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Pink1−/− rats demonstrate swallowing and gastrointestinal dysfunction in a model of prodromal Parkinson disease

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Abstract

Early motor and non-motor signs of Parkinson disease (PD) include dysphagia, gastrointestinal dysmotility, and constipation. However, because these often manifest prior to formal diagnosis, the study of PD-related swallow and GI dysfunction in early stages is difficult. To overcome this limitation, we used the *Pink1−/−* rat, a well-established early-onset genetic rat model of PD to assay swallowing and GI motility deficits. Thirty male rats were tested at 4 months (*Pink1−/−* = 15, wildtype (WT) control = 15) and 6 months (*Pink1−/−* = 7, WT = 6) of age; analogous to early-stage PD in humans. Videofluoroscopy of rats ingesting a peanut-butterbarium mixture was used to measure mastication rate and oropharyngeal and pharyngoesophageal bolus speeds. Abnormal swallowing behaviors were also quantified. A second experiment tracked barium contents through the stomach, small intestine, caecum, and colon at hours 0–6 post-barium gavage. Number and weight of fecal emissions over 24-hours were also collected. Compared to WTs, *Pink1−/−* rats showed slower mastication rates, slower pharyngoesophageal bolus speeds, and more abnormal swallowing behaviors. *Pink1−/−* rats demonstrated significantly delayed motility through the caecum and colon. *Pink1−/−* rats also had significantly lower fecal pellet count and higher fecal pellet weight after 24 hours at 6 months of age. Results demonstrate that swallowing dysfunction occurs early in *Pink1−/−* rats. Delayed transit to the colon and constipation-like signs are also evident in this model. The presence of these early swallowing and GI deficits in *Pink1−/−* rats are analogous to those observed in human PD.

Keywords

Parkinson disease; PINK1; rat; gastrointestinal; videofluoroscopy; dysphagia

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Introduction

Parkinson disease (PD) is a PNS and CNS degenerative disease [1]. Because a definitive diagnosis is typically verified postmortem, the current PD diagnostic process is primarily based on the presence of hallmark motor signs, *i.e.* tremor at rest, muscle rigidity, and bradykinesia, response to pharmacological dopamine replacement, and imaging tests [2–4]. However, non-motor or 'other motor' signs of disease appear significantly earlier in the disease process and prior to a formal diagnosis [5–7]. Specifically, swallowing and gastrointestinal (GI) dysfunction often clinically manifest in prodromal-stage PD and may serve as early biomarkers.

Impairments in swallowing (dysphagia) and GI functioning are highly prevalent in PD. Dysphagia affects over 80% of patients and contributes to weight loss, malnutrition, dehydration, medical costs, social isolation, and decrease in quality of life [8–12]. PD-related swallowing deficits include, but are not limited to, impaired mastication, piecemeal deglutition, increased oral transit time, delayed pharyngeal swallow initiation, and delayed airway closure [13,14]. These ultimately compromise swallowing safety [8] and increase the risk of developing aspiration pneumonia, the leading cause of death in PD [8,15–20]. Similarly, GI deficits, such as delayed gastric emptying, compromised GI motility, and constipation (including changes in fecal output, *i.e.* frequency and weight), affect 70–100% of patients and emerge as early as 20 years prior to the onset of motor impairments [5,21–24]. Despite their prevalence, swallowing and GI dysfunction in PD are not typically reported or treated until later in the disease progression. Furthermore, in the absence of hallmark motor signs, the attribution of these deficits to PD is difficult and limits our understanding of the pathophysiology in prodromal PD.

One way to overcome this challenge is to use animal models. Germane to this work is the *Pink1−/−* rat. This model is based on one of the most common autosomal recessive forms of PD [25,26], in which mutation/deletion of PINK1 results in decreased cellular protection against oxidative stress and apoptosis, and leads to signs of disease nearly clinically identical to sporadic PD, albeit in early adulthood. The *Pink1−/−* rat model recapitulates onset and progression of sensorimotor deficits in PD as the rat ages and pathology worsens [27–30], making it a highly valid translational tool for studying prodromal and early PD. Pathology found in this model include phosphorylated alpha-synuclein aggregation in the substantia nigra pars compacta, locus coeruleus, periaqueductal gray, and nucleus ambiguus [29], as well as loss of nigral dopaminergic neurons, increased norepinephrine concentrations in the substantia nigra, decreased norepinephrine concentration in the locus coeruleus, and decreased tyrosine hydroxylase immunoreactivity (TH-ir) in the locus coeruleus at 8 months of age [29,31,32]. Peripherally, *Pink1−/−* rats also show pathology in tongue and laryngeal muscles, including alpha-synuclein aggregates in the genioglossus muscle, an increase of myosin heavy chain isoform 2a in the styloglossus muscle, and decreased myofiber size in the vocalis division of the thyroarytenoid [33,34]. Behaviorally, *Pink1−/−* rats show limb sensorimotor, vocal motor, and oromotor deficits [29,30], including increased variability in tongue press force and lick rate and inability to sustain licking behavior during drinking, at 6 months of age [33], and slower mastication rate at 8 months of age [35]. Furthermore, *Pink1−/−* rats show swallowing deficits at 8 months of age, specifically increased bolus

area and oropharyngeal bolus speed compared to wildtype (WT) controls, suggesting difficulty with bolus control [35]. Relationships between brain and behavior have also been established in this model, including a positive correlation between locus coeruleus TH-ir and vocal intensity [29], a positive correlation between TH-ir counts in the locus coeruleus and mastication rate [35], and negative correlations between locus coeruleus TH-ir and oromotor functioning as measured by tongue-press force and spontaneous activity (hindlimb steps) [29].

The onset and progression of these deficits and associated pathology are consistent with Braak staging of PD, where caudal brainstem regions are affected earlier in the disease progression than rostral ones [36]. The dorsal motor nucleus of vagus, which is responsible for parasympathetic innervation of the gut, and swallow-related nuclei, including the nucleus ambiguus, nucleus tractus solitarius, and hypoglossal nucleus (among others), are arguably some of the first brainstem regions impacted by disease (Braak stages 1 and 2), earlier than more rostral regions like the substantia nigra (Braak stage 3), which is responsible for hallmark motor disturbances key for diagnosis. As such, behavioral deficits related to these structures would be expected to occur in the early stage of disease in this model, and thus were chosen for this present study.

The purpose of this study was to assay prodromal swallow functioning and GI motility using fluoroscopy, a real-time x-ray examination technique most commonly used in clinical practice for the assessment of swallow and upper/lower GI function in humans. Given that 8 months in the *Pink1−/−* rat is analogous to mid-stage disease/time of diagnosis, we chose to assess earlier timepoints to elucidate *prodromal* (dys)function. The 4- and 6-month timepoints were chosen as they correspond to the prodromal stage of disease in humans, when impaired swallowing and non-typical motor signs such as delayed gastric emptying and constipation are evident in the absence of hallmark motor impairments (*i.e.* tremor). We tested six central hypotheses: (1) *Pink1−/−* rats would demonstrate deficits in oropharyngeal swallowing at 4 months of age compared to healthy WT control rats (specifically, decreased mastication rate, increased oropharyngeal bolus speed, and decreased pharyngoesophageal bolus speed); (2) *Pink1−/−* rats would show presence of abnormal behaviors during swallowing such as dorsal head movement and stasis in the pharynx and esophagus; (3) These deficits would worsen (i.e. significant decrease in mastication rate, increase in oropharyngeal bolus speed, and decrease in pharyngoesophageal bolus speed) from 4 to 6 months of age; (4) *Pink1−/−* rats would demonstrate a decrease in GI motility (specifically, in the stomach, small intestine, caecum, and colon) and (5) that motility would further decrease from 4 to 6 months of age. Lastly, consistent with decreased motility and constipation, we expected to see (6) change in fecal output (decreased number of fecal emissions, decreased weight of fecal emissions) at 6 months of age.

Methods

Animals and Housing

This was a prospective study with all procedures approved by the University of Wisconsin School of Medicine and Public Health Animal Care and Use Committee (IACUC) and conducted in accordance with the National Institutes of Health Guide for the Care and

Use of Laboratory animals. Thirty male Long-Evans rats were tested at 4 months of age (*Pink1*−/− *n* = 15, WT n = 15) and 13 of the same rats were tested via videofluroscopy at 6 months of age (*Pink1*−/− *n* = 7, WT n = 6). A second cohort of 6-month-old male rats (*Pink1*−/− *n* = 10, WT n = 10) was used to examine fecal output. Rats were obtained from Envigo™ Research Labs (Boyertown, PA, USA) at 6 weeks of age. Standard polycarbonate cages (290mm × 533mm × 210mm) with corncob bedding were used to house rats in pairs. Following arrival, rats were acclimated to handling by experimenters for two weeks prior to testing. Body weight was recorded weekly and overall animal health was monitored twice per week. A 12:12 hour reverse light:dark cycle was implemented with lights off at 7AM. Thus, all handling, experimental procedures, and testing were completed under red light illumination during the dark cycle. Rats received food and water *ad libitum* until testing (see below). Videofluoroscopic swallow and GI studies occurred on separate days.

Videofluoroscopic Swallow Study

Prior to testing, rats had a 23-hour food restriction and *ad libitum* access to water. Testing occurred at the University of Wisconsin-Madison Institute for Medical Research. Each rat was tested individually in the home cage. The cage was positioned within the radiographic field in the lateral plane. An L-shaped platform affixed to the cage was used for size calibration. Five g of peanut butter (Jif, Orrville, OH) and 5 mL of liquid barium (Varibar Nectar Barium Sulfate, CMX Medical Imaging, Tukwila, WA) were mixed for a final concentration of 1g/mL and placed onto the platform. Each rat was video recorded for 5 minutes while ingesting the mixture (Fig. 1), with the goal of obtaining three visually unobstructed swallows. Digital videos were obtained at 30 frames per second with a C-ARM fluoroscope, model OEC 9800 (GE Medical Systems, Salt Lake City, UT).

