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Effects of supplemental oxytocin on feeding and swallowing maturation in rats

Shakera K. Fudge*

Comparative Medicine Program, University of Missouri, Columbia, MO, USA

ABSTRACT

Background: Pediatric dysphagia is a prominent feature of neurodevelopmental disorders, such as Prader–Willi syndrome (PWS). Dysphagia increases the risk of malnutrition, aspiration, and subsequent respiratory infections, highlighting the importance of improving the understanding and management of dysphagia.

Aim: The present study investigated the potential role of oxytocin (OXT) in advancing feeding and swallowing behaviors in postnatal day 0 (P0) to P42 (6-week-old) rats, with potential therapeutic implications for PWS. We hypothesized that OXT administered subcutaneously in Sprague Dawley rats within 12 to 24 hours of birth would accelerate the maturation of feeding and swallowing behaviors compared to naïve and saline-treated controls.

Methods: Importantly, the videofluoroscopic swallow study (VFSS) protocol was successfully adapted to evaluate rats as young as P21, broadening the application of this protocol beyond the previous limitation of 6-week-old rats. Using an adapted VFSS protocol for juvenile (P21–P35) and peripubertal (P36–P42) rats, feeding and swallowing maturation was objectively characterized using custom JawTrack™ software. Protocol adaptations included the refinement of oral contrast formulations for liquid and solid foods and the optimization of fluoroscope settings and equipment. Body weight and developmental milestones (e.g., crawling, walking, self-feeding) were also recorded.

Results

OXT modulated specific feeding behaviors in juvenile rats (i.e., lick rate and inter-lick interval). However, OXT did not significantly accelerate the attainment of developmental milestones in rats, and the selective effects on feeding behaviors were not observed to extend into the peripubertal stage.

Conclusion

The present study establishes a useful methodology for future research using our enhanced VFSS protocol. In light of these results, future research is well-positioned to expand our understanding of the potential of OXT to treat dysphagia in neurodevelopmental disorders.

Keywords: Behavior development, Neurodevelopment, Rats, Swallowing physiology.

Introduction

Pediatric dysphagia (difficulty swallowing) is a potentially life-threatening symptom prevalent among numerous neurodevelopmental genetic disorders (Lawlor and Choi, 2020) that elevates the risk of malnutrition (Miller *et al.*, 2011), respiratory infections, (Tan and Urquhart, 2017), and reduced quality of life (Mazaheri *et al.*, 2013). To address these challenges, in-depth research is necessary to improve our understanding of dysphagia in these complex disorders (LaMantia *et al.*, 2016, Malandraki and Arkenberg, 2021). The scope of this paper focuses on Prader–Willi Syndrome (PWS) as a representative example, in which clinical manifestations arise due to a deletion within the paternal chromosome 15, denoted as 15q11-q13 (Ledbetter *et al.*, 1981; Bittel and Butler, 2005; Butler, 2023). Although PWS features a spectrum of clinical abnormalities, profound hypotonia and hyporeflexia resulting in feeding and swallowing difficulty are defining characteristics (Zellweger and Schneider, 1968; Cassidy *et al.*, 2012; Angulo *et al.*, 2015; Butler *et al.*, 2019).

Dysphagia in PWS can manifest as delayed and/or weakened suckling and swallowing reflexes essential for survival during early development (Illingworth, 1969; Gross *et al.*, 2017; Salehi *et al.*, 2017; Butler *et al.*, 2019). The neuromuscular coordination involved in blocking the trachea while opening the esophagus during swallowing is particularly affected (Gross *et al.*, 2017). Dysfunctional coordination of swallowing and breathing limits the success of bottle/breastfeeding and often necessitates tube feeding to ensure adequate weight gain for development and survival (Bacheré *et al.*, 2008). As affected infants grow older, a gradual improvement in feeding and growth is often observed (typically around 9 months of age; Miller

***Corresponding Author:** Shakera K. Fudge. Comparative Medicine Program, University of Missouri, Columbia, MO, USA.
Email: *sfudge@bioqual.com*

et al., 2011). Additional symptoms associated with dysphagia become increasingly evident at this stage, including residual food/liquid in the pharynx, chronic aspiration during swallowing, and delayed esophageal transit (Gross *et al.*, 2017). Unfortunately, the precise mechanisms underlying PWS pathophysiology remain uncertain, and as such, effective treatments targeting dysphagia in PWS are lacking (Calandra-Buonaura *et al.*, 2021). However, recent research suggests that oxytocin (OXT), a neuropeptide primarily synthesized by the hypothalamus, may play a pivotal role in feeding and swallowing during early development (Schaller *et al.*, 2010; Meziane *et al.*, 2015; Miller *et al.*, 2017; Althammer *et al.*, 2022; Muscatelli *et al.*, 2022).

Clinical studies of patients with PWS (Swaab *et al.*, 1995) and histological studies of hypothalamic tissue in a mouse model of PWS (Schaller *et al.*, 2010) revealed decreased OXT levels and reduced numbers of OXT-producing neurons, respectively. Additionally, administration of a single subcutaneous injection of OXT within 24 hours of birth in PWS mice not only rescued them from early mortality but also enhanced sucking activity and encouraged normal weight gain (Schaller *et al.*, 2010). Similarly, Tauber *et al.* (2017) studied 18 infants (under 6 months of age) with PWS and observed that intranasal OXT administration for 7 consecutive days was associated with improved suckling and swallowing function in 88% of cases, as evidenced by assessments such as the Neonatal Oral-Motor Scale (NOMAS) and the videofluoroscopic swallow study (VFSS) (Tauber *et al.*, 2017). The NOMAS scoring system utilizes a standardized checklist of oral motor patterns (categorized as normal, disorganized, or dysfunctional) and facilitates objective evaluation through structured observation, with higher scores denoting more severe dysfunction in infants' oral motor patterns (Palmer *et al.*, 1993; da Costa and van der Schans, 2008). The aforementioned study of 18 infants with PWS reported a significant decrease in NOMAS scores (from 16 to 9; $p < 0.001$), signifying improved oral motor skills vital for effective ingestive behaviors. VFSS is recognized as the gold standard for diagnosing oropharyngeal dysphagia (Hamrang-Yousefi and Goyal, 2023) and employs a scoring system of nine pertinent items routinely used in clinical practice to objectively document the safety and mechanics of the oral and pharyngeal stages of swallowing (Tauber *et al.*, 2017). Tauber *et al.* (2017) reported that intranasal OXT was associated with improved VFSS testing results, as evidenced by decreased scores from 18 to 12.5 ($p < 0.001$), where a score of 11 indicates normal swallowing and 29 indicates maximum dysfunction (Tauber *et al.*, 2017). This promising preclinical and clinical evidence suggests a link between OXT and early developmental feeding and swallowing activities, underlining the need for further exploration in this area.

The methodology of the three-part, present study (Fig. 1) was designed to investigate the effects of OXT supplementation on the maturation of feeding and swallowing behaviors in rats across sequential developmental stages: neonatal (postnatal day 0 [P0] to P7), infantile (P8–P21), juvenile (P22–P35), and peripubertal (P36–P42). This preclinical study spanned from P0 to P42 (6 weeks) of age in rats, corresponding from birth to 14 years (neonatal to peripubertal stages) in humans (Ghasemi *et al.*, 2021). This parallel allows for a focused investigation into feeding challenges commonly experienced during early developmental stages in PWS, such as low birth weight, hypotonia, and poor suckling reflex (Grootjen *et al.*, 2022). During later developmental stages (akin to the juvenile and peripubertal phases seen in rodents), individuals with PWS often experience hyperphagia and obesity (Miller *et al.*, 2011; Hong *et al.*, 2021). The goal of our investigation is to establish methodological tools for use in healthy rodents that facilitate a deeper understanding of feeding and swallowing behaviors across various developmental stages. Given that dysphagia typically presents during the crucial early stages of development, we aimed to emphasize this time period during which the role of OXT may have the most impact.

