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ORIGINAL ARTICLE

Genes, Brain and Behavior

The role of cerebellar *FOXP1* in the development of motor and communicative behaviors in mice

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Abstract

The gene *FOXP2* is well established for a role in human speech and language; far less is known about *FOXP1*. However, this related gene has also been implicated in human language development as well as disorders associated with features of autism spectrum disorder (ASD). FOXP1 protein expression has also recently been identified in the cerebellum—a neural structure previously shown to express FOXP2 protein. The current study sought to elucidate the behavioral implications of a conditional knock-out of *Foxp1* using an *En1-Cre* driver, which is active in the entirety of the cerebellum and a subset of neurons in the midbrain and spinal cord, in mice using a test battery including motor tasks associated with cerebellar dysfunction, as well as communicative and autistic-relevant behaviors. Male and female mice with a conditional knock-out (cKO, $n = 31$) and wildtype littermate controls (WT, $n = 34$) were assessed for gross and orofacial motor control, motor-coordination learning, locomotion, social behavior, anxiety, auditory processing and expressive vocalizations. Overall results suggest *Foxp1* plays a specific role in the development of communicative systems, and phenotypic expression of disruptions may interact with sex. Robust motor deficits associated with Foxp1 protein loss may particularly affect vocalizations based on significant orofacial motor deficits in cKO subjects could also contribute to vocalization anomalies. In summary, the current study provides key insights into the role of *Foxp1* in cerebellar function and associated behaviors in mice, with implications for an improved understanding of communicative and motor-based neurodevelopmental disabilities in humans.

KEYWORDS
behavior, cerebellum, conditional knockout, en1 cre, foxp1, genetics, motor, mouse, sex differences, transgenic

1 | INTRODUCTION

FOXP genes encode proteins that belong to a subfamily of the forkhead-box containing transcription factors. FOXP proteins regulate essential developmental processes in various organs, including the lung, heart and nervous and immune system.[1–5] Systemic loss of *FOXP* gene products often results in embryonic lethality showing the critical role of FOXP genes.[6] Unlike the other FOX proteins, members of the FOXP subfamily can form homo- and hetero-dimers to bind to DNA at forkhead box-binding sequences.[2,4,6,7] *FOXP1* and *FOXP2* are also

known to interact with *TBR1*, a neural transcription factor implicated in autism spectrum disorder and related developmental language disorders.[4] *FOXP1* disruption has been directly tied to autistic-like phenotypes such as global developmental delay, impaired speech and language abilities and impaired intellectual disabilities.[8] Mutations of *FOXP2*, which was once labeled as "the language gene," are linked to disruptions in human speech and language.[6,9,10]

At the molecular level, FOXP1 protein is specifically involved in monocyte differentiation and macrophage function,[5] and complete systemic loss of FOXP1 is associated with significant heart defects that are prenatally fatal.[7] *FOXP1* is categorized as a gene-dosage-sensitive gene.[2] Heterozygous mutations of *FOXP1* are associated with broad neurodevelopmental symptoms including but not limited to global developmental delay, intellectual disability, features of autism spectrum disorder and impaired speech and language abilities.[3] In contrast, a murine homozygous knockout of *Foxp1* is embryonically fatal in mice,[7,11] a more severe outcome. *FOXP1* is also considered to be one of the top five most prevalent autism spectrum disorder (ASD) risk genes[12] and typically affects expressive language (speech and writing abilities) at a higher rate than receptive language (listening and interpretation[1]). Interestingly, these effects are seen despite a lack of obvious structural brain anomalies.[1,2,4–7,13,14]

In humans, the mutation is referred to as FOXP1 Syndrome, characterized by facial deformities, developmental delay and mild to moderate intellectual disorders.[1,2,5] This syndrome is further associated with fine and gross motor coordination deficits,[1] expressive speech delay of up to 1 year of age[5,15] and articulation problems leading to minimally verbal patients and specific trouble in pronouncing consonants.[5,15] FOXP1 Syndrome is typically considered to fall along the autism spectrum disorder spectrum based on the presence of repetitive behaviors and reduced social activity.[7] Patients also display clinically significant attention deficit hyperactivity disorder (ADHD) symptoms, including inattention, hyperactivity and impulsivity.[2,5,7,16] FOXP1 Syndrome results from a specific mutation, however, other mutations of the gene can cause manifestation of some, yet not all, of these pathologies. Therefore, it is important to characterize the function of the *FOXP1* gene throughout different brain systems.