ImageJ (National Institutes of Health, Bethesda, MD) software was used to analyze digitalized videos in a frame-by-frame manner [35,37–39]. Only unobstructed swallows from each rat were analyzed, which were defined by: 1) continuous positioning of the rat in the sagittal plane for the entirety of the swallow (from procurement to passing through the proximal 1/3 of the esophagus) and 2) complete visualization of all anatomical coordinates necessary for analysis throughout all frames of the swallow. A doctoral speech pathology student with expertise in videofluoroscopy and rat anatomy assessed all videos. A second rater, also trained and reliable in videofluoroscopic assessment and rat anatomy, analyzed videos for reliability. This rater, blinded to experimental condition, analyzed swallows from each rat for the following variables: (A) mastication rate (cycles per sec), measured in a frame-by-frame manner through five jaw openings and subsequent closings prior to bolus transfer to the oropharynx; mastication rates were averaged for each rat; (B) bolus area (mm^2), measured after swallow initiation and before the bolus reached C4 of the spinal cord; bolus areas were used as covariates for oropharyngeal and pharyngoesophageal bolus speeds; (C) oropharyngeal bolus speed (mm/sec), calculated from the frame at which the head of the bolus entered the oropharynx to the frame at which the head of the bolus reached the pharyngoesophageal segment (PES); (D) pharyngoesophageal bolus speed (mm/sec), calculated from the frame at which the head of the bolus reached the PES to the frame at which the bolus fully exited the PES; (E) percentage of abnormal swallowing behaviors, calculated as $\left(\frac{number\ of\ abnormal\ behaviors}{total\ number\ of\ opportunities}\right) \times 100$. Abnormal swallowing behaviors included the

following: 1) a forced expiration, 2) compensatory head movement to help facilitate bolus propulsion towards the pharynx, 3) stasis in the pharynx at the level of the PES, and 4) stasis in the proximal 1/3 of the esophagus. Stasis was defined as no advancement of the bolus until a subsequent bolus was swallowed. Each of these behaviors was assessed per swallow. If the animal moved out of frame (*e.g.* head was not fully visible for dorsal head movement), that abnormal swallowing behavior was not assessed for the respective swallow. Once all swallows per rat were analyzed, the number of abnormal behaviors was summed, divided by the total number of opportunities, and multiplied by 100 to obtain a percentage of abnormal swallowing behaviors per rat. All frames used to calculate bolus speeds contained a stable reference marker at approximately C2 of the spinal cord to account for gross body movement.

Gastrointestinal Motility

Prior to testing, animals were fasted overnight. Each rat was orally gavaged with 2mL of liquid suspended barium (2.38 g/mL; E-Z-HD Barium Sulfate for Suspension, CMX Imaging, Seattle, WA). Individual rats were held by the examiner so that the ventral surface was positioned within the radiographic field in the lateral plane parallel to the fluoroscope. Three second videos were taken at 30 frames per sec with a C-ARM fluoroscope, model OEC 9800 (GE Medical Systems, Salt Lake City, UT) to visualize the gastrointestinal tract. Videos were obtained for the following timepoints: hour 0 (immediately following oral-gavage), hour 1, hour 2, hour 3, hour 4, hour 5, and hour 6.

As with the swallow studies, ImageJ (National Institutes of Health, Bethesda, MD) software was used to analyze digitalized videos of the GI tract in a frame-by-frame manner. Four continuous regions of interest were analyzed within the gastrointestinal system—stomach, small intestine, caecum, and colon (Fig. 2, 3, and 4). The parameters for analyses included: portion of the organ labelled (0–4), intensity of label (1–4), profile of the organ (1–2), and homogeneity of label (1–2), as described in Cabezos et al., 2008 [40]. These scores were summed to create a composite score for each region of interest at each hour, allowing for the construction of motility curves.

Fecal Output

Rats were housed in pairs during this time to ensure that separation stress did not influence results. Body weight was documented for each rat individually. Fecal pellets from each cage (n = 5 cages per genotype) were collected to measure the following variables: total fecal output (g), fecal pellet count, and average weight of fecal pellets (g) over a 24-hour period. Additionally, food and water consumption were also documented.

Statistics

R (version 3.6.2) was used for statistical analyses. For mastication rate, a two-way mixed-model Analysis of Variance (ANOVA) was used, with age (4 months, 6 months) and genotype (*Pink1*−/−, WT) as independent variables. To test oropharyngeal and pharyngoesophageal bolus speeds, two-way mixed-model Analysis of Covariance (ANCOVA) were used, with age, genotype, and bolus area included in the model. Bolus area was used as a covariate, as this factor can have significant impacts on bolus speed.

To assess the effects of age and genotype on abnormal swallowing behaviors (%), a two-way mixed-model ANOVA was used. Three-way mixed-model ANOVAs were used to analyze gastrointestinal motility for each GI region (stomach, small intestine, caecum, colon) with age, genotype, and timepoint (hours 0–6) as independent variables. When interactions were not significant, full and reduced models were compared, and the reduced model was used when appropriate. Post-hoc analysis was performed with Fisher's Least Significant Difference. Finally, two-tailed t-tests were used to assess food intake, water intake, and fecal output (total fecal output, number of fecal emissions, average weight of fecal emissions). Critical level of significance was set a priori at 0.05. Intraclass correlations (ICC) were performed to determine intra- and inter-rater reliability on 10% of dependent variables from videofluoroscopy of swallowing and GI motility. Prior laboratory studies have demonstrated that the re-analysis of 10% of data is sufficient for determining inter- and intra-rater reliability.

Results

Rater Reliability

Rater reliability was determined with intraclass correlation coefficients (ICC). Intra- and inter-rater reliabilities for mastication rate, bolus area, abnormal swallowing behaviors, and gastrointestinal motility were greater than 0.90.

Swallowing

Mastication Rate—We did not observe a significant interaction effect between age and genotype for mastication rate [$F(1,9) = 0.78$, $p = 0.401$]. There was a main effect of genotype [$F(1,27)=52.82$, $p < 0.0001$], regardless of age [$F(1,9) = 0.3$, $p = 0.599$] (Fig. 5). *Pink1*−/− rats had slower rates of mastication compared to WTs.

Oropharyngeal Bolus Speed—ANCOVA controlling for bolus area did not identify a significant interaction effect between age and genotype for oropharyngeal bolus speed [$F(1,11) = 0.03$, $p = 0.86$]. There were also no main effects of genotype [$F(1,28) = 0.28$, $p = 0.6$] or age [$F(1,11) = 0.14$, $p = 0.72$] for oropharyngeal bolus speed.

Pharyngoesophageal Bolus Speed—ANCOVA controlling for bolus area did not identify a significant interaction effect between age and genotype for pharyngoesophageal bolus speed [$F(1,11) = 0.81$, $p = 0.39$]; however, there was a significant main effect of genotype [$F(1,28) = 7.73$, $p = 0.0096$], regardless of age [$F(1,11) = 0.7$, $p = 0.42$] (Fig. 6). *Pink1*−/− rats had slower pharyngoesophageal bolus speeds compared to WTs.

Abnormal Swallowing Behaviors—We did not observe a significant interaction effect between age and genotype for abnormal swallowing behaviors [$F(1,11) = 0.10$, $p = 0.757$]. There was a main effect of genotype [$F(1,28) = 14.19$, $p < 0.001$], regardless of age [$F(1,11) = 0.01$, $p = 0.938$]. Overall, *Pink1*−/− rats had a higher percentage of abnormal swallowing behaviors than WTs (Fig. 7).

Descriptive statistics for abnormal swallowing behaviors are summarized in Tables 1 and 2. No rats in this study of either genotype showed signs of forced expiration while swallowing.

Compensatory head movements were only observed in *Pink1−/−* rats (4% of trials), not WTs (0% of trials). Stasis in the pharynx was observed in *Pink1−/−* rats in 16% of trials, while in WTs in only 2% of trials. Lastly, stasis in the proximal 1/3 of the esophagus was the most frequently occurring abnormal swallowing behavior, observed in *Pink1−/−* rats in 27% of trials and in WTs in 5% of trials.

Gastrointestinal Motility

Stomach—In the absence of any significant interaction effects, the reduced model revealed significant main effects of age [$F(1, 317.34) = 68.736$, $p = 3.23e\text{-}15$, Fig. 8] and hour [$F(6, 297.11) = 167.875$, $p < 2.2e\text{-}16$]. 4-month-old rats had more contents in their stomachs than 6-month-old rats. There were no main effects for genotype [$F(1, 31.68) = 0.825$, $p = 0.371$].

Small Intestine—In the absence of a significant three-way interaction effect, the reduced model revealed a significant interaction effect between genotype and age [$F(1, 311.907) = 4.117$, $p = 0.043$]. However, pairwise comparisons revealed no significantly different findings between groups ($p > 0.05$, Fig. 9). There was also a main effect of hour [$F(6, 292.080) = 108.657$, $p < 2e\text{-}16$].

Caecum—In the absence of a significant three-way interaction effect, the reduced model revealed no significant interaction effect between genotype and age [$F(1, 296.112) = 0.297$, $p = 0.586$]; however, there was a significant interaction effect between genotype and hour [$F(6, 285.446) = 3.699$, $p = 0.001$]. Pairwise comparisons revealed *Pink1−/−* rats had fewer contents in the caecum at hour 2 ($p = 0.002$) and hour 3 ($p = 0.035$) compared to WTs (Fig. 10a). The interaction effect between age and hour was also significant [$F(6, 285.480) = 3.836$, $p = 0.001$]. Pairwise comparisons revealed 4-month-old rats to have fewer contents in the caecum compared to 6-month-old rats at hour 2 ($p < 0.0001$, Fig. 10b).

Colon—In the absence of a significant three-way interaction effect, the reduced model revealed a significant interaction effect between genotype and hour [$F(6, 291.873) = 2.987$, $p = 0.008$]. Pairwise comparisons revealed *Pink1−/−* rats had fewer contents in their colon at hour 3 ($p = 0.001$) and hour 4 ($p = 0.047$) compared to WT controls (Fig. 11). There was no main effect of age [$F(1, 311.032) = 1.115$, $p = 0.292$].

Fecal Output

Food Intake—There was no significant difference in 24-hour food intake between *Pink1−/−* and WT rats at 6 months of age ($p = 0.488$). The mean food intake of *Pink1−/−* cages was of 56.72 grams with SD 8.841 and SEM 3.954, while WT cages had an average food intake of 53.480 grams with SD 4.581 and SEM 2.049.

Water Intake—There was no significant difference in 24-hour water intake between *Pink1−/−* and WT rats at 6 months of age ($p = 0.162$). The mean water intake of *Pink1−/−* cages was 85.440 mL with SD 10.424 and SEM 4.662, while WT cages had an average water intake of 72.340 mL with SD 15.893 and SEM 7.108.