Methods and Materials

Animals

Animal care was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Research, 2011). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Missouri (MU), which is USDA-approved and AAALAC-accredited. The full bodies of naïve Wistar cryopreserved rats ($n = 5$) were utilized during the pilot study to refine the technical settings of our fluoroscopic imaging system. The use of cryopreserved specimens enabled us to circumvent complications associated with the movement of live animals. In addition, the pilot study involved an in-house litter of Wistar rats ($n = 16$) to characterize the developmental stages and swallowing-related behaviors essential for refining our VFSS protocol. Following the pilot study, three Sprague Dawley (SD) rat breeder pairs were purchased from Envigo (Fredrick, MD) to generate eight litters totaling 103 offspring. For all live animals included in this study, the date of birth = P0. From the first 3 litters ($n = 46$), a subsample of 30 SD rats was used to refine the experimental protocol for VFSS testing. Rats were selected based on participation and performance during VFSS priming (i.e., behavioral conditioning). During the experimental phase of our study, five litters of SD rats ($n = 57$) were utilized for weekly VFSS testing, of which 42 were allocated to the OXT experimental study: OXT ($n = 19$), saline ($n = 10$), and naïve ($n = 13$) groups. The remaining 15 naïve rats were used to capture additional body weight and developmental milestone data (i.e., hair growth,

mature crawling, mature walking, ear and eye-opening, spout/bowl drinking, and solid food eating). These 15 naïve rats were also used to further optimize the VFSS protocol for enhanced visualization of bolus transit, including during the esophageal phase of swallowing. At P42 (6 weeks) following the final VFSS test, rats were humanely euthanized via exsanguination after intraperitoneal administration of ketamine and xylazine anesthesia. Lung tissue samples were collected to investigate dysphagia-related aspiration and potential links to observed feeding behaviors and respiratory health in rats. After histological processing, the samples were encased in paraffin blocks and stored in a dry area in the laboratory for future histological analysis.

Husbandry

Rats were housed in conventional caging with aspen shaving bedding (#322 Aspen Shavings 2.6 cu ft, NEPCO) under standard vivarium conditions (20.0°C–26.0°C, 30%–70% relative humidity, and a 12:12-hour standard light:dark cycle). Postweaned rats were group-housed (3–7 rats per cage, based on sex and weight) throughout the study and were fed *ad libitum* (Laboratory Rodent Diet 5008, Purina) and acidified water, except for overnight water restriction (8–12 hours) prior to VFSS testing to increase participation. Postweaning enrichment consisted of a polyvinyl chloride pipe or transparent red cubicle and one wooden chew block per cage. All rats were monitored daily to ensure overall health and well-being. Furthermore, standardized rodent health monitoring via our sentinel program was performed quarterly to detect unwanted pathogens. The testing panel of the sentinel program explicitly excluded the following pathogens: rodent coronavirus, sendai virus, pneumonia virus of mice, parvovirus, Theiler's murine encephalomyelitis virus, *Pneumocystis carinii, Mycoplasma pulmonis*, fur mites, and pinworms. All rats were housed in the same room for the duration of the study, but behavioral assays were conducted in a separate laboratory outside the vivarium.

The pilot study with cryopreserved and live Wistar rats

Establishing fluoroscopic settings and VFSS chamber size matching for preweaned cryopreserved Wistar rats

For the initial phase of the pilot study, cryopreserved Wistar rats (n = 5, ranging from ages P4–P21) were utilized to establish fluoroscopic settings and VFSS chamber sizes for preweaned rat pups. This strategy effectively minimized the challenges associated with the active movement of live neonatal and infant rats. The fluoroscope miniature block camera was also upgraded (XBC-KX10, Korea) with 30 and 60 frames per second (fps) capability, providing a timely assessment of the visual resolution needed for JawTrack™ analysis.

The two major fluoroscopic parameters adjusted were the source–image distance (SID) and the kilovoltage peak (kVp). The SID, which represents the distance from the X-ray source to the detector, is a pivotal factor influencing distortion and magnification in radiographs (Carla, 2022). The kVp is the peak voltage applied to the X-ray tube that accelerates electrons from the cathode to the anode in X-ray image generation (Carla, 2022). It determines the quantity and quality of the generated photons and influences the image contrast and penetration of the X-ray beam (Carla, 2022).

Adjustments to the SID required testing of the cryopreserved specimens at various distances (in centimeters) from the camera. The kVp setting was modified by incremental increases and decreases in voltage until the desired image quality was achieved. Various zoom levels and fluoroscope intensity settings were explored to determine the most favorable conditions for high-quality VFSS imaging to facilitate a detailed analysis of feeding and swallowing behaviors.

The second phase of the pilot study involved VFSS chamber selection, as the previously established VFSS protocol was designed to study adult rats aged P42 (6 weeks) and older (Lever *et al.*, 2015; Lind *et al.*, 2018; Welby *et al.*, 2020; Mueller *et al.*, 2022; Murphy *et al.*, 2022). We identified suitably sized chambers for preweaned rats from the existing collection of VFSS chambers for rats and mice. The selected chambers (Fig. 2), which were composed of translucent and radiolucent materials, were positioned on a remote-controlled lift table within a low-energy miniaturized fluoroscope (Glenbrook Technologies, Randolph, NJ). Each chamber was secured by two endcaps, creating a controlled environment for enhanced voluntary drinking and eating behaviors while reducing disturbances from behavioral distractions and exploratory tendencies. Rat age and size were both considered during the chamber selection process (Fig. 2). For rats up to P21 (3 weeks), mouse chambers were utilized because they were the most appropriate size for rat pups at this developmental stage.

Syringe feeding trials in Wistar rats

In the third stage of the pilot study, a single litter of in-house Wistar rats (n = 16) was used to characterize the developmental stages and swallow-related behaviors from (P1) to P42 (6 weeks) of age. To mitigate early life stress in pups and prevent stress-induced alterations in maternal behavior (Orso *et al.*, 2019), we cautiously introduced external elements to the environment. From P1 to P3, the rat pups and dam were exposed to two nestlet squares saturated with five ml of puppy milk replacer (Pet-Ag Inc., Esbilac Puppy Milk Replacer, Hampshire, IL) and placed at opposite ends of the home cage. Concurrently, 1–2 pieces of barium sulfate kibble (40% weight/volume, manufactured in collaboration with AFB International, St. Charles, MO) per rat were scattered across the home cage floor. Both the milk replacer and pellets were used to facilitate olfactory acclimation in preparation for subsequent syringe feeding trials and VFSS testing. Observations were avoided at P0 to allow sufficient time for the pups to

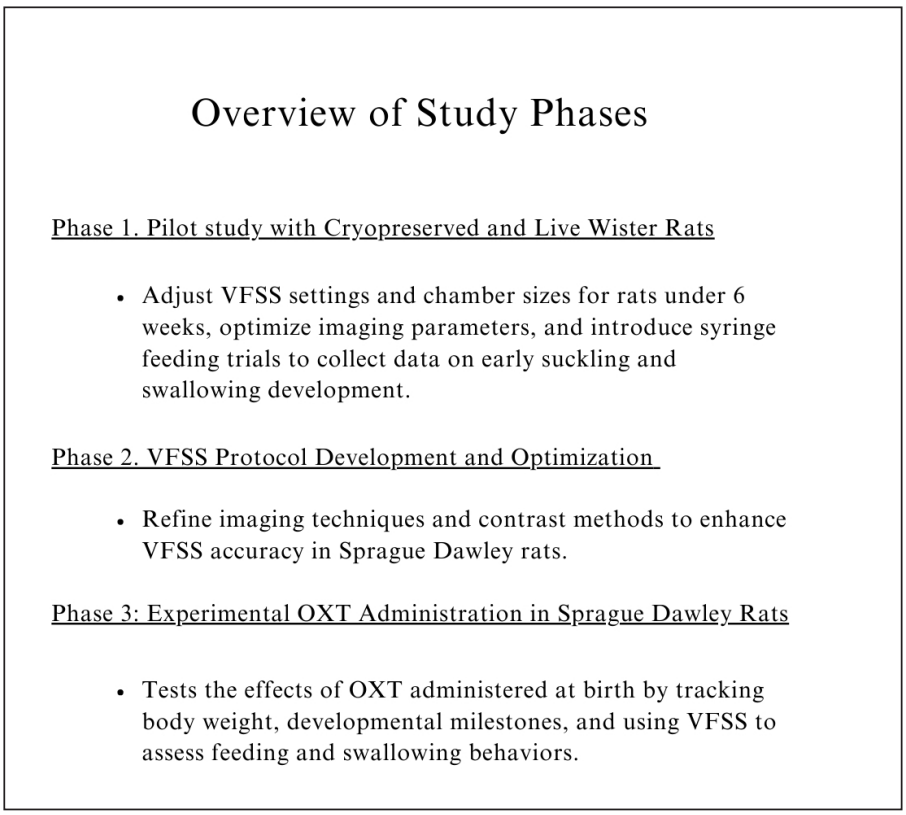

Fig. 1. Overview of the three-part study. This figure illustrates a three-part study to adapt and refine our established videofluoroscopic swallow study protocol for rats (up to P42 or 6 weeks) and assess the impact of oxytocin administration on feeding and swallowing development.

experience natural breastfeeding before commencing syringe-feeding trials.