Mouse genetic studies have shown that *Foxp1* is required for motor neuron specification,[6] migration[5] and differentiation[2] in the motor system. *Foxp1* also influences motor neuron migration to various efferent targets,[2] and may play additional roles in motor neuron development via other genetic pathways. For example, FOXP1 affects motor neuron diversification by modulating HOX proteins and PITX3 protein and influences neuronal migration by gating reelin signaling pathways.[14] *Foxp1* is also thought to be involved in pathways which regulate embryonic morphogenesis,[7] and in humans, some studies show a trend toward macrocephaly in FOXP1 syndromic patients.[5] Finally, conditional knockout of *Foxp1* in motor neurons of mice causes impairments in limb coordination during motor movement.[17] We have recently discovered that *Foxp1* is expressed in a subset of Purkinje cells and deep cerebellar nuclear neurons,[18] hinting at Foxp1's importance to the development and function of sensorimotor circuits.

Other rodent studies indicate strong pre-pulse inhibition deficits reflecting impaired sensorimotor integration in animals with knockdown of *Foxp1*.[7] In birds, *Foxp1* knockdown prevented juvenile birds from learning and mimicking adult songs but did not affect song learning acquired prior to gene manipulation.[12] Investigators also found a significant sex difference in gene effects on communication in another avian sample consistent with sexual dimorphism already present in the species, wherein males contain more FOXP1 protein within the cerebellum than females and therefore are more affected by a *Foxp1* mutation.[19] This suggests that the role of *Foxp1* in the communication of these species is linked to the integrity of the gene. These studies taken all together support a key role for *FOXP1* in human speech and language development,[5] and suggest specific FOXP1 modulation of cerebellar motor circuitry is critical to these functions.

In the current study, we aimed to explore the behavioral implications resulting from a regionally specific deletion of *Foxp1* in the developing mouse brain. Specifically, we targeted our deletion to regions co-expressing the gene *En1*—an important gene in hindbrain development—by combining a *Foxp1*-flox line with an *En1*-cre line to generate cKO subjects. These genes co-express pre- and postnatally predominantly in the mouse pre-pontine hindbrain (Allen Developing Mouse Brain Atlas, Seattle, WA 98109), but also show low levels of developmental co-expression in the midbrain. The resulting cKO model exhibits Foxp1 protein loss in all of the cerebellum, along with several other hindbrain (e.g., periaqueductal gray, reticular formation) and midbrain (e.g., tegmentum, inferior colliculus, superior colliculus) structures. Given *Foxp1*'s influence on motor neuron differentiation and migration, combined with complete cerebellar deletion, we hypothesized that *Foxp1* cKO animals would show deficits in motor tasks such as rotarod, wire hang and oral-motor lickometer tasks. We were also interested in the secondary impact(s) of protein loss on communication, given communication deficits in FOXP1 syndromic patients, as well as the gene's involvement in speech and language disorders in humans. For example, the cerebellum has become a focus of researchers who are looking at the error-correction mechanisms of language,[20,21] and in dyslexia patients, decreased cerebellocortical connectivity suggests a link between the cerebellum and the ability to correctly identify spelling and grammar mistakes.[22] Results showing concurrent motor and vocalization deficits in our cKO mouse model could support hypotheses that communicative impairments associated with disruption of FOXP1 may specifically relate to motor aspects of human speech and language. On the other hand, given the regional specificity of our KO mainly to the pre-pontine hindbrain, we hypothesized a lack of cognitive or social behavioral impairments in our cKO mice. Such findings could aid in dissociating cognitive, social and motor contributions to communicative deficits associated with FOXP1.