Total Weight of Fecal Output—There was no significant difference in total fecal output weight between *Pink1−/−* and WT rats at 6 months of age ($p = 0.829$). The mean total fecal output weight of *Pink1−/−* cages was 15.660 grams with SD 2.832 and SEM 1.266 while WT cages had an average total fecal output weight of 15.360 grams with SD 0.999 and SEM 0.447.

Fecal Pellet Count—There was a significant difference in the number of fecal pellets between *Pink1−/−* and WT rats at 6 months of age (p = 0.00006) (Fig. 12). On average, *Pink1−/−* rats had fewer fecal emissions over 24 hours than WTs. The mean fecal pellet count of *Pink1−/−* cages was 54.6 with SD 4.037 and SEM 1.806, while WT cages had an average fecal pellet count of 74 with SD 4.062 and SEM 1.817.

Average Weight of Fecal Pellet—There was a significant difference in average weight of individual fecal pellets between *Pink1−/−* and WT rats at 6 months of age (p = 0.006) (Fig. 13). On average, the weight of individual *Pink1−/−* fecal pellets was greater than that of WTs. The mean fecal pellet weight of *Pink1−/−* cages was 0.286 grams with SD 0.046 and SEM 0.021, while WT cages had an average fecal pellet weight of 0.208 grams with SD 0.012 and SEM 0.005.

Discussion

Because swallowing and GI dysfunction are highly prevalent but understudied in prodromal and early-stage PD, the primary purpose of this study was to use fluoroscopy to characterize oropharyngeal swallowing and GI impairments in the *Pink1−/−* rat. Overall, significant findings reveal that *Pink1−/−* rats have slower mastication rates, slower bolus speeds through the PES, and a larger percentage of abnormal swallowing behaviors. Additionally, *Pink1−/−* rats show delayed entry of contents into the caecum and colon, with significant differences between genotypes across several testing hours, as well as fewer fecal pellet emissions and increased fecal pellet weight. Overall, these results suggest that the *Pink1−/−* rat shows swallowing and GI deficits that occur in prodromal/early PD.

Oropharyngeal Swallowing

Findings from this study show that *Pink1−/−* rats have significantly slower mastication rates compared to WT controls. Overall, this is consistent with human PD findings, as patients often experience impaired masticatory and oromotor function [41]. Previous findings have shown *Pink1−/−* rats have slower mastication rates compared to WTs at 8 months of age. The present study suggests that mastication rates may be slower in *Pink1−/−* rats in early timepoints, representing prodromal PD. Furthermore, previous findings have shown that *Pink1−/−* rat mastication rates decrease from 4 to 8 months of age, signifying a progressive worsening of oromotor deficits over time. Our findings did not show worsening of deficits from 4 to 6 months of age, which may indicate that the testing timepoints are too close to detect changes in oromotor dysfunction.

This study was the first to look at bolus transit through the PES in *Pink1−/−* rats. As with mastication rate, there were no changes in bolus speeds for *Pink1−/−* rats from 4 to 6 months, suggesting that these deficits are static between 4 and 6 months. Adjusting

for bolus area, pharyngoesophageal bolus speeds were significantly slower in *Pink1−/−* rats compared to WTs. Similar to other behavioral dysfunctions in the *Pink1−/−* rat, slower pharyngoesophageal bolus speeds may represent neurologic and/or physiologic disruption at the level of the PES. In human populations, studies have shown PD patients to have reduced upper esophageal sphincter (*i.e.* PES) relaxation, reduced upper esophageal sphincter diameters, and high intrabolus pressures [42]. Slower bolus speeds through the PES, therefore, may suggest impaired cricopharyngeal muscle quiescence or disruption in timing during a swallow. Slower bolus transit through the PES can also increase the risk of airway invasion [43]. In this study, stasis at the level of the PES was shown in 16% of trials in *Pink1−/−* rats, further indicating impairment at this phase of swallowing.

Previous swallow findings in the *Pink1−/−* rat have shown increased oropharyngeal bolus velocities at both 4 and 8 months of age compared to WT controls. The present study did not find this difference between genotypes; however, it should be noted that this study accounted for bolus size, whereas previous studies did not. Inconsistency in findings could also demonstrate swallow variability in earlier stages of disease. Furthermore, it has been reported that swallow dynamics change with different measured bolus volumes in humans [44–48]. Because our design incorporated ad libitum eating, bolus volume was not controlled.

Abnormal Swallowing Behaviors

Similar to previous work, this study also demonstrated significant differences between genotypes in the percentage of abnormal swallowing behaviors observed [37]. Overall, *Pink1−/−* rats showed more compensatory head movement and stasis of the bolus in the pharynx and esophagus compared to WTs. In human PD, patients often experience swallowing abnormalities such as residue in the pharynx and/or delay in the initiation of swallow [49]. As such, patients may employ voluntary and involuntary compensatory strategies to aid in swallowing such as repeated swallows to clear the bolus from the pharynx or postural maneuvers to aid in swallowing [50,51].

Gastrointestinal Motility

Over the past three decades, the PD literature has expanded to include studies of gastrointestinal motility. Previous work has shown impaired gastric emptying, prolonged transit through the small intestine, and pathologically prolonged transit times through the colon [52–56]. This led us to hypothesize that transit times through the GI tract of *Pink1−/−* rats would also be impaired. In this study, no differences were noted between *Pink1−/−* and WT controls in either the stomach or small intestine. However, lower GI regions – the caecum and the colon – revealed the most salient findings.

In this study, the caecum was the first region of interest where *significant* differences between genotypes were found. Specifically, *Pink1−/−* rats showed significantly fewer contents in the caecum compared to WTs, particularly at earlier hours when contents began entering the caecum. Findings are in line with human work, as previous studies indicate that PD patients have prolonged caecum transit times [57]. Impaired motility into and within the colon was also shown in *Pink1−/−* rats. WT rats showed presence of barium in the

colon an hour earlier than *Pink1−/−* rats, and significantly more barium in the colon at later timepoints as well, suggesting delayed entry into the colon and impaired motility over time in *Pink1−/−* rats. This has also been shown in humans, whereby time to first propagating colonic movement in PD patients is significantly longer than that of healthy adults [57]. Additionally, PD patients have significantly prolonged colonic transit times compared to healthy adults [56,58]. Colonic motility issues have also been found in MPTP [59], 6-OHDA [60], and rotenone models [61]. Given that no differences were noted between *Pink−/−* and WT rats in the small intestine or stomach, impairment may possibly be occurring at the level of the caecum or lower in the GI tract earlier in disease progression. Similar findings were demonstrated in the MPTP mouse model, whereby colonic motility was impaired, yet no changes in gastric emptying or small intestine transit were noted [62]. Overall findings from this study demonstrate impairment in gastrointestinal motility at early timepoints, analogous to prodromal PD; future work will assess pathology.

Fecal Output

At 6 months of age, *Pink1−/−* rats had fewer fecal pellet emissions over 24 hours compared to WT controls. This is indicative of slower colonic motility and constipation-like signs. Similar findings were reported in the 6-OHDA model where fecal output was significantly decreased in compared to controls [63]. These findings are analogous to human PD, as constipation is common and perhaps one of the earliest signs to manifest. Additionally, the longer feces remain in the colon, the more water may be extracted, making the pellets harder and more difficult to excrete.

Lastly, we analyzed average fecal pellet weight. Because food and water intake did not significantly differ between *Pink1−/−* and WT rats, fecal pellet weight was likely not influenced by amount ingested. Contrary to our hypothesis, *Pink1−/−* rats had a higher average fecal pellet weight compared to WTs. The walls of the small intestine, caecum, and colon absorb fluids as contents travel through the GI tract. Slower movement through the GI tract could suggest more time for water absorption, leading to dryer, and thereby lighter, feces. This has been shown in PD patients with confirmed constipation, as their stool weights were found to be lower compared to non-constipated patients with PD [64]. The alpha-synuclein overexpressing mouse model of PD has also shown fewer fecal emissions with significantly less water content. However, this was only true for older (9–12 month) mice; younger (2–4 month) mice showed no changes in fecal water content compared to WT controls [65], suggesting earlier timepoints are less likely to result in altered fecal properties, such as weight. Similarly, MPTP mouse models of PD exhibit decreased stool frequency but no difference in stool weight [66]. As such, changes to fecal output ought to be assessed at later timepoints. Age and progression of disease may influence fecal properties such as weight. Findings from this study demonstrate that, as in prodromal PD, the *Pink1−/−* rat model demonstrates early signs of constipation.

Our understanding of PD pathogenesis is evolving. Traditional view of the disease has heavily focused on degeneration of dopaminergic neurons in the substantia nigra pars compacta. When nigrostriatal pathways are reduced by approximately 80%, hallmark Parkinsonian motor signs are robust [67,68]. Confirmation of striatal dopamine neuron loss

and response to dopamine and basal ganglia circuitry remains the standard of care for these classical signs [2]; however, this does not address other signs/symptoms of PD [69,70]. After decades of basic, translational, and clinical research, PD is now believed to be a multisystem disorder [71]. Leading hypotheses in pathophysiology point to inflammation, accumulation of alpha-synuclein, and mitochondrial dysfunction as mediators of cell death [72–75], implicating central and peripheral nervous systems and target tissues, and including other neurotransmitter systems. An abundance of evidence points to the GI tract as a potential site of PD pathogenesis [76–83], with pathology spreading from the periphery to the CNS via the vagus nerve [76–78,80,82]. In line with this directionality of spread, lower brainstem regions are affected earlier than substantia nigra [36,84,85]. Several brainstem nuclei critical for GI and/or swallow functioning, such as the dorsal motor nucleus of the vagus, nucleus ambiguus, and nucleus tractus solitarius, are some of the first brain regions to be implicated in PD [36,84,85]. Given that GI and swallow deficits appear in the prodrome, prior to nigrostriatal dopamine depletion, other systems outside of nigrostriatal pathology likely underlie prodromal dysfunction.