The syringe feeding trials were initiated with rat pups during the neonatal and infancy stages, spanning from P4 to P10. The timing of this decision was made to further minimize maternal stress from early separation and to allow a gradual introduction of the pups to alternate feeding methods. The study protocol refrained from imposing fluid restrictions or separating rat pups from the dam before the feeding trials began. However, when a rat pup displayed reduced interest in syringe feeding, temporary fluid restriction (1–3 hours) was implemented to encourage suckling during syringe feeding trials. For the feeding trials, 0.5 ml of the puppy milk replacement was drawn into a 1-ml syringe for each rat pup. This volume was chosen to account for potential spillage and waste, even though the expected consumption per pup was only between 0.1 and 0.2 ml (Dunsey, 2021). A nipple (The Miracle Nipple, Mini PKG, Chris's Squirrels and More, LLC, Somers, CT) was securely attached to each filled syringe, followed by warming the filled syringe in a water bath on a stirring hotplate (Fisher Scientific, 11-600-49SH, Waltham, Massachusetts) set to 40°C in order to closely mimic the temperature of dam milk (37.7°C) (Otto *et al.*, 2015). Setting the water bath temperature slightly above the dam's body temperature accounted for slight cooling during each feeding trial. Each rat pup underwent 3 brief feeding trials lasting approximately 1 minute (within a maximum 1-hour feeding session).

During feeding trials, each rat pup was gently handled, and body temperature was measured using infrared thermometry in the abdominal area (HoMedics No Contact Infrared Digital Thermometer, TIE 240, Commerce Township, Michigan). The pups were placed in sternal recumbency on the palm of the hand (Fig. 3). The syringe nipple was positioned parallel to the rat pup's mouth, and a single drop of the warmed puppy milk replacer was slowly administered to the lips to gauge their interest at the start of each 1-minute feeding trial. Throughout this process, special care was taken to prevent overfilling the pup's mouth and to avoid inadvertent aspiration of milk through the nostrils. Body temperature was measured before and after feeding trials, and pups were returned to their home cage immediately following the final feeding trial. The experimental procedure commenced with the first trial for all rats, proceeded with the second, and concluded with the third within the allocated maximum

Fig. 2. Videofluoroscopic swallow study priming and testing chambers. At P21 (3 weeks) timepoint, the chamber used had dimensions of 14.4 x 5.0 x 5.0 cm. For the P28 (4 weeks) timepoint, the dimensions of the chamber were increased to 15.2 x 5.5 x 11.5 cm to suit the subjects' developmental needs. At the P35 (5 weeks) and P42 (6 weeks) timepoints, the dimensions were further expanded to 25.5 x 8.0 x 10.5 cm.

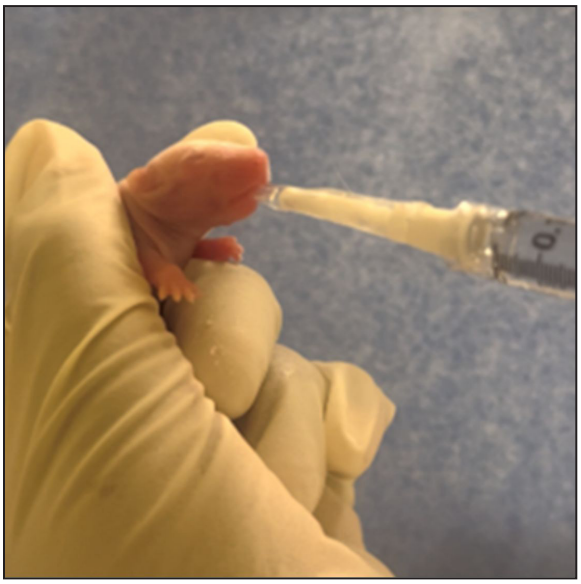

Fig. 3. Custom-made silicone nipple for syringe feeding trials. The image features the thin, transparent construction of the nipple, sized to accommodate the mouths of rat pups during neonatal and infant developmental stages.

timeframe of 1 hour. To ensure accurate tracking and identification of individual pups across the three trials, each pup was marked with a nontoxic indelible ink marker (Sharpie, Atlanta, GA). The process was systematically repeated for each pup in the litter, with gloves replaced between handling to avoid any stickiness caused by the milk replacer. Additionally, the dam's abdomen and mammary glands were gently rubbed with fresh gloves and the syringe nipple for scent adaptation prior to each syringe feeding trial for each rat pup.

Once the critical milestone of eye-opening was observed in the Wistar rats, VFSS priming was initiated to investigate the early onset of independent feeding and swallowing behaviors. The objective was to determine the earliest developmental stage at which the transition from suckling to bowl drinking occurs. The selected timeframe aimed to capture the initial instances of bowl drinking that emerge post-eye-opening. In addition, initially guided by observations from cryopreserved rats for the first P21 (3 weeks), efforts to size-match chambers continued during this period. At 4 weeks of age, a mouse chamber was deemed suitable for the size of the rat pups at this developmental stage (Fig. 2). At P35 (5 weeks) and P42 (6 weeks) ages, the rat pups were transitioned to the standard VFSS rat chamber (Fig. 2). The progressive increase in chamber size was correlated with the growth of the rats and was essential for adequate containment of the rats during VFSS assessment. Once consistent bowl drinking was demonstrated by the Wistar rats, weekly VFSS testing was performed to systematically evaluate drinking behavior as the rats progressed toward the peripubertal stage. The P11 timepoint was compared to the P(42) (6 weeks) timepoint within the same litter to track developmental progression and changes in feeding and swallowing behaviors over this period.

VFSS priming

Incorporating a silicone nipple for syringe feeding optimization

The pilot study revealed limitations with commercially available nipples, which were found to be unsuitably large and excessively firm for the rat pups. To overcome this challenge, the first 3 litters of SD rats were utilized to optimize the syringe feeding protocol using a customized silicone nipple from P4 until the onset of eye-opening. The custom nipple was inspired by previous research (Hoshiba, 1996; 2004) and involved the use of silicone (KE1950-A/B, Shin-Etsu Silicones of America, Inc., Akron, Ohio) to create flexible, soft, and size-matched nipples for the rat pups (Fig. 3). The process began by preheating the oven to 150°C. Equal portions (50:50) of the silicones KE1950 A and KE1950 B were mixed in a weigh boat. A 200 µl beveled pipette tip was then coated with this mixture using a cotton swab, ensuring that a thin layer without holes was present and that excess silicone beyond the pipette lip was avoided. The coated tip was heat-treated in the oven at 150°C for approximately 5 minutes, and the silicone drying process was verified by testing detachability from the pipette tip before it was

carefully peeled off. A 22-gauge needle was then used to puncture an opening at the tip, creating a functional hole to facilitate feeding.