2 | METHODS

2.1 | Subjects

Mice (total $n = 61$; cKO M/F = 15/14, WT M/F = 19/13) were bred at the University of Connecticut Farmington Campus Cell and

Genome Sciences Building (400 Farmington Ave, Farmington, CT 06032). *En1*$^{cre/+}$ (JAX:007916) and *Foxp1*$^{F/F}$ (JAX:017699) mice were obtained from the Jackson Laboratory. Intercrosses between *En1*$^{Cre/+}$; *Foxp1*$^{+/F}$ and *Foxp1*$^{F/F}$ mice were carried out to generate *En1*$^{Cre/+}$; *Foxp1*$^{F/F}$ (*Foxp1*-cKO, referring as cKO) and littermate controls (phenotypically wild-type, referring as WT). Foxp1 and En1 developmentally co-express in only two regions—midbrain and the prepontine hindbrain (including the cerebellum; Allen Developing Mouse Brain Atlas, Seattle, WA 98109). Thus, gene deletion in cKO offspring was restricted to these regions. Newborn pups were housed together with the dam and littermates in an *Optimice* rack (Animal Care Systems Inc., Centennial, CO) until weaning (postnatal day (P)21). Pups were individually identified through foot tattoos prior to performing vocalization recordings (below) using animal-safe tattoo ink (Ketchum Animal Tattoo Ink, Ketchum Mfg. Co., Lake Luzerne, NY). At weaning, ear tissue samples were obtained for genotyping. All experimental subjects (cKO or WT) were single-housed in standard mouse tubs at P35 (12 h/12 h light/dark cycle) with food and water ad libitum. Heterozygous weanlings were returned to the breeding colony. All behavioral experiments and animal manipulations were performed according to protocols approved by the University of Connecticut Institutional Care and Use Committee (IACUC).

2.2 | Pup vocalization recording (P5–15)

Starting at 5 days after birth (P5), distress vocalizations during separation from the dam were recorded for each pup, using an ultrasonic microphone (Brüel & Kjær Type 4954-B microphone, Nærum, Denmark). To obtain the 5-min recording, pups were individually removed from their mother and placed in a standardized mouse tub with shredded pine bedding and a cotton nestlet. Pups were settled atop a heating pad to prevent hypothermia. The microphone was set at 192,000 Hz, connected to an RME Fireface UC audio interface (RME Audio, Haimhausen, Germany), and was placed 5 cm above the top of the Plexiglass tub. Sound files (.wav) were recorded using DIGICheck 5.92 (RME Audio, Haimhausen, Germany). This procedure was repeated for three other sessions on P7, 9, 15. Pups were identified based on tattoos which were refreshed following each recording session to avoid fading. Data were collected and analyzed through the use of MUPET (Mouse Ultrasonic Profile ExTraction),[23] including number of vocalizations, duration of vocalizations and types of vocalizations based on Heckman et al. (2016).[24]

2.3 | Rotarod (P40)

Mice were assessed for motor coordination and motor learning using a rotarod task. Subjects were placed on a cylindrical drum (6 cm diameter; 7.5 cm length, 20 cm from top of the table) which rotated at an accelerating rate, starting at 4 rotations per minute (RPM) and increasing up to 40 RPM over a period of 2 min. This task continued for 5 days, with four trials per subject per day. The latency to fall from the rotating drum was recorded in seconds and averaged across trials per day.

2.4 | Wire hang (P47)

Mice were assessed for paw strength and coordination through a wire-hang task. In this task, a standard mouse tub hopper was flipped upside down so the mouse was sitting on the grate with all four paws on the metal. The apparatus was then gently shaken for ~1 s to ensure that the mouse was gripping onto the metal. Then, the hopper was flipped so that the mouse was hanging upside down from the metal grating, which sits ~25 cm from the top of a table. The latency to fall from the metal grating was recorded in seconds.

2.5 | Lickometer (P48)

Mice were assessed for orofacial coordination and movement using a lickometer task. Here, mice were placed on top of a wire mesh floor with an affixed metal alligator clip. Inside the chamber, a graduated water bottle (filled with sugar water) was affixed to the side of the chamber and connected to the other end of the alligator clip. When the mouse touched the spout of the water bottle it completed a circuit, generating a recording (Acknowledge 4.1 program, Biopac Systems, Goleta, CA). The number of times the mouse touched the water-spout, the duration of each bout of licking (seconds) and how much water was consumed (milliliters) over the 10-min recording session were recorded.

2.6 | Open field (P51)

An open field task was used to assess anxiety-like behavior and spontaneous locomotor behavior. During the task, subjects were placed in the center of a clean square box with grid floor, high side walls, and no top (50 cm × 50 cm × 50 cm). Subjects were videotaped from above and allowed to roam freely throughout the box for 10 min. TopScanLITE (CleverSys, Reston, VA) was used to process video feed, virtually segregating the floor grid into four regions (center-inner, center-outer, outer-inner, outer-outer) starting from a box in the center and moving concentrically outward (each region taking up a quarter of the entire area of the box). A map record of subject movement was generated, and TopScanLITE extracted time spent, distance traveled, velocity and latency to enter each of the four virtually defined regions for the session.

2.7 | Three-chamber social task (P61)

Mice were assessed on social interest using a modified three-chamber social task. This task tests a mouse's innate preference for a conspecific over a novel object over two trials. After completing a 5-min

habituation period, subjects were placed in the center of a three-chambered testing box (overall: 40.5 cm × 62 cm × 23 cm; individual chambers: 40.5 cm × 20 cm × 23 cm) and allowed to freely explore all chambers for 10 min. During this session, a novel same-sex conspecific was contained within one of the chambers in a small, cylindrical cage (7.5 cm diameter; 8 cm height) while the other chamber on the opposite side of the box contained a novel toy in an identical cage (Trial 1: colored legos; Trial 2: orange torpedo toy). The middle chamber, where the subject was initially placed in the box, was empty. Using video tracking and TopScan LITE (Clever Sys Inc., Reston, VA), the percentage of time spent interacting with the novel objects (mouse vs. toy), as well as the number of entries and the amount of time spent in the chamber without interacting with the objects, was recorded and analyzed for each subject. This was repeated the following day with the mouse and toy chambers swapped to control for side bias. In this second trial, a new mouse and toy were used to prevent habituation. Time spent interacting with each object, versus the amount of time spent in each chamber *regardless of interaction* was recorded in seconds.

2.8 | Adult vocalization recording (P65)

Vocalizations were recorded again for all subjects once they reached adult age. These vocalizations were used to assess socio-communicative ability. For female subjects, a single female subject was placed in a clean standard mouse tub (28 cm × 16.5 cm × 12 cm), along with another female subject of the same genotype. Recorded vocalizations were attributed 50% to each mouse. Vocalizations were recorded using an ultrasonic microphone (Brüel & Kjær Type 4954-B microphone, Nærum, Denmark) over 5 min. During the recording the microphone was set at 192,000 Hz and connected to an RME Fireface UC audio interface (RME Audio, Haimhausen, Germany) and placed 5 cm above the top of a Plexiglass tub. Sound files (.wav) were recorded using DIGICheck 5.92 (RME Audio, Haimhausen, Germany). For male subjects, a non-littermate WT female was placed in the standard mouse tub along with each male subject. In this configuration, females typically do not vocalize, allowing us to infer that all recorded vocalizations were emitted from the male.[25] Vocalization sound-files were analyzed in MATLAB (MathWorks) using MUPET (Mouse Ultrasonic Profile ExTraction)[23] to extract attributes such as a total number of vocalizations, durations of each syllable and type of syllable (see Heckman et al., 2016 for review and definitions of each syllable category).[24]

2.9 | Water escape (P79)

Subjects then underwent a water escape task to evaluate any confounding results when analyzing those of the 4/8 radial swim test, a more complex task than water escape. In this task, mice were placed in a galvanized oