhang et al 2021). To examine the efficiency of area postrema $GLP1R^+$ neuron ablation, we used *in situ* hybridization to detect *Glp1r* mRNA levels. In addition, to control for ablation specificity, we detected another non-overlapping neuronal marker *Calcr* mRNA, as these neurons do not express GLP1R. While area postrema *Glp1r* and *Calcr* expression was not affected by SAP injection, *Glp1r* expression was significantly reduced in mice injected with Ex4-SAP. However, Ex4-SAP injection did not affect *Calcr* expression in the area postrema, suggesting specific ablation of the $GLP1R^+$ but not $CALCR^+$ neurons (Figure 1B and C). Together, we conclude that Ex4-SAP can effectively and specifically ablate GLP1R-expressing cells *in vitro* in a human cell line and *in vivo* in mice.

3.2. Ex4-SAP as a tool to ablate shrew area postrema $GLP1R^+$ neurons

One of the goals of characterizing Ex4-SAP is to test whether it can specifically ablate defined neural types in an emetic animal model, for example, the house musk shrew, to study the roles of $GLP1R^+$ neurons in emesis. Hence, we tested the ability of Ex4-SAP to ablate shrew area postrema $GLP1R^+$ neurons *in vivo*. We injected Ex4-SAP and control SAP into the shrew area postrema based on the modified stereotaxic injection (Figure 2A) (Zhang et al 2021). A fluorescent dye was added to the injection mixtures to mark injection sites for *post hoc* analysis. To assess ablation efficiency, we cloned a shrew *Glp1r* cRNA probe and used *in situ* hybridization to detect *Glp1r* mRNA expression. Shrews with Ex4-SAP targeted in the area postrema showed significant loss of $GLP1R^+$ neurons as indicated by loss of *Glp1r* mRNA expression, compared with control SAP injected shrews (Figure 2B–C). We also observed intact area postrema *Glp1r* mRNA expression in shrews where the fluorescent dye-marked injection sites were off-targeted in the cerebellum or other regions of the brainstem, such as the hypoglossal nucleus (8/11 shrews, more details in methods). In short, Ex4-SAP can mediate ablation of shrew $GLP1R^+$ neurons *in vivo*.

In the previously published mouse study on GLP1R agonist-induced nausea, loss of area postrema $GLP1R^+$ neurons abolished conditioned flavor avoidance while preserving the feeding suppression imposed by exendin-4 (Zhang et al 2021). In shrews, exendin-4 induced a dose-dependent emetic response, including retching and vomiting and suppression of acute food intake (Figure 3A), consistent with previous observations (Chan et al 2013). As

proof of principle, we tested whether loss of area postrema GLP1R$^+$ neurons affected the nausea and emesis responses caused by exendin-4 in shrews. Shrews with either SAP or Ex4-SAP injections recovered fully and showed no abnormal activities. We tested emesis and feeding responses in these shrews after full recovery. Loss of area postrema GLP1R$^+$ neurons resulted in a significantly delayed onset of emesis behaviors (49.0 ± 15.4min vs. 104.7 ± 13.6min, $p<0.0001$) and overall reduced total emetic episodes, including retching and vomiting (11.9 ± 6.9 vs. 3.7 ± 3.5, $p=0.0698$) (Figure 3B). Despite the reduced nausea, both ablate and non-ablate shrews exhibited reduced food intake 2 hours after exendin-4 injections (0.08 ± 0.13g vs 0±0g, $p=0.45$; compared to saline 0.57±0.7g) (Figure 3A–B), suggesting that likely additional GLP1R$^+$ neuron types mediate the feeding suppression imposed by exendin-4. While we lacked power in this proof of principle feeding and emesis tests to conclude the roles of GLP1R$^+$ area postrema neurons in nausea and feeding, we demonstrated that Ex4-SAP provided a specific method to manipulate the area postrema GLP1R$^+$ neurons that could be used to address related behavioral functional questions.

4. Discussion

Nausea is one of the top adverse side effects of GLP1R agonists-based therapeutics, which are widely used to treat type 2 diabetes and obesity. Understanding how nausea is induced is critical to help develop better treatments and improve patient care. While genetic studies in mice have revealed fundamental area postrema neural mechanisms that contribute to nausea-associated behaviors, emetic animal models, including the house musk shrew, offer additional advantages and critical insights into nausea physiology. Tools that allow specific ablation or manipulation of neural pathways in non-model organisms are very limited. Classical nausea-related studies in shrews using the lesion approach can result in damage to the nearby nuclei or passing afferent fibers, which can confound interpretations. Hence, there is an important need to develop better strategies and tools for manipulating defined neural pathways for functional analysis in the shrews. Here we characterized the Ex4-SAP as a new tool to manipulate GLP1R-expressing neurons *in vitro* in a human cell line, *in vivo* in mice, and *in vivo* in house musk shrews. We showed proof of principle emesis and feeding behavioral analysis after the loss of area postrema GLP1R$^+$ neurons in the shrews. While we focused our characterization on the GLP1R$^+$ cell type in this study, saporins could be conjugated to other receptor ligands to target defined cell types, providing powerful strategies to manipulate distinct neural populations in organisms that lack genetic tools.

A widely used ablation approach in mice involves diphtheria toxin (DT) and *Cre*-guided expression of the DT receptor (DTR) (Buch et al 2005), which can often achieve highly specific and efficient ablation (often >95% reduction). Compared to this approach, Ex4-SAP achieves ~60% reduction in mice (Figure 1C) and ~90% reduction in shrews (Figure 2B). Incomplete ablation may contribute to the remaining emetic events after exendin-4 injection that we observed in shrews, or alternatively, other cell types may be involved. To make meaningful conclusions, a large cohort of animals needs to be involved to increase the statistical power considering the ablation efficiency in future works. Ex4-SAP may also bind to axon terminals if GLP1R is present and ablate neurons that project to the injection site. Careful analysis of ablation of the nodose ganglia and hypothalamus needs to be done for area postrema injections to eliminate possible off-target events. The Ex4-SAP tool could be

used to address important questions, for example, whether partially distinct neural circuits are responsible for the appetite suppression and nausea effects of exendin-4 mediated by the area postrema GLP1R neurons. Dissociating the desirable therapeutic effects from the negative nausea effects of GLP1R agonist-based therapeutics can potentially provide a mechanistic foundation to optimize drug development.

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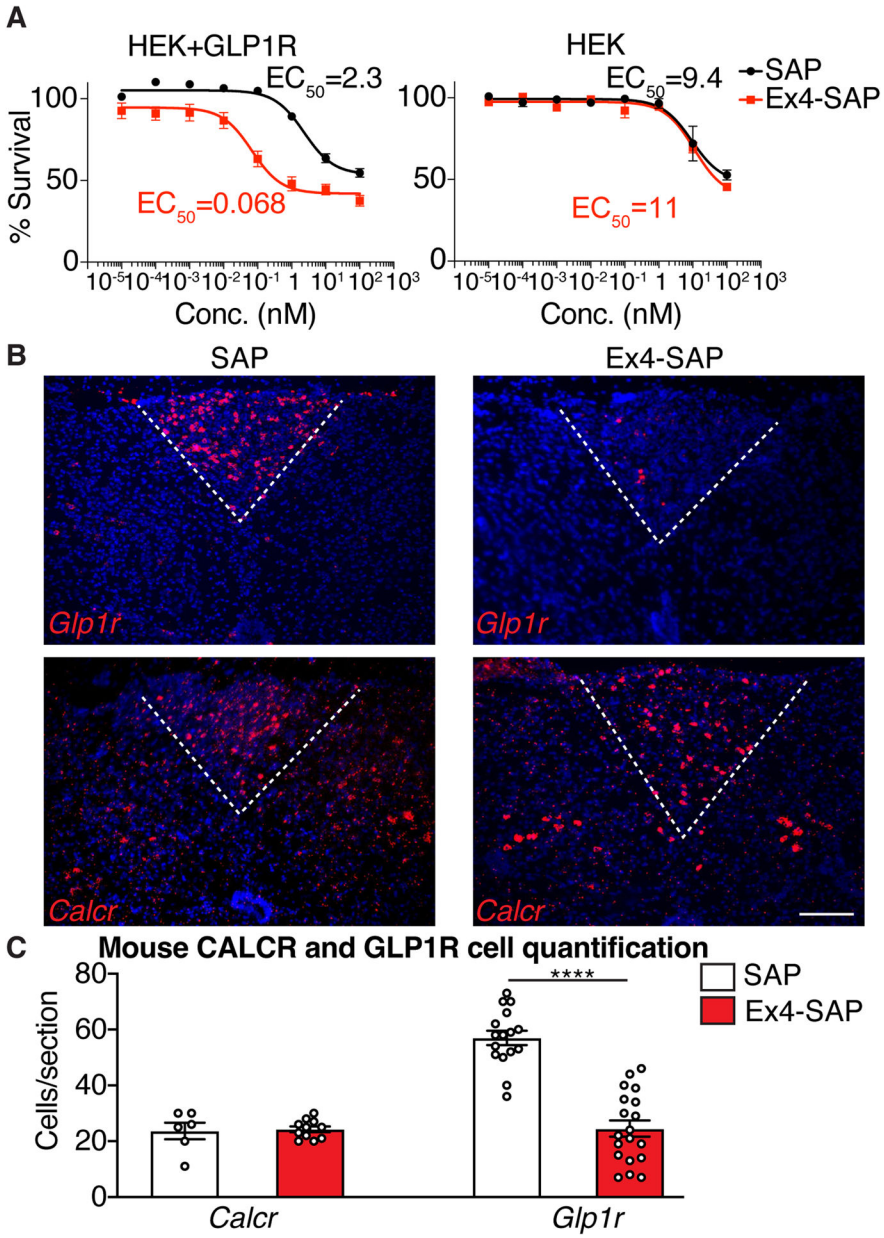

Figure 1. Ex4-SAP demonstrates cytotoxicity *in vitro* in a human cell line and *in vivo* in mice.

(A) Cytotoxicity assay of Ex4-SAP and control SAP in HEK293T cells transfected with human GLP1R versus HEK293T cells without transfection. (B) Fluorescent RNA *in situ* hybridization for *Glp1r* and *Calcr* in SAP (200 ng/μl) or Ex4-SAP (200 ng/μl) injected wildtype mouse area postrema coronal brainstem cryosection (25 μm), scale bar: 100 μm. (C) Quantification of area postrema cells displaying fluorescence signals per coronal brainstem cryosection (25 μm), N=6, 11, 16, 19 sections from 3 (SAP) or 6 (Ex4-SAP) mice (from left to right), mean ± sem, circles: individual section, ordinary one-way ANOVA test with Tukey's multiple comparisons test, ****p<.0001.

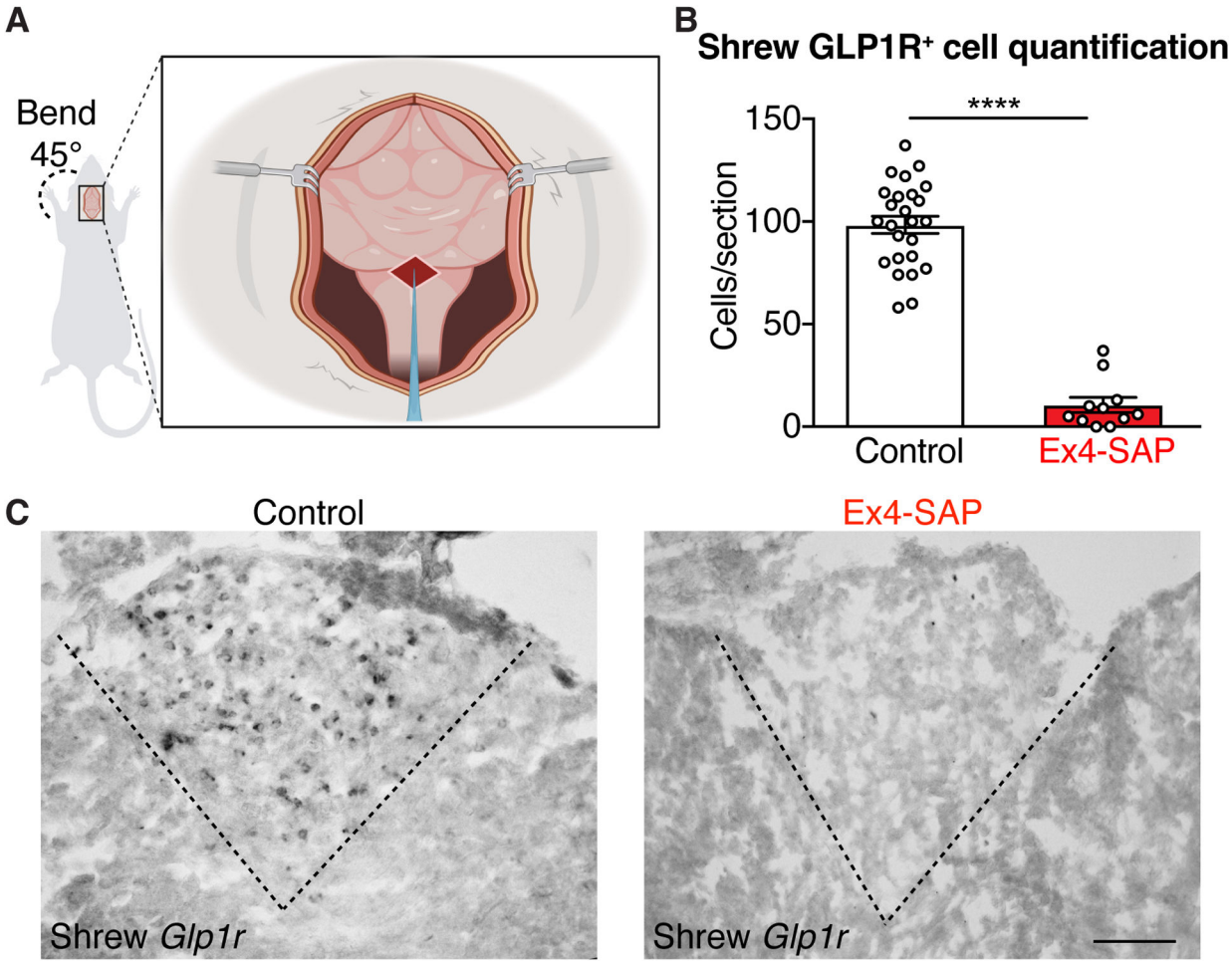

Figure 2. Ex4-SAP demonstrates cytotoxicity *in vivo* in shrews.

(A) Cartoon demonstrating a modified stereotaxic injection into the shrew area postrema, which involves bending the head at 45 degrees and surgically exposing the area postrema for Nanoject needle injection. (B) Quantification of area postrema cells displaying colorimetric signals per coronal brainstem cryosection (25 μm), N=25, 11 sections from 13 (SAP) or 3 (Ex4-SAP) shrews (from left to right), mean ± sem, circles: individual section, unpaired t-test, ****p<.0001. (C) Colorimetric RNA *in situ* hybridization for *Glp1r* in SAP (500 ng/μl) or Ex4-SAP (500 ng/μl) injected shrew area postrema coronal brainstem cryosection (25 μm), scale bar: 100 μm.

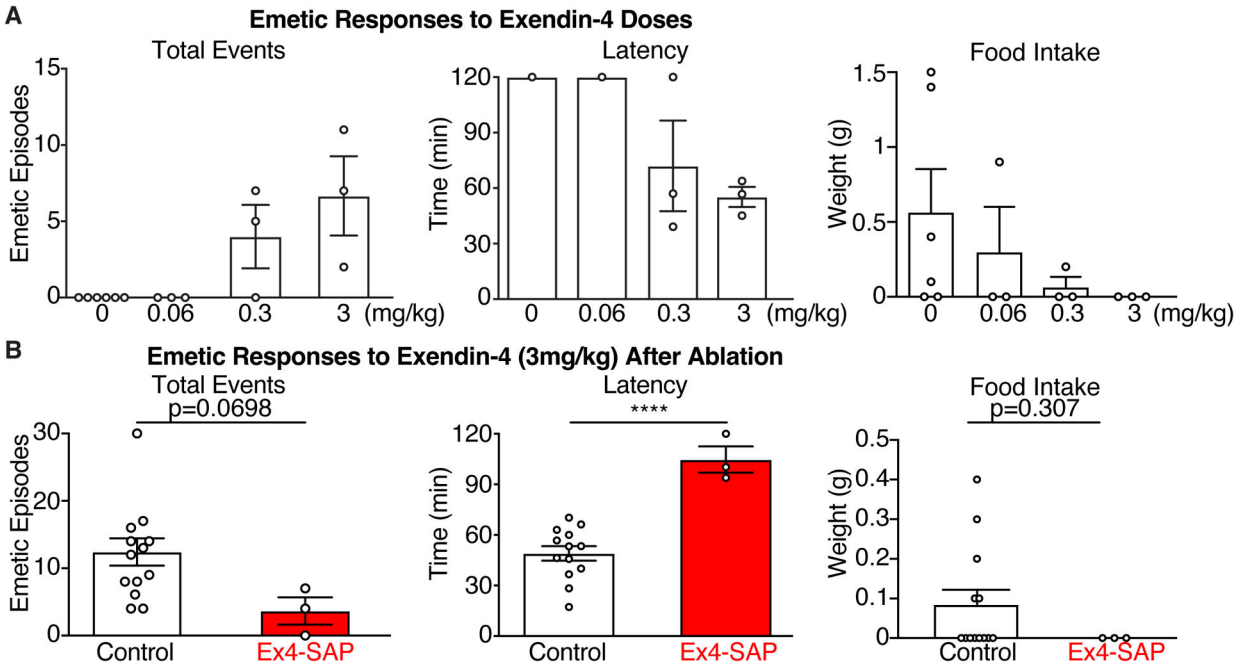

Figure 3. Emetic and feeding responses after ablating shrew area postrema GLP1R^{+} neurons

(A) Behavioral characterization of exendin-4 injection (2 hours) at different doses (0, 0.06, 0.3, and 3 mg/kg) in shrews, including total emetic episodes (retching and vomiting), latency to the first emetic episode, and food intake. Number of shrews from left to right N= 6, 3, 3, 3; mean ± sem, circles: individual shrew. (B) Behavioral characterization of exendin-4 injection (2 hours) at 3 mg/kg in shrews that have received SAP or Ex4-SAP area postrema injections 10 days earlier, including total emetic episodes (retching and vomiting), latency to the first emetic episode, and food intake. Number of shrews from left to right: N=13, 3; mean ± sem, circles: individual shrew, unpaired t-test, ***p<0.001.