VFSS protocol development and optimization in SD rats

Recalibration of fluoroscopic settings

Challenges with bolus visibility during VFSS analysis led to the re-evaluation of the camera used. During this phase of the study, we utilized the original version of the miniature block camera for the fluoroscope (FCB-IX11a; Sony Corporation of America, New York, NY) with 30 fps capability, which differed from the one used in the pilot study. Various SID and kVp settings were tested using the first 3 litters of SD rats so that optimal fluoroscopic parameters were tailored to the camera's imaging capabilities. These efforts ensured that high-quality imaging was obtained during the subsequent experimental study for VFSS data collection and analysis.

Optimization of contrast agents: exploration of iohexol and barium concentrations

The final naïve cohort of SD rats ($n = 15$) was utilized for the experimental exploration of varying concentrations of iohexol and barium sulfate within the thin liquid and dry, crunchy food consistencies, respectively. The goal was to improve bolus visualization and tracking during VFSS analysis. The existing barium sulfate kibble was manufactured via an extrusion process in collaboration with AFB International (St. Charles, MO), using the industry standard 40% weight/volume barium sulfate concentration. However, the manufacturing process markedly degraded the barium concentration in the final kibble product, as evidenced by the poor visibility during VFSS testing. Thus, in the present study, we collaborated with the Department of Grain Science and Industry at Kansas State University (Manhattan, KS) to formulate molded barium kibble with markedly improved contrast density. Similarly, the concentration of iohexol in the liquid contrast recipe was increased from 25% to 50% to improve X-ray visibility during VFSS testing and analysis.

Experimental OXT administration in SD rats

In the final phase of the study, we utilized three experimental groups of SD rats: naïve, OXT-treated, and saline-treated. The decision was made to begin the experiment on the day of birth (at P0). Although this approach diverged from the pilot study (where experiments were initiated at P1), the decision to begin at P0 more closely mirrors prior OXT rodent research (Schaller *et al.*, 2010). Furthermore, given our extensive experience with the SD rat strain, developed through frequent in-house breeding, we felt confident in our decision to temporarily separate dams from their pups for drug administration at P0.

Body weight and developmental milestones were important outcome measures for assessing the effects of OXT administration. Additionally, the introduction of a custom-made VFSS chamber at cage level (Fig. 4A) allowed us to identify the earliest instances of independent bowl drinking and pellet eating. Once these behaviors were observed, VFSS testing and analysis were conducted to investigate swallowing and feeding dynamics.

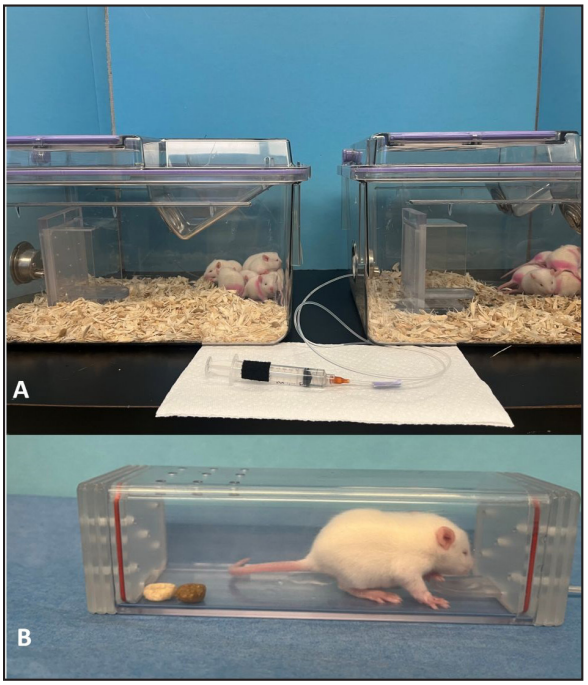

Fig. 4. Experimental setup for videofluoroscopic swallow study (VFSS) Priming. Representative images of in-cage group priming (A) and individual chamber priming (B) in preparation for VFSS testing. The NexGen™ Rat 1800 Allentown cages depicted in image (A) were lined with aspen shavings and included our customized rat pup priming chamber with peg bowl attached to a syringe delivery system for dispensing liquid. The custom mouse chamber (14.4 x 5.0 x 5.0 cm polycarbonate tube with removable endcaps) shown in image (B) was used for individual chamber priming.

Drug delivery

Twelve to 24 hours after birth (P0), SD rat pups were administered a single 20 µl subcutaneous injection of either isotonic saline or OXT (2 µg dissolved in isotonic saline; No.051-01, Phoenix Pharmaceuticals, Inc., Strasbourg, France) (Schaller *et al.*, 2010). This uniform dosage, irrespective of individual body weights, is consistent with veterinary practice in which OXT dosages are often based on species-specific needs rather than body weight (Papich, 2016). A total of 29 SD rat pups were injected. For the first experimental litter, all rats were administered OXT as a precautionary measure to avoid potential human error in experimental group assignment, as well as to ensure that all pups survived without adverse effects before proceeding with the study. For the 2 subsequent litters, the rats were assigned to either the OXT or saline group was conducted randomly for unbiased distribution. This allocation scheme resulted in 19 and 10 rats assigned

to the OXT group and 10 rats assigned to the saline group. To accurately track and identify individual rats, daily marking with a non-toxic indelible ink marker (Sharpie, Atlanta, Georgia) was employed until sufficient fur growth allowed for permanent marking using Carbol Fuchsin stain.

Body weight and developmental milestone monitoring

The SD rats underwent daily weighing for the first 8 days after birth (P0–P7), allowing for regular and frequent assessments to closely monitor for potential adverse effects of OXT administration. Although major adverse reactions of OXT were not expected, there were potential risks of bleeding from the subcutaneous injection as well as overall sedation secondary to OXT toxicity (Uvnäs-Moberg *et al.*, 1994). From P7 onward, rats were weighed every other day until P21, followed by weekly body weight monitoring until P42 (6 weeks) of age. In addition, the rats underwent daily monitoring from P1 to P21 for assessment of developmental milestones such as hair growth, mature crawling, mature walking, eye-opening, transition to spout/bowl drinking, and solid food eating. The developmental milestones were documented at first observation to ascertain progression and response following subcutaneous injections (OXT or saline).

Cage-level VFSS priming trials with experimental SD rats

Following the onset of eye-opening, priming trials were conducted once daily. The experimental setup during VFSS priming included temporary separation from the dam, after which the rats were group-housed according to the assigned treatment groups. During instances where participation in bowl drinking was not observed within 15 minutes, a temporary separation from the dam (up to 4 hours) was implemented to promote the pups' active involvement in the priming process. Leveraging insights from the pilot study with Wistar rats, we endeavored to identify the onset ages of developmental milestones in SD rats. To assess the onset of drinking and eating solid food at the cage level, a customized priming chamber was developed (approximately 1/3 of the standard rat chamber length with the associated rat standard peg bowl), as shown in Figure 4A. During the priming sessions, rats were introduced to this custom chamber designed for easy administration of a 15% sucrose liquid solution. Additionally, 1–2 barium sulfate kibble and 1–2 Cheerios per rat were provided to enable the observation of mastication. Rats that did not demonstrate in-cage bowl drinking were subjected to individual chamber priming sessions (Fig. 4B), each lasting 5 minutes. During individual priming sessions, rats were provided the same drinking solution and feeding material used during in-cage priming. Rats that engaged in mastication behavior without drinking from the bowl during priming sessions were not subjected to VFSS testing, as the primary focus of the study was to capture drinking events. Starting at weaning (P21), rats underwent VFSS testing at 7-day intervals, specifically on P21, P28, and P35, with the final test on P42, which marks 6 weeks of age, for subsequent JawTrack™ analysis.

VFSS testing

For VFSS testing, each rat was individually confined within the same appropriately size-matched chamber as used for VFSS priming. In order to minimize radiation exposure, the fluoroscope was activated only when the rat approached the bowl, as confirmed through a webcam positioned above the chamber (C920 HD Pro Webcam; Logitech International S.A., Lausanne, Switzerland). The fluoroscope was deactivated immediately after the rat turned away. Each rat underwent fluoroscopic assessment in the lateral plane. Approximately 30 kVp and 0.2 mA were utilized for up to 5 minutes, contingent on participation in bowl drinking and pellet eating. During the assessment, rats had access to th- oral contrast agent solution (30% sucrose and 100 µl of vanilla extract mixed with 25% iohexol. GE Healthcare, Inc., Princeton, NJ) and one barium sulfate kibble (40% weight/volume, manufactured in collaboration with AFB International, St. Charles, MO). Rats drinking and eating pellets were visualized via fluoroscopy in real-time and recorded as AVI videos at 30 fps using Pinnacle Studio 18 (Corel Corporation, Ottawa, Canada). Each video was trimmed into 3–5 second clips of uninterrupted drinking using Pinnacle Studio 24 (Corel Corporation, Ottawa, Canada), for a total of 10–15 seconds per rat. The VFSS clips were subsequently analyzed using JawTrack™ software as demonstrated in Figure 5.

Statistical approach

Statistical analyses were performed using SAS® software version 9.4 (SAS Institute Inc., Cary, NC, USA). Data outliers for each variable were identified and rechecked for accuracy but were not removed from the dataset. VFSS-dependent variables were analyzed using a generalized linear model (GLM) repeated measures ANOVA (RMANOVA; 3 groups X 2 timepoints), following initial verification of the ANOVA assumptions as well as univariate analysis confirmation that sex was not a contributing factor (i.e., no sex differences within groups, $p > 0.05$). Statistically significant main effects from the ANOVA model were investigated using Bonferroni-adjusted pairwise comparisons with one-sided two-sample *t*-tests, in alignment with our a priori directional research hypotheses. For body weight data, a generalized estimating equation (GEE) with an autoregressive working correlation structure was used to obtain the main effect of sex, group, and time on body weight, which is normally expected to be higher for males (*vs*. females) and older (*vs*. younger) animals. Statistically significant differences between group Least Squares Means (LSMeans) in the GEE model were investigated using Tukey-Kramer-adjusted pairwise comparisons for unbalanced data. Developmental milestone data were analyzed using the log-rank test. As a final

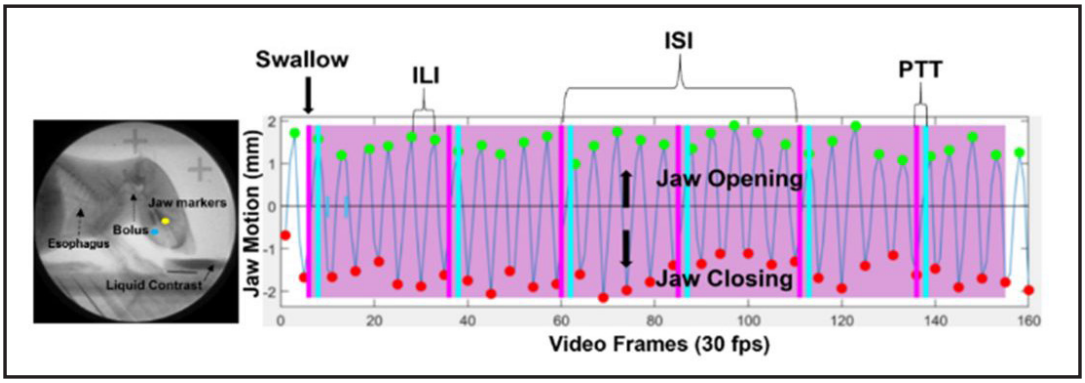

Figure 5. Quantitative analysis of videofluoroscopic swallow study (VFSS) metrics using JawTrack™ software. Radiographic images captured a Sprague Dawley (SD) 3-week-old rat drinking liquid contrast voluntarily from a bowl. Jaw tracking markers in yellow (upper jaw) and blue (lower jaw) were applied via JawTrack™ software. Representative jaw tracking plots displayed key events, including jaw maximum open (green dots) and closed (red dots) positions, within a 5-second analysis window following a swallow event (pink line). Manual event markers were used to indicate "swallow onset" (pink line) and "end of pharyngeal transit" (blue line). Additionally, the unlabeled metric, esophageal transit time (ETT) was evaluated in SD 3-week-old rats. VFSS metrics, such as inter-swallow interval (ISI), inter-lick interval (ILI), and pharyngeal transit time (PTT), were automatically derived from event marker data. Within each purple analysis window, the average values for each VFSS metric were calculated, with this process repeated for two separate video clips per rat (captured at 30 frames per second).

statistical approach, PASS 2022 (Software 2022) was used for sample size estimation as needed to guide future studies.

Results

For the pilot study, all 16 Wistar rats were retained to evaluate continuous suckling behavior, a critical component of VFSS testing in preweaned rats. Although we observed a modest increase in feeding participation (particularly in pups aged P8 to P10), the degree of improvement was not substantial. We encountered difficulties when encouraging the pups to sustain suckling for the required duration of one-minute (necessary for VFSS imaging). Furthermore, the pups displayed a marked disinterest in the puppy milk replacement administered during feedings. Overall, rat pup reluctance or inability to fully engage with the substitute feeding for the requisite one-minute duration hindered our ability to collect VFSS data.

This challenge prevented further development of a syringe-feeding delivery system for VFSS testing. Until the pups demonstrate a willingness to drink via hand syringe feeding, spending time on developing a hands-free syringe feeding system seems unwarranted, as the primary challenge remains the pups' acceptance of any alternative feeding method. The preliminary findings from VFSS testing**,** however, indicate age-dependent variability in feeding and swallowing behaviors among healthy Wistar rats. Notably, at P11 (which was the earliest point we could capture bowl drinking upon eye opening), rat pups (n = 4) exhibited a considerably slower lick rate compared to the 6-week timepoint. However, due to study design limitations, which did not involve radiographic contrast administrations at timepoint P11, we were unable to measure other VFSS outcome measures, such as swallow rate, inter-swallow interval, lick-to-swallow ratio, and pharyngeal transit time (PTT) (Table 1). The decision to forgo contrast at the P11 timepoint was made to minimize potential gastrointestinal symptoms (i.e., diarrhea) and subsequent weight loss during development. Although animal welfare was secured, this methodological decision limited the ability to obtain detailed quantitative data for these specific metrics during the neonatal and infancy stages of development. Additionally, regarding the preliminary results of pellet eating, further refinement of JawTrack™ is required for semiautomated analysis of mastication-related swallowing behaviors.

Gender comparisons did not reveal any significant differences in feeding and swallowing function in Wistar rats**,** suggesting that the maturation of these behaviors is consistent across both sexes at these developmental timepoints. However, it is important to note that due to the limited sample size, statistical power to detect subtle differences is reduced. Consequently, statistical analysis was performed using paired t-tests to accommodate the small sample size, allowing for a more sensitive assessment of within-subject changes over time.

VFSS protocol development and optimization with SD rats

Radiological parameters (such as SID and kVp) were optimized for juvenile and prepubertal-age rats (P21 to P42) as opposed to adult rats (P42 or 6 weeks and older). The SID for rats in the juvenile and prepubertal

age ranges was set at 13 cm, which is shorter than the SID of 14 cm used for adult rats. Correct scaling within the imaging field is essential for precise anatomical representation and minimal image distortion, particularly for younger rats.(Carla, 2022) The kVp for all age groups was uniformly set at 30 kVp. Troubleshooting the kVp is essential for image quality, improved contrast in soft tissues, and adequate photon penetration and exposure (Carla, 2022) in VFSS analysis of rats within the P21 to 6-week age range. However, difficulties encountered during syringe feeding attempts in neonatal and infant rats impeded the ability to gather VFSS data during the earliest developmental stages. Despite these limitations, the visibility of anatomical markers (i.e., jaw bones and molars) in radiographic images at P6 (Fig. 6B) is a promising sign. Overall, once the aforementioned feeding issues in pre-weaned rats are resolved, methodological improvements have established a foundation to support the expansion of VFSS testing in neonatal and infant rats.

During VFSS priming, rats showed a marked preference for sucrose vanilla solution, with the majority of pups actively participating in drinking it. This preference in dietary choice was further highlighted by a distinct favoritism for Cheerios over barium sulfate kibble. Such preferences influenced data collection at the P21 VFSS testing timepoint and in subsequent sessions. Notably, only a few rats attempted to eat barium sulfate pellets (20% concentration by weight) during both the VFSS priming and testing phases. The manufacturing process of the molded kibble enhanced the contrast density, thereby improving the visibility of the kibble on X-ray imaging. Additionally, this kibble was flavored with molasses and bacon to increase palatability for the rodents. The difference in manufacturing processes between extruded and molded kibble was a key factor in achieving the desired contrast densities for effective X-ray imaging.

The results of the VFSS imaging enhancements are shown in Figure 7. The use of a 50% iohexol concentration demonstrated superior imaging results compared to 25% iohexol, providing clearer and more detailed radiographic visualization of swallowing during VFSS. The original extruded barium sulfate kibble (with a 40% weight/volume concentration) exhibited a relatively faint appearance on X-ray imaging, posing challenges during attempts to track the bolus. Despite a reduction in barium content to 20% weight/volume, the kibble underwent a specialized molding process that compacted the barium and resulted in a dense composition. This manufacturing improvement allowed the bolus to be more easily

Table 1. Metrics and operational definitions for videofluoroscopic swallow studies (VFSS).

Lick rate (#/s):	Average number of jaw fully opened/closed cycles within a 3–5 seconds VFSS video clip.
Inter-lick interval (ms):	Average duration between consecutive lick cycles throughout the 3–5 seconds VFSS video clip.
Swallow rate (#/s):	Average frequency of swallows throughout the 3–5 seconds VFSS video clip.
Inter-swallow interval (ms):	Average time between consecutive swallows throughout the 3–5 seconds VFSS video clip.
Pharyngeal transit time (ms):	Average duration for the bolus to traverse the pharynx during the pharyngeal phase of swallowing.
Esophageal transit time (ms):	Average duration for the bolus to traverse the esophagus. Start frame: "rest frame" immediately preceding visible transfer of the bolus from the swallow trigger point. End frame: when the bolus tail enters the stomach.

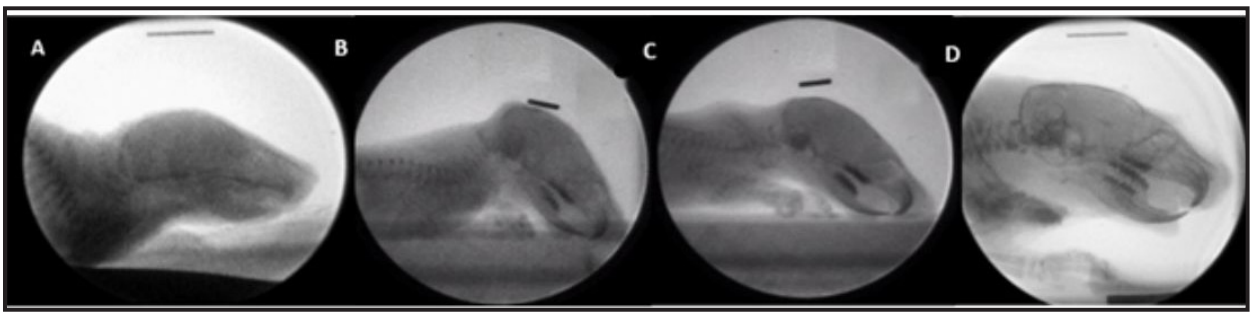

Fig. 6. Radiographic images of cryopreserved Wistar rat pups at key developmental milestones: P4 (A), P6 (B), P11 (C), and P21 (D), with external fiducial marker implants (0.6 x 0.8 mm stainless steel) evident in B and C. Images A and D also feature a 10 mm standard videofluoroscopic swallow study calibration marker for scale.

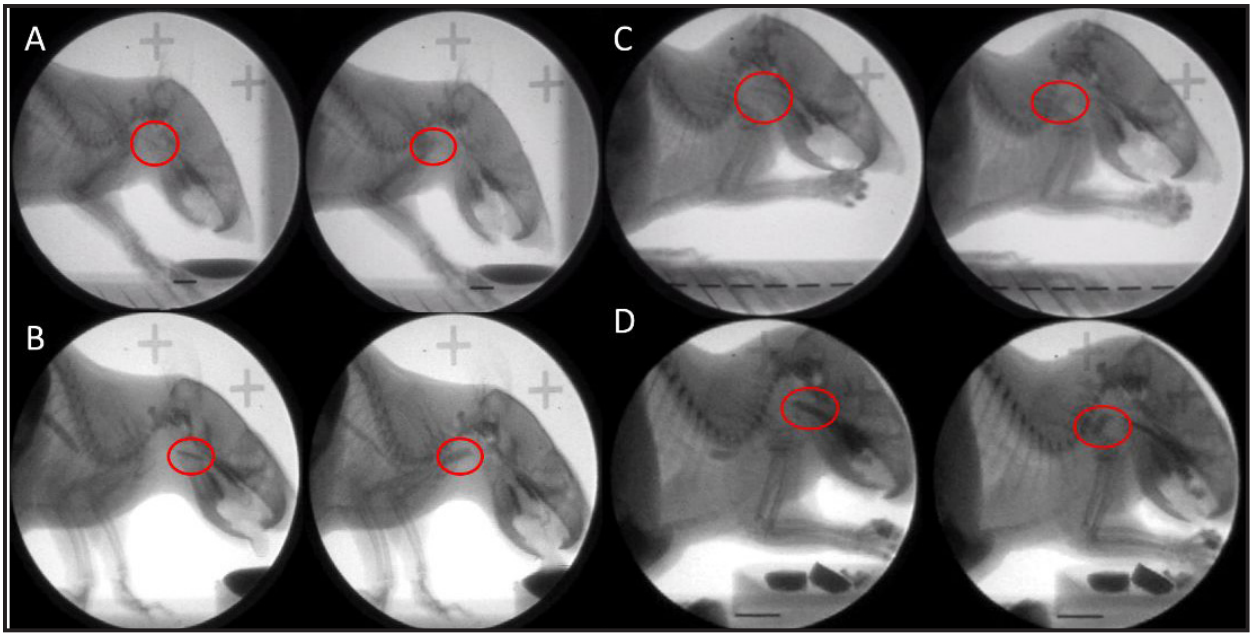

Fig. 7. Comparative visualization of enhanced contrast agents in videofluoroscopic swallow study. (A) and (B) Images contrast the effectiveness of 25% and 50% iohexol concentrations, respectively. The 50% iohexol demonstrates superior contrast during the visualization of swallowing mechanisms. (C) Image illustrates the original extruded barium sulfate kibble (40% weight/volume) and the limited visibility on X-ray imaging. (D) Image displays the improved barium kibble formulation with enhanced contrast density, offering clearer visualization on X-ray imaging.

detectable on X-ray. Lastly, the optimal SID and kVp settings necessary to capture the esophagus in 5 and 6-week-old rats have not yet been identified. Although the SID was adjusted to 23 cm and the kVp was set at 30 kVp to accommodate the larger size of these older rats, these settings were not fully effective and require further refinement to achieve quality imaging for this age group.

OXT supplementation in SD rats

Animals

A total of 42 rats were utilized for weekly VFSS testing. The study was structured into three groups: naïve, saline-treated, and OXT-treated. The naïve group originally consisted of 13 rats but was reduced to 8 due to technical challenges during software analysis. Specifically, we encountered corrupted files when attempting to open videos, experienced poor tracking, and faced challenges in placing markers for significant swallowing events, compromising the validity of the assessment and review process for these five rats. Ten rats were also originally assigned to the saline group; however, a reduction of two rats was necessary due to the small litter size (n = 2),which was critical to preserve experimental homogeneity. The OXT-treated group initially consisted of 19 rats, but it also underwent several adjustments: four subjects were excluded due to injection site leakage, four were removed due to insufficient VFSS data, and three were excluded due to software-related technical issues. These modifications resulted in uniform sample sizes across all experimental groups, with each group containing eight rats. Each group had an equal distribution of four males and four females, ensuring a balanced representation of both sexes. This uniformity across all experimental groups allowed for a more controlled and comparable analysis within the study, ensuring that the variations observed could be more confidently attributed to the treatment effects rather than sample size disparities.

OXT and developmental milestones

Differences in the attainment of developmental milestones (i.e., crawling, walking, and the emergence of a full coat of fur) were observed across the three experimental groups as indicated by the log-rank test. Although OXT treatment did not accelerate the overall developmental process (in comparison to saline-treated and naïve controls), certain nuances were observed. Both OXT and saline groups demonstrated a mean onset of crawling at 6 days, which was slightly later than the naive group, which had a mean onset at 5 days. Walking was observed earlier in the OXT group (at approximately 14 days), compared to a mean onset of walking at 15 days in both the saline and naive groups. The timing of eye-opening, with a mean occurrence at approximately 16 days, was consistent across all groups. The development of a full coat of fur in the OXT group occurred at 12 days: one day earlier than in saline and naive groups (at 13 days). All groups demonstrated bowl drinking and eating

of food on the floor at 17 and 19 days, respectively. Overall, the OXT group achieved the developmental milestones of walking and a full coat of fur earlier than saline-treated and naïve controls. Other milestones were achieved within a similar or slightly delayed timeframe compared to the saline-treated and naive groups. Despite minor variations in the attainment of developmental milestones, OXT treatment does not markedly hasten the overall progression of early development in young healthy rats.

An interesting observation during these experiments with SD rats revealed the potential impact of litter size on the achievement of developmental milestones. Rat pups born to small litters (e.g., two rats per litter) demonstrated variable developmental progression for behaviors such as crawling, eye-opening, and mature walking. One litter was evaluated prior to the decision to exclude small litters from the analysis due to these observed variations. This observation prompted us to standardize litter sizes within a range of 11 to 16 pups to simulate milk competition and ensure uniform development across all study subjects included in the experimental groups.

OXT and body weight

Statistical analyses (GEE; LSMeans) demonstrated no significant effect of OXT on body weight. An increased (albeit non-significant) amount of body weight was observed in OXT-treated rats compared with saline-treated and naïve controls (LSMeans = 5.02 *vs.* 2.89 and 2.13, respectively). The mean weight of OXT-treated rats at P0 was 6 g, which was identical to the saline group and slightly less than the 7 g recorded for the naïve group. At the outset of development, OXT treatment was not associated with a significant difference in body weight.

VFSS analysis: effect on swallowing function for naïve, OXT, and saline-treated rats

The GLM RMANOVA model revealed statistically significant results for only 3 of the 10 oropharyngeal-based VFSS variables: lick rate, lick-swallow-interval, and PTT. For lick rate, there was a statistically significant group X time interaction effect ($p = 0.027$) as well as a main effect of time ($p < 0.0001$) but not group ($p = 0.0898$). For inter-lick-interval (ILI), there was a statistically significant group X time interaction effect ($p = 0.0071$) as well as main effects of time ($p < 0.0001$) and group ($p = 0.0447$). Thus, both lick rate and inter-swallow-interval matured over time for all groups, as expected. However, ILI appears to be more sensitive than lick rate for detecting changes in tongue/jaw function. For PTT, there was a statistically significant group X time interaction effect ($p = 0.0004$) but no main effects of group or time ($p < 0.05$), indicating a significant difference in the regression line slopes among the three groups. This suggests that although there might not be an overall difference when comparing the groups at a single timepoint, a significant difference emerges when observing the change over time. In other words, even though the groups might start or end at similar levels, the rate of change (slope of the regression lines) is significantly different. We further investigated the observed group differences in lick rate, ILI, and PTT at only P21 (3 weeks) of age, as the boxplots (Fig. 8) suggested this earlier timepoint was the source of the main effect of the group. Indeed, pairwise comparisons showed a significantly higher (i.e., better/improved) lick rate for OXT-treated rats compared with saline-treated rats (mean difference of 0.525), as well as a higher but not significant lick rate for OXT-treated rats compared to naïve rats (mean difference of 0.35). Similarly, pairwise comparisons showed a significantly shorter (i.e., better/improved) ILI for OXT-treated rats compared to saline-treated rats (mean difference of 14.5) but not naive rats (i.e., a smaller but not significant mean difference of 8.38). Regarding PTT, no significant pairwise group comparison results were identified ($p > 0.05$). Power analyses showed that at least 14 rats per group would be needed for lick rate and 16 rats per group for ILI to detect a minimum of 0.35 and 8.5 mean difference, respectively, with a Bonferroni-adjusted significance level of 0.016 using one-sided two-sample equal-variance *t*-tests. In contrast, >70 rats per group would be needed to detect a minimum of 2.7 differences for PTT.

Discussion

In the present study, the VFSS protocol was successfully adapted to evaluate rats as young as P21, broadening the application of this protocol beyond the previous limitation of 6-week-old rats. The expanded scope of the VFSS protocol allows for a more detailed exploration of feeding and swallowing development in juvenile rats, especially during younger ages than previously investigated. This represents a significant contribution to pediatric neurological research and the study of dysphagia in this population. The VFSS protocol in the present study was able to detect essential anatomical structures (e.g., jaw bones and molars) from as early as P6 (Fig. 6). As a result of this finding, accurate tracking and visualization of jaw movements during swallowing are now possible at the neonatal stage of development. Early detection of swallow dysfunction via VFSS analysis is instrumental for phenotyping disease models of dysphagia, particularly for conditions such as PWS and other pediatric neurological disorders. These advancements in the VFSS analysis protocol offer the capacity for detailed phenotyping, setting new standards in dysphagia research and improving early detection and comprehensive disease management. Moreover, there is significant translational potential for this research, as it can inform new therapeutic approaches for pediatric dysphagia. Overall, our VFSS protocol's adaptation to juvenile rats represents a critical stride toward understanding and addressing the complexities of pediatric feeding and swallowing