A range of neurotransmitters and neuromodulators are suggested to be involved in swallow and GI physiology. Noradrenaline is a catecholamine that functions as a neurotransmitter and hormone. In PD, disruption to the noradrenergic system is common, especially early in the disease progression [36,84,86–91]. Noradrenaline is synthesized in the locus coeruleus, a brain region associated with arousal, attention, anxiety, behavior, cognition, learning and memory, sensory processing, and feeding [32,92–94]. The locus coeruleus projects to cortical and subcortical regions, including many of those involved in swallow and GI function, such as the nucleus ambiguus and the dorsal motor nucleus of the vagus [95]. In the periphery, noradrenaline plays an inhibitory role on the enteric nervous system, affecting GI motility, sphincter contraction, and secretion of digestive enzymes [96].

Serotonin in particular is a monoamine neurotransmitter involved in affective and motor functioning [97–101], suggested to be involved in the development of non-motor and other motor signs and complications associated with PD, like swallow and GI dysfunction [102–106]. Serotonin produced in the gut epithelium accounts for over 90% of the body's serotonin and controls smooth muscle contraction, muscle relaxation, and visceral sensitivity [107,108]. In PD, patients often experience serotonin-mediated dysfunction such as delayed gastric emptying, impaired intestinal peristalsis/intestinal secretion, compromised colonic tone, and nausea [96,109]. Serotonin also plays a role in the swallowing mechanism. In the CNS, for example, the hypoglossal nucleus receives serotonergic inputs that control the complex movements of extrinsic and intrinsic tongue muscles [110,111]. Moreover, the release of serotonin in the nucleus tractus solitarius, a key component of the swallow central pattern generator, has been shown to affect swallow inhibition [112].

Dopamine is implicated in GI motility (gastric contractile activity, colonic motility, gastroduodenal motility, GI transit) from esophagus to colon [113]. In PD, degeneration of dopaminergic neurons in the enteric nervous system has been linked to GI dysmotility issues in both humans and animal models [62,114]; though, with multiple neurotransmitters regulating GI function, it is difficult to disambiguate this dysfunction in PD. More importantly, how dopamine is affected in the prodrome or whether dopamine is implicated in

the periphery *prior* to the CNS, is still not fully understood. Better understanding how these neurotransmitters and neuromodulators modulate central and peripheral function across disease stages will advance identification and treatment of disease.

Lastly, with no yet known etiology and such heterogeneity in the presentation of disease, it is possible that PD may encompass several subtypes with different mechanisms underlying each [115]. Animal models are therefore useful for studying different aspects of PD on both behavioral and pathophysiological levels. The rat model chosen for this work has a global knockout of the *Pink1* gene and, therefore, mitochondrial dysfunction is *systemic* and occurs throughout the whole body. Yet, certain deficits still manifest earlier than others. This study in particular revealed that swallowing and GI dysfunction manifest at prodromal timepoints. Prior work also demonstrated early and progressive vocal dysfunction [29], as well as affective deficits [32], prior to the manifestation of limb-motor deficits and significant nigrostriatal dopamine implication [27,29]. This suggests that some systems may be more susceptible to loss of Pink1, mitochondrial dysfunction, and therefore pathology, than others. The study of these prodromal systems may provide unique and valuable insight into the etiology of PD.

Limitations

Videofluoroscopic images of swallowing were acquired in the sagittal plane, restricting the assessment of swallowing to a two-dimensional plane, which is a known limitation. This means that bolus area was assessed rather than bolus volume. Bolus volume was not controlled as the design used *ad libitum* access to food to assess natural swallowing behaviors. Another limitation was that videofluoroscopic swallow and GI studies were performed on separate testing days. As such, we did not assess the same bolus as it transited from the mouth to excretion. Further, our study only assayed GI motility until 6 hours post-gavage. Although delayed entry into the caecum and colon was observed in this study, 6 hours was deemed insufficient time to assess *full clearance* of contents from each organ. Our study was designed *a priori* to assess GI motility until 6-hours post-gavage, which was deemed enough time to assess amount contents present in all organs, including the last organ of interest – the colon. It was also deemed sufficient time to obtain meaningful data without exposing rats to more radiation than necessary. However, future work should consider extending testing beyond the 6-hour mark, while still maintaining ethical and scientific integrity with regard to radiation exposure.

Overall conclusions

This work expanded on previous research that assayed oromotor functioning and oropharyngeal bolus kinematics during swallowing and added novel assessment of bolus transit through the PES and GI system. Overall, results from this study demonstrate that the *Pink1−/−* rat model recapitulates the swallowing and gastrointestinal deficits that are similar to those that occur in early human PD.

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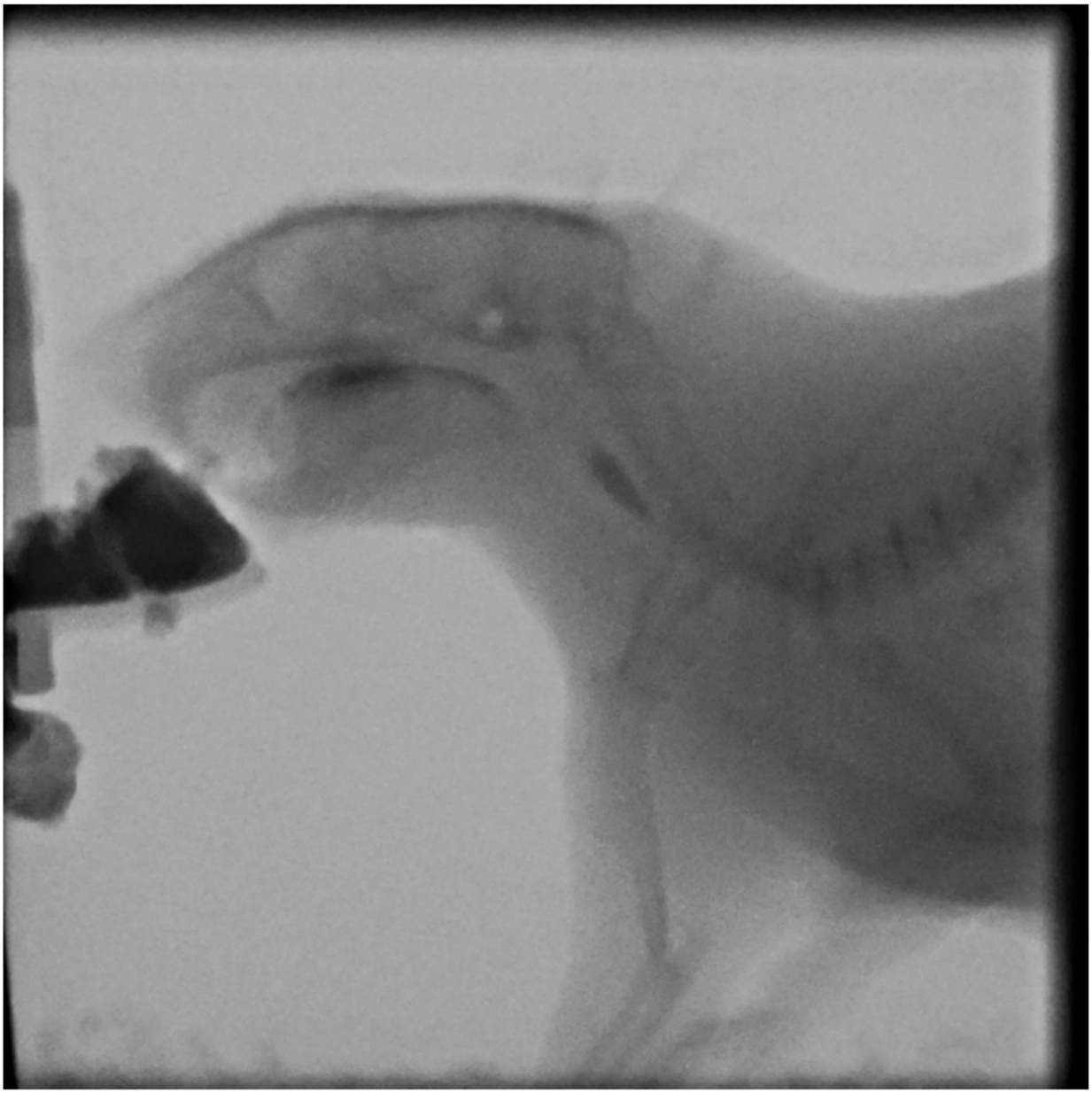

Fig. 1.
Still frame of a rat undergoing videofluoroscopy. Bolus is seen in the pharynx. Peanut butter mixed with barium seen on platform affixed to cage

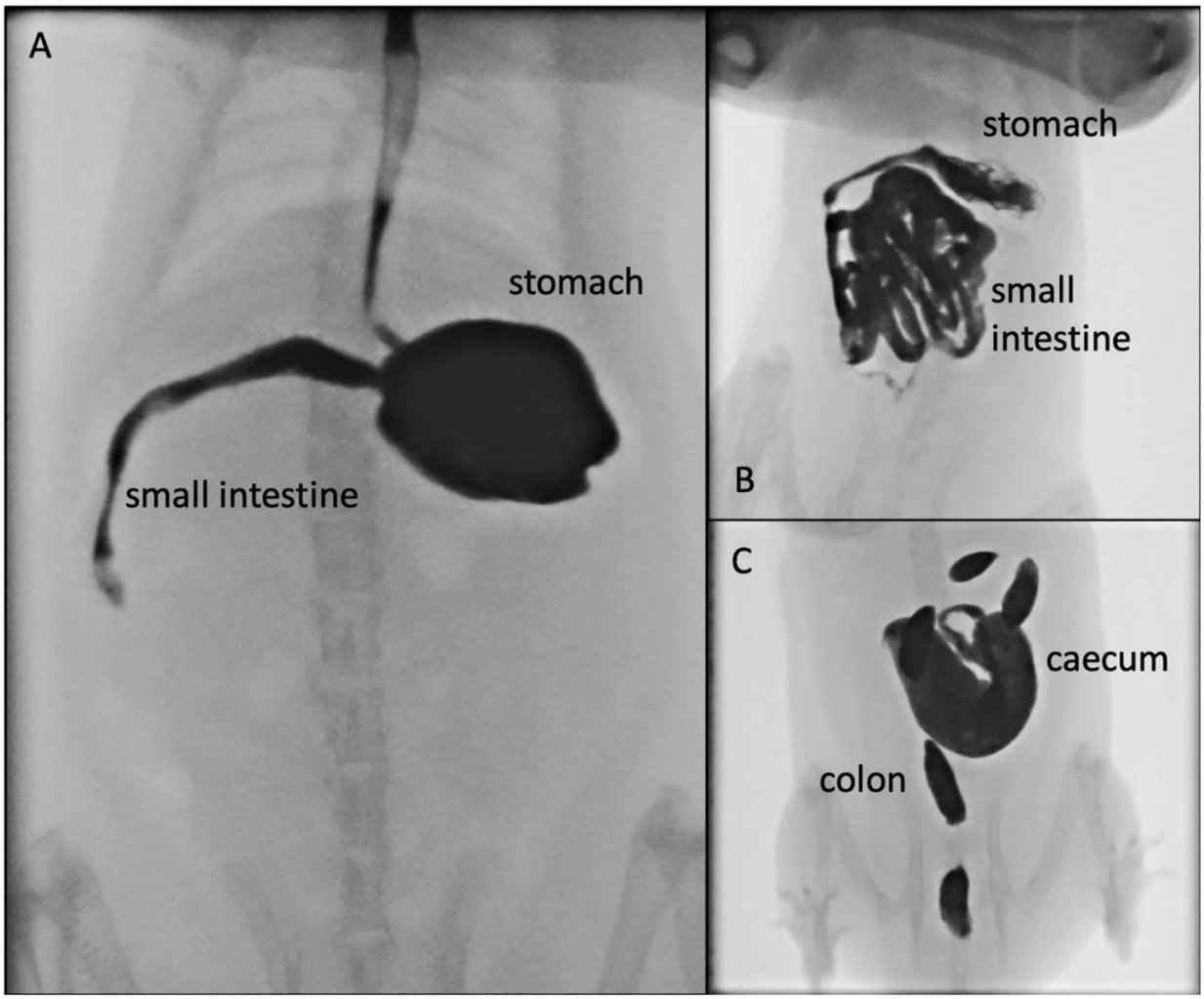

Fig. 2.

Still frames from videofluoroscopic study of gastrointestinal organs filled with barium post-oral gavage. a. Barium visualized in stomach and entering the small intestine. b. Barium in stomach and advancing further into small intestine. c. Barium in caecum and colon

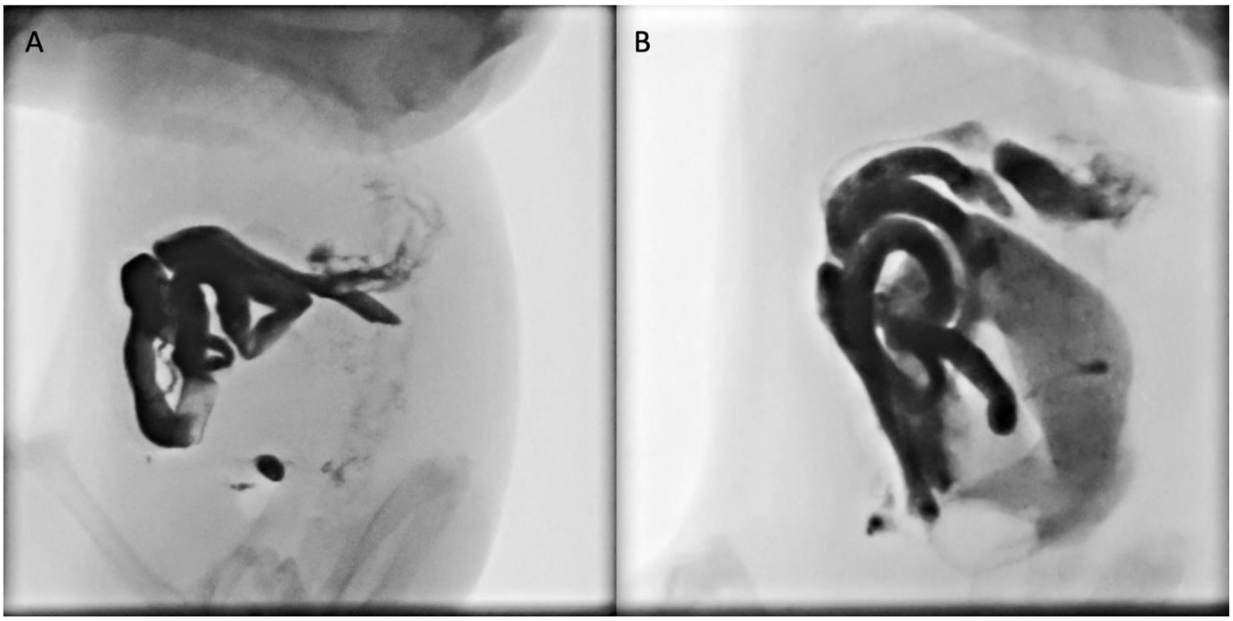

Fig. 3.

Still frames from videofluoroscopic study of gastrointestinal organs filled with barium 2 hours following oral gavage. a. *Pink1−/−* rat with barium coating the stomach and barium in the small intestine. b. WT rat with barium in stomach, small intestine, and caecum

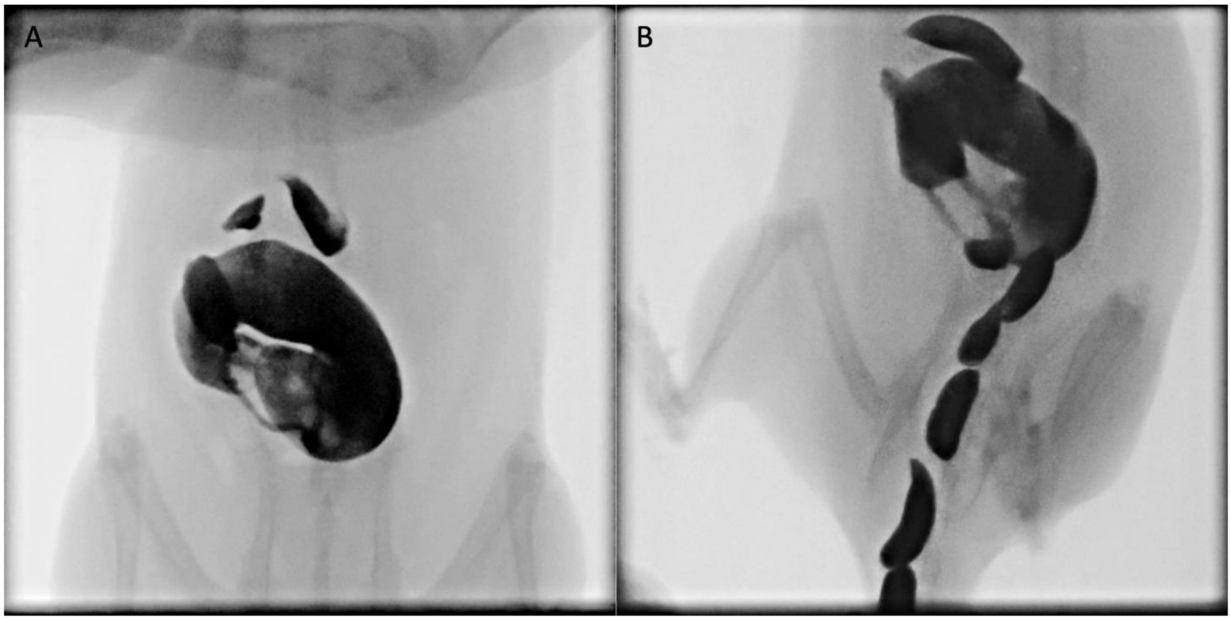

Fig. 4.

Still frames from videofluoroscopic study of gastrointestinal organs filled with barium 6 hours following oral gavage. a. *Pink1−/−* rat with barium coating the caecum and few pellets in the colon. b. WT rat with barium in caecum and full colon with pellets

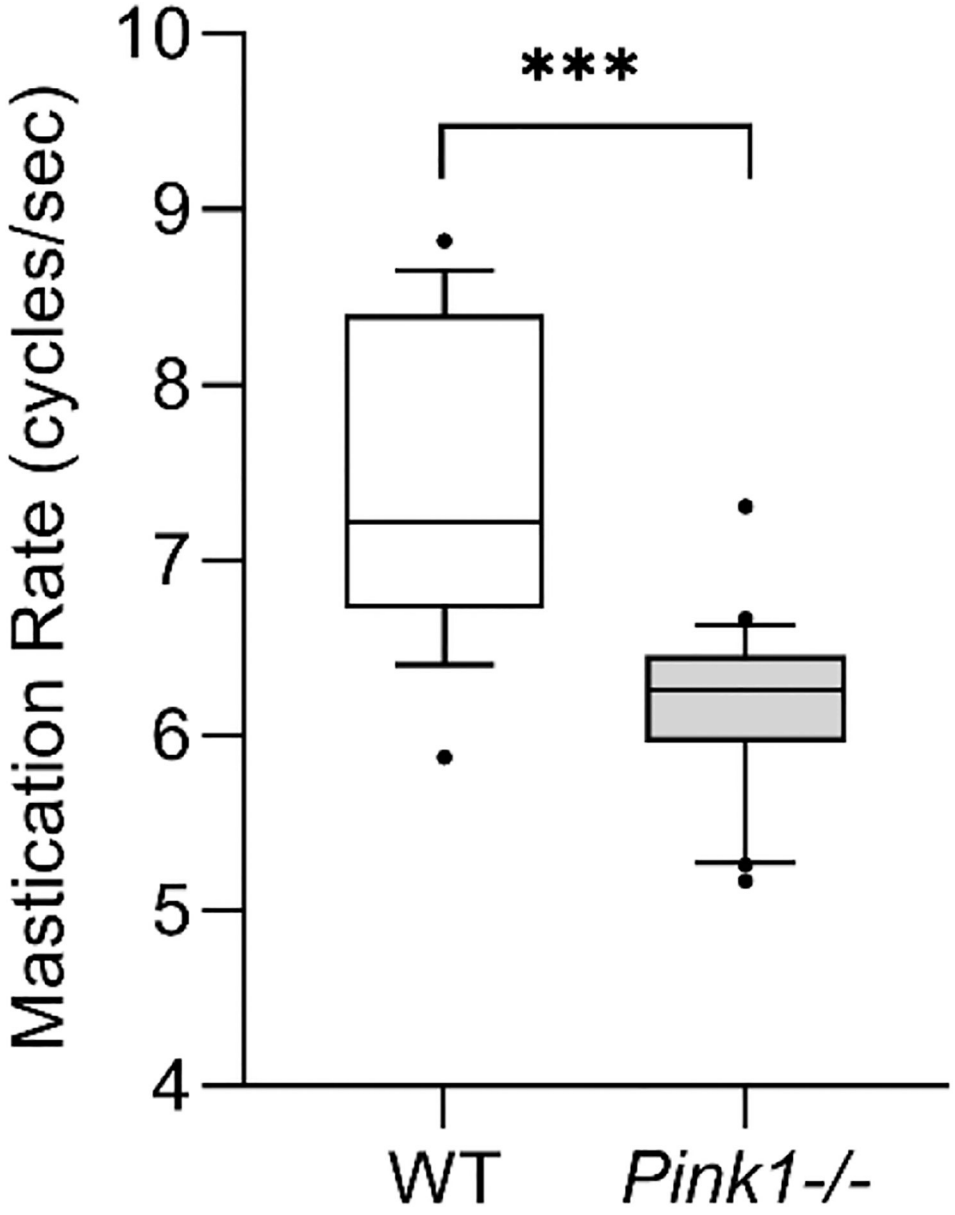

Fig. 5.

Pink1−/− rats (gray bar) had a significantly slower rate of mastication compared to WT rats (white bar). From bottom to top, box plots indicate the 10th percentile (whisker below box), the 25th percentile (lower box boundary), the median (line within the box), the 75th percentile (upper box boundary), and 90th percentile (whisker above box). Values outside the 10th and 90th percentiles are indicated by dark circles. Triple asterisk represents statistical significance (***p<0.001)

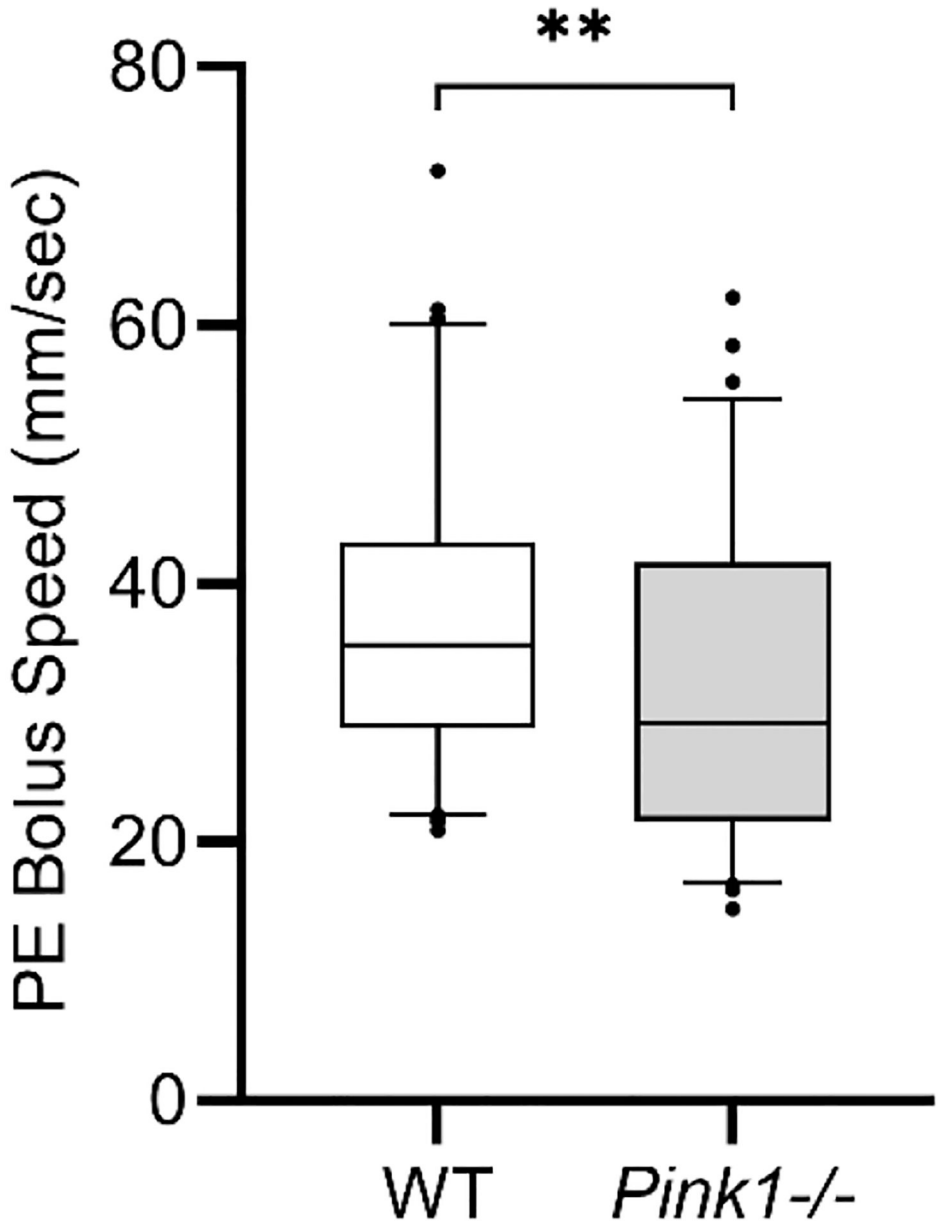

Fig. 6.

Pink1−/− rats (gray bar) had significantly slower bolus speeds compared to WT rats (white bar). From bottom to top, box plots indicate the 10th percentile (whisker below box), the 25th percentile (lower box boundary), the median (line within the box), the 75th percentile (upper box boundary), and 90th percentile (whisker above box). Values outside the 10th and 90th percentiles are indicated by dark circles. Double asterisk represents statistical significance (**p<0.01)

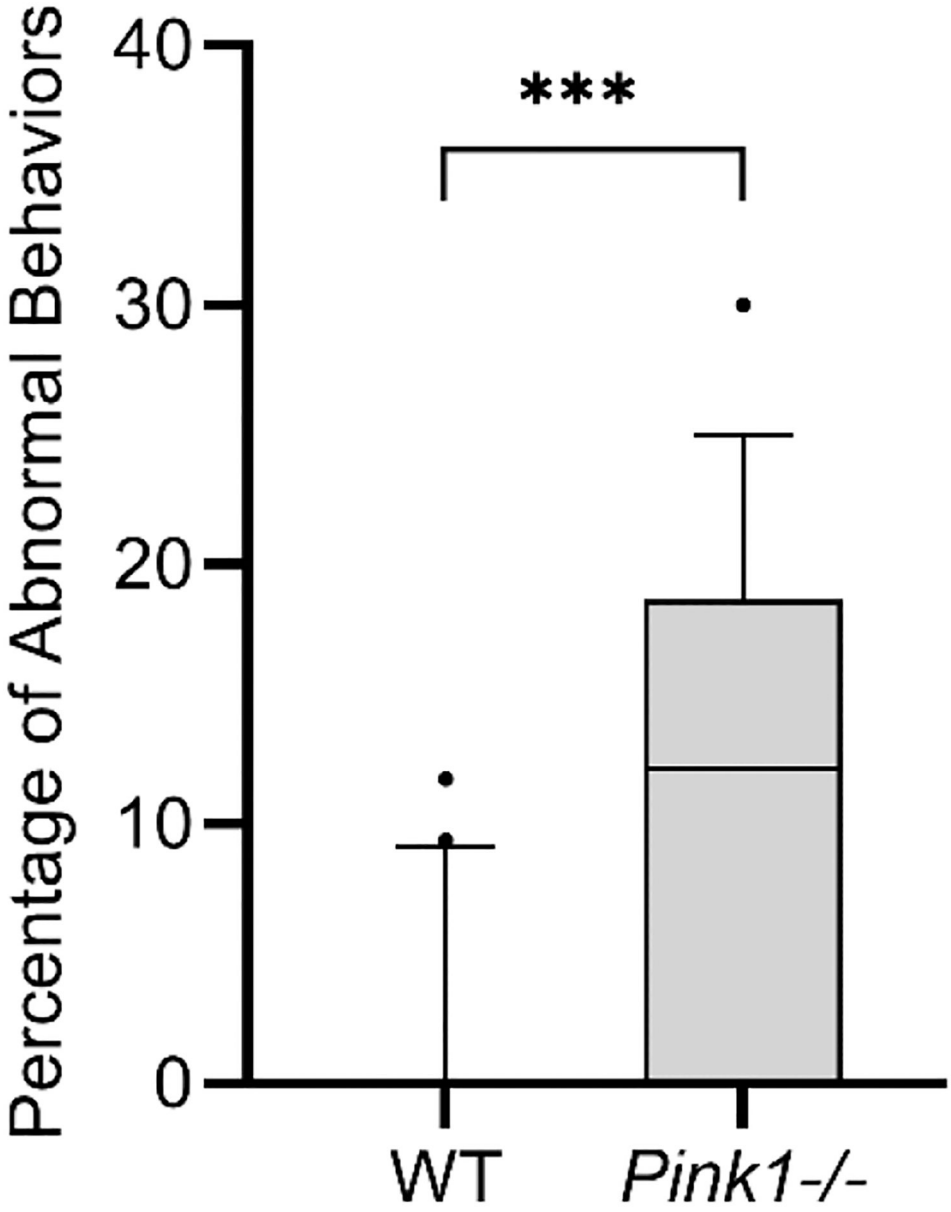

Fig. 7.

ink1−/− rats (gray bar) had a significantly greater percentage of abnormal swallowing behaviors compared to WT rats (white bar). From bottom to top, box plots indicate the 10th percentile (whisker at 0), the 25th percentile (lower box boundary), the median (line within the box), the 75th percentile (upper box boundary), and 90th percentile (whisker above box). Values outside the 10th and 90th percentiles are indicated by dark circles. Triple asterisk represents statistical significance (***p<0.001)

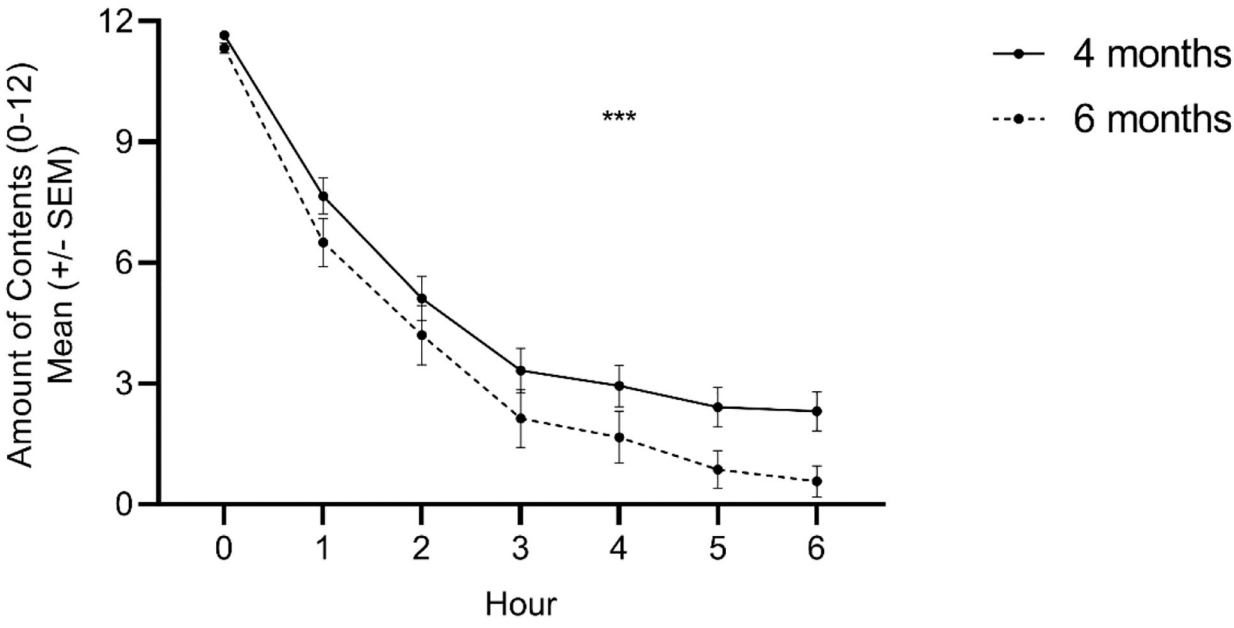

Fig. 8.

Overall, 4-month-old rats (solid line) had more contents in their stomach compared to 6-month-old rats (dashed line). Error bars indicate standard error of the mean. Asterisks indicate statistical significance (*** p<0.001) and denote a main effect of age in this graph

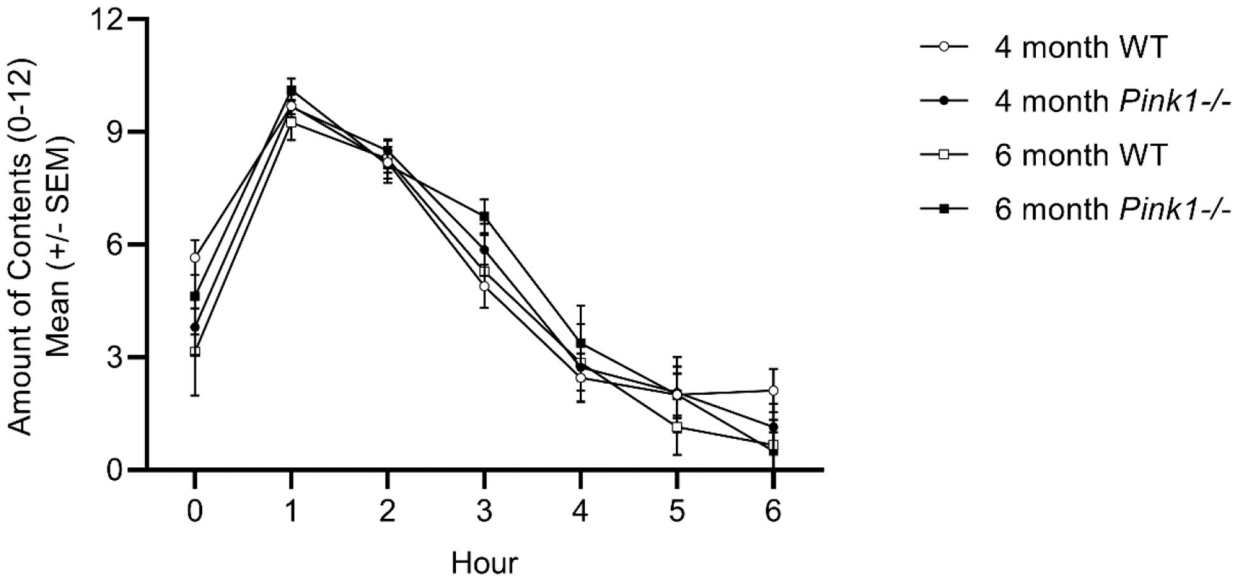

Fig. 9.

Contents in the small intestine did not differ between *Pink1−/−* and WT rats at either 4 or 6 months of age. Error bars indicate standard error of the mean

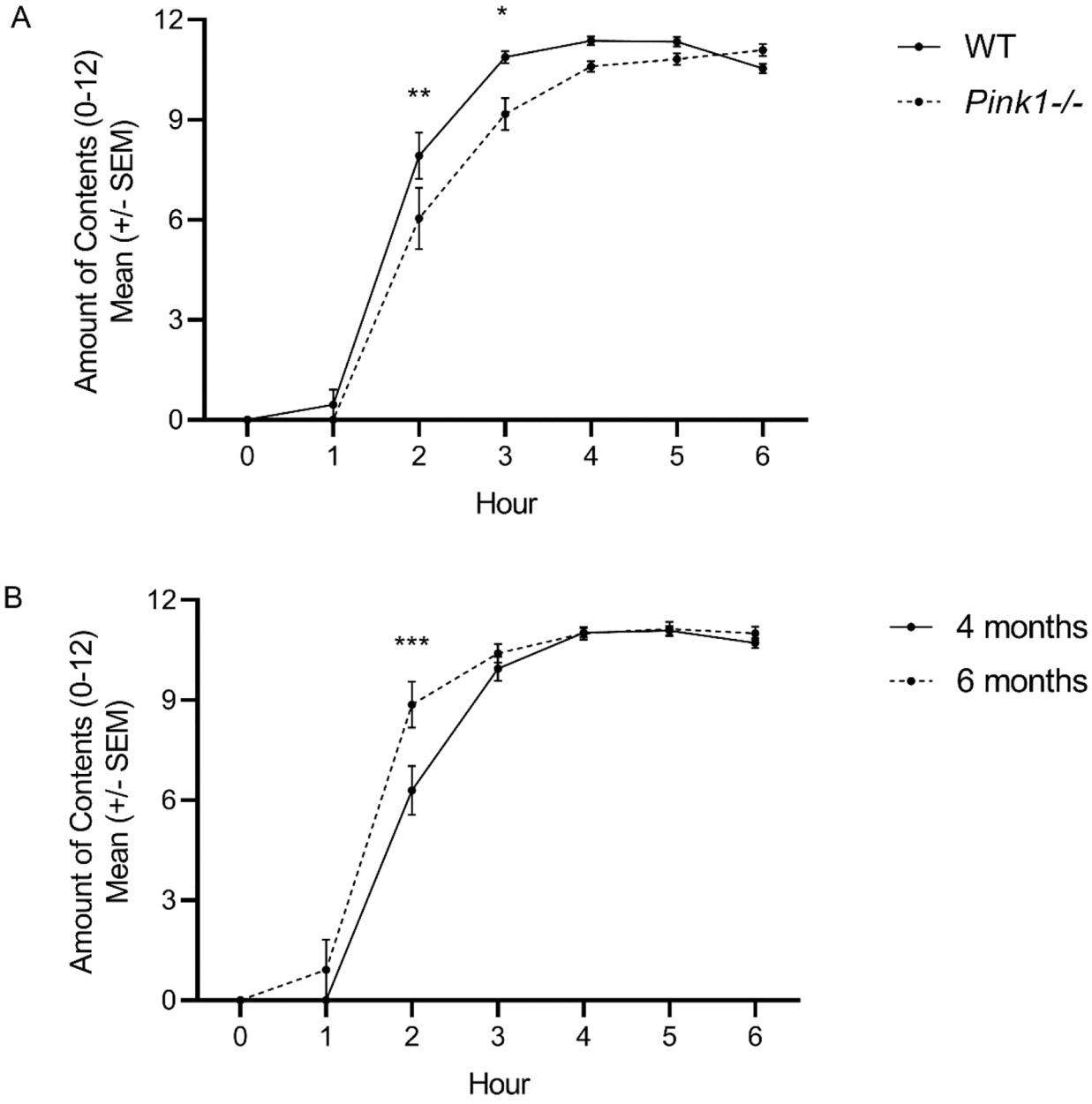

Fig. 10.

a *Pink1−/−* rats (dashed lines) have fewer contents in the caecum at hours 2 and 3 compared to WTs (solid lines). **b** 4-month-old rats (solid lines) have fewer contents in the caecum compared to 6-month-old rats (dashed lines) at hour 2. Error bars indicate standard error of the mean. Asterisks indicate statistical significance (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

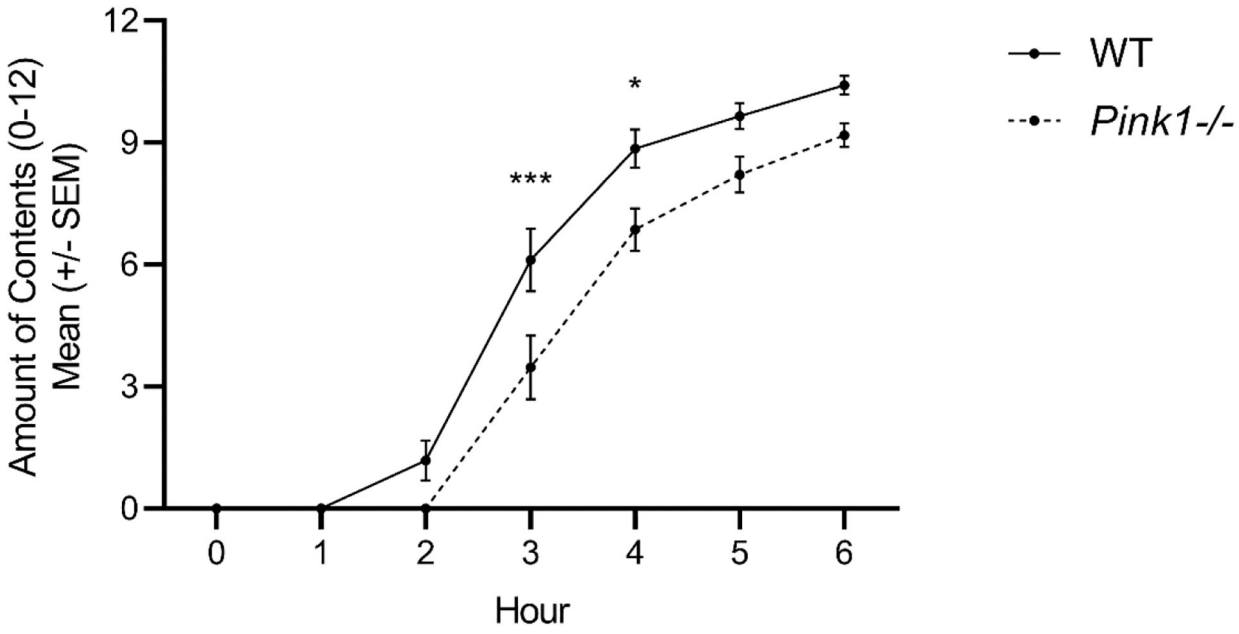

Fig. 11.

Pink1−/− rats (dashed lines) had fewer contents in the colon at hours 3 and 4 compared to WTs (solid lines). Error bars indicate standard error of the mean. Asterisks indicate statistical significance (* $p<0.05$, *** $p<0.001$)

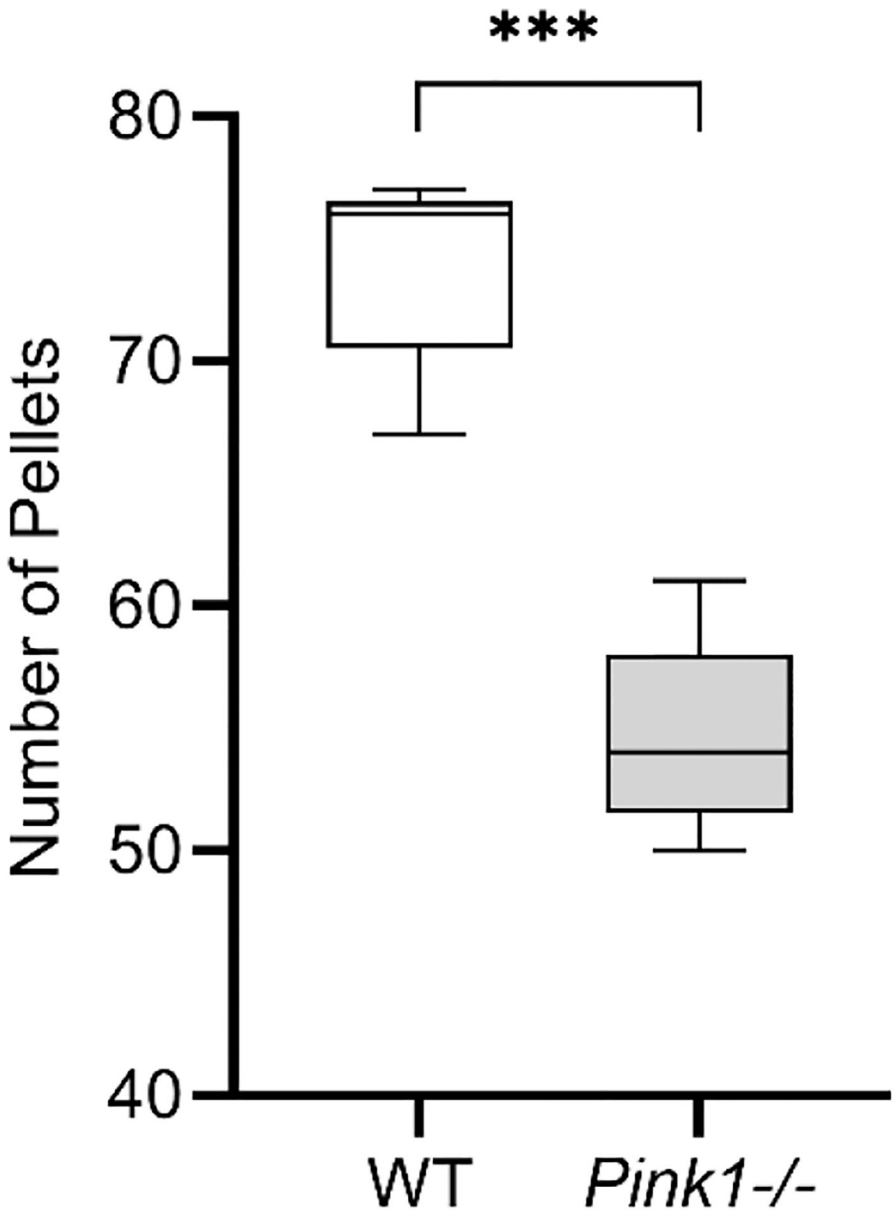

Fig. 12.

Pink1−/− rats (gray bar) had a significantly lower fecal pellet count compared to WT rats (white bar). From bottom to top, box plots indicate the 10th percentile (whisker below box), the 25th percentile (lower box boundary), the median (line within the box), the 75th percentile (upper box boundary), and 90th percentile (whisker above box). Triple asterisk represents statistical significance (***p<0.001)

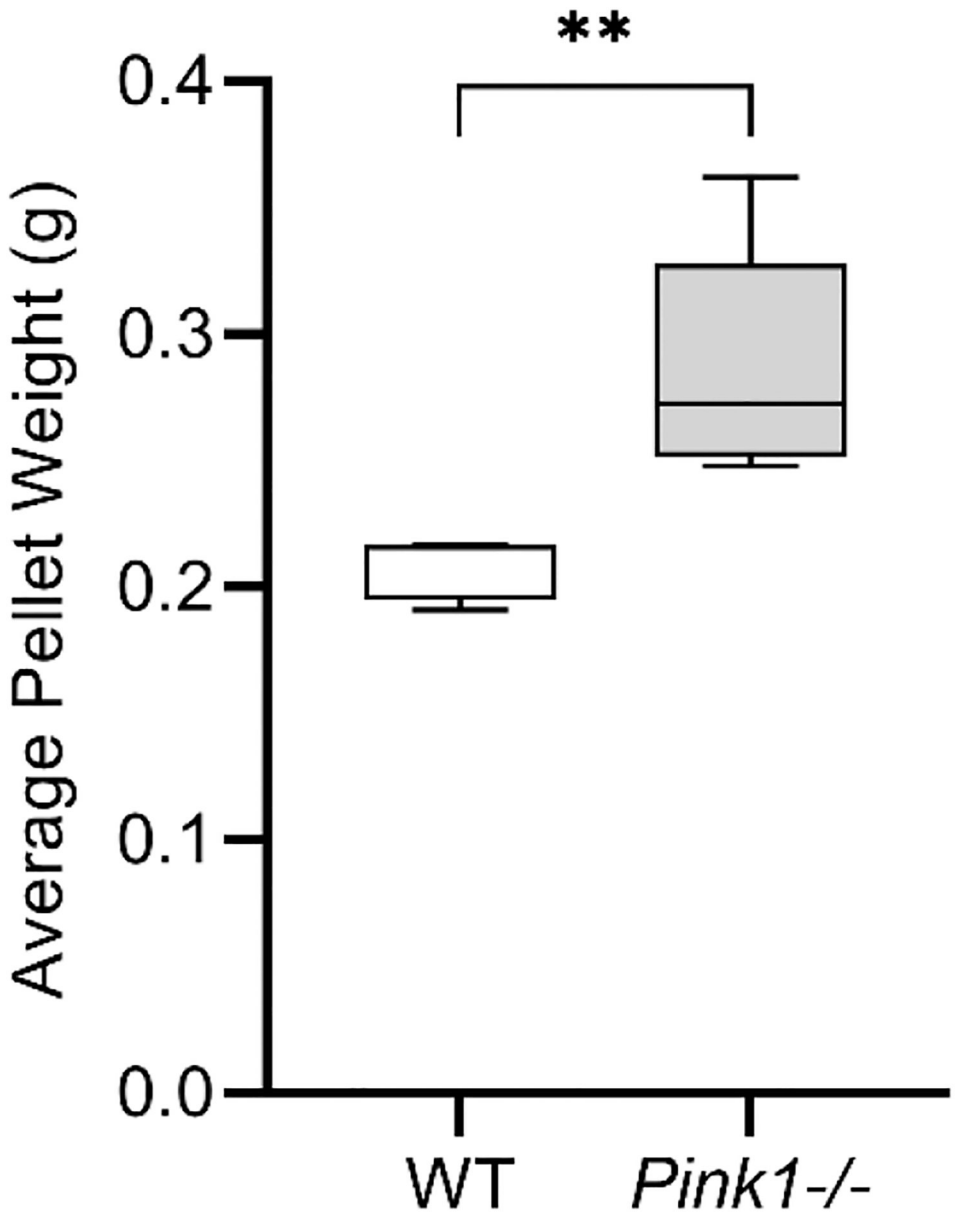

Fig. 13.

Pink1−/− rats (gray bar) had significantly higher average weight of fecal emissions compared to WT rats (white bar). From bottom to top, box plots indicate the 10th percentile (whisker below box), the 25th percentile (lower box boundary), the median (line within the box), the 75th percentile (upper box boundary), and 90th percentile (whisker above box). Double asterisk represents statistical significance (**p<0.01)

Table 1

Summary table of percent abnormal swallowing behaviors.

Age (months)	Genotype	N	Mean	Standard Deviation	Minimum	Maximum
4	*Pink1*−/−	15	11.56	10.22	0	30.00
	WT	15	1.18	3.12	0	9.38
6	*Pink1*−/−	7	11.36	8.51	0	21.43
	WT	6	1.96	4.80	0	11.76

WT = wildtype

Table 2

Summary table of specific abnormal swallowing behaviors for *Pink1−/−* and WT rats.

Measures	*Pink1−/−* Abnormal/total trials (% abnormal trials)	WT Abnormal/total trials (% abnormal trials)
Cough/gag	0/152 (0%)	0/97 (0%)
Head compensation	6/152 (4%)	0/98 (0%)
Stasis in pharynx	24/149 (16%)	2/98 (2%)
Stasis in proximal 1/3 esophagus	35/130 (27%)	4/84 (5%)

Values correspond to the number of abnormal trials out of all trials (% abnormal trials). WT = wildtype; % = percent