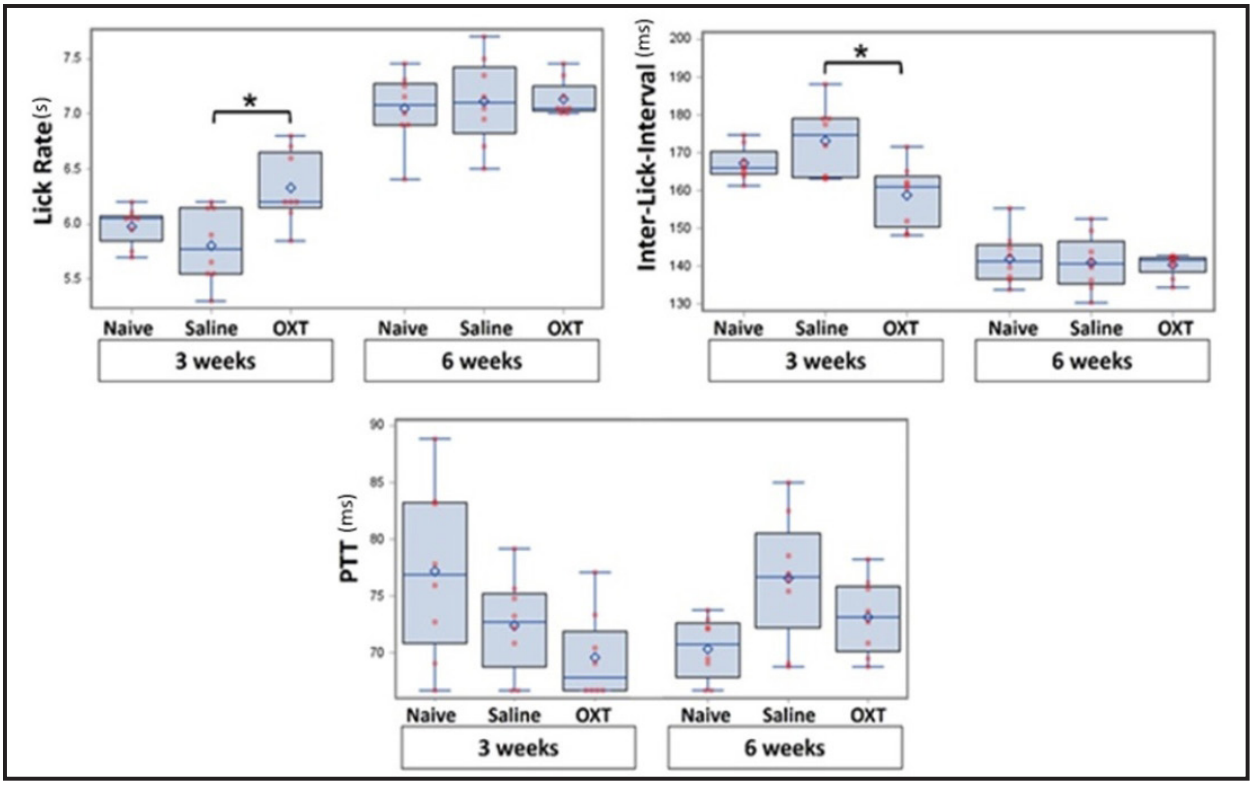

Fig. 8. Effect of oxytocin (OXT) on feeding and swallowing parameters. This figure highlights differences in lick rate, inter-lick-interval (ILI), and pharyngeal transit time (PTT) between experimental groups (n = 8 for each group). OXT treatment increased lick rate and reduced inter-lick-interval at P21 (3 weeks) of age, in comparison to saline-treated rats. Although PTT demonstrated significant group–time interaction effects, no distinct pairwise group differences were observed.

disorders, offering hope for earlier and more effective interventions in the future.

A key challenge during the development of the VFSS methodology was establishing the differentiation of radiographic contrast agents from bone and soft tissue. It was observed that rat skeletal elements are fully ossified just 2 days after birth, which is consistent with existing research (DeSesso and Scialli, 2018). As such, the appearance of bone on X-ray imaging does not change significantly with age beyond P6 (Fig. 6), suggesting that VFSS testing is possible as early as P6 in rats. However, the structure and behavior of neonatal and infant rats presented considerable limitations to the use of fluoroscopy. The small anatomical size necessitates the optimization of fluoroscopic settings (particularly the SID and kVp) to enable accurate visualization of essential anatomical structures. The inherent restlessness and spontaneous movements characteristic of neonatal and infant rats challenge the clarity and consistency of VFSS imaging.

Significant advancements were made to enhance the visibility of the contrast agent on VFSS. The initial utilization of extruded barium sulfate kibble proved to be suboptimal due to a relatively faint appearance on X-ray. This observation prompted the adoption of a denser, more detectable formulation of molded barium kibble. Bridging these advancements with future research objectives, a key direction will be the inclusion of mastication analysis. The additional focus on mastication is important given the unique biomechanical and neuromuscular complexities involved, distinct from liquid swallowing. The aforementioned advancements concerning the visibility of the bolus utilizing the barium sulfate kibble will allow for successful VFSS tracking and analysis of masticatory behaviors. However, it is important to note that the analysis of eating behaviors requires further innovation, which highlights a limitation of our current JawTrack™ software. Developed primarily for analyzing drinking behaviors, this software necessitates additional development to effectively analyze eating behaviors.

Future directions and limitations

Administration of OXT antagonist for VFSS

The utilization of an OXT antagonist is a logical next step in our research, as it could offer valuable insight into the role of OXT on developmental feeding behaviors such as suckling, bowl drinking, and self-feeding. Such an approach may not only shed light on the role of OXT during development but could also

suggest potential pathways for targeted intervention toward specific deficits observed in developmental disorders related to feeding and swallowing. Employing pathological models, such as those with neurological challenges (i.e., PWS), could provide a more responsive context for observing OXT's effects. The use of an OXT antagonist (i.e., Atosiban) with a short half-life (~18 minutes) (Albrecht *et al.*, 1999) will allow for the investigation of OXT's role during feeding and swallowing behaviors. It is beneficial to use a compound with a short half-life such as Atosiban because of its temporary effects, particularly in experiments with healthy rats. This characteristic guarantees that any changes in behavior or physiological responses caused by the antagonist are short-lived. Consequently, the healthy rats can quickly return to their normal state after the experiment.

Due to the short half-life, the experimental timing of OXT antagonist administration requires careful planning during VFSS testing. We propose that administration of the antagonist ~5 minutes prior to VFSS will allow for the observation of acute effects of OXT modulation and the impact on feeding patterns and swallow frequency under the antagonist's influence. The initial focus will be on rats at P21, a stage where feeding and swallowing behaviors are decisively established, albeit still evolving. This choice of developmental stage builds on previous positive findings that demonstrate an effect of OXT on licking behaviors at this timepoint and subsequently provides a platform for observing the effects of OXT antagonism. Moreover, we aim to expand our research to encompass earlier developmental stages (including the neonatal and infant phases) to fully characterize the role of OXT in feeding behaviors during the full trajectory of development.

However, there are unique challenges specific to neonatal and infant rats, such as injection site leakage. This problem became apparent when administering subcutaneous OXT (and saline) to pups aged 12 to 24 hours: the active movements intrinsic to these rat pups resulted in solution leakage at the injection sites, which challenged the consistency of dose delivery. This difficulty is not uncommon in biomedical research involving neonatal models (Linakis *et al.*, 2016). Small anatomical size, increased fragility, and inherent physical activity make the safe and precise administration of drugs a demanding task. As we progress with plans to administer experimental OXT antagonists to preweaned rodents, we will need to refine our current subcutaneous injection techniques or explore alternative routes of administration, such as intraperitoneal, intranasal, or intramammary (for lactational transfer through the dam) to achieve consistent and reliable drug delivery.

Refining syringe feeding techniques in preweaned rats

In the present study, the VFSS protocol aimed to observe the feeding and suckling behaviors of preweaned rat pups. A pilot study with Wistar rats investigated the utility of syringe feeding for VFSS data collection and initially utilized commercially available nipples. Unfortunately, the artificial nipples were less effective than intended as rat pups demonstrated a marked preference for normal nursing behavior with the dam. Unlike protocols for orphaned rats (Dunsey, 2021) where syringe feeding was the sole option, the experimental protocol in the pilot study presented rat pups with a choice, negatively impacting the success of syringe feeding. The rat pups' instinctive comfort and familiarity with natural nursing adversely affected the ability to gather VFSS data during the neonatal and infant stages. However, efforts are underway to enhance the syringe feeding methodology by exploring the integration of a pressure/squeeze sensor within a custom silicone nipple, drawing inspiration from technologies that have proven successful in feeding applications for human infants (Barlow *et al.*, 2010). The sensor is expected to provide additional measurements of rat pup feeding dynamics, such as the pressure exerted during suckling and the frequency of feeding sessions. This non-invasive experimental approach presents an alternative to the regular use of VFSS. By minimizing our reliance on VFSS, we aim to significantly reduce radiation exposure commonly associated with these studies.

Conclusion

In conclusion, our preliminary work sheds light on the multifaceted role of OXT during the development of feeding and swallowing behaviors in SD rats. The impact of OXT on specific behavioral metrics such as lick rate and ILI, highlights its potential influence on developmental processes. However, the transient nature of these findings highlights the complexity of OXT's role, emphasizing the need for further in-depth research to unravel its broader implications and applications. Moreover, our work has been instrumental in establishing a foundation for future research, especially due to the optimization of VFSS methodologies and related assessments in young rodent models. In particular, the ability to distinguish contrast agents from bone and soft tissue represents meaningful steps forward for VFSS optimization.

The present study, therefore, not only sheds light on the impact of OXT in developmental biology but also provides tools to open new avenues for future research. The potential applications of these findings in therapeutic contexts, especially for neurodevelopmental disorders and conditions like PWS, are particularly promising. As we continue to explore and refine our methodologies, we anticipate that future studies will build upon this groundwork and continue to enhance the understanding of OXT's role during development

and characterize the therapeutic potential of OXT for various medical and behavioral conditions.

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Conflict of interest

The author has no conflicts of interest to declare.

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Data availability

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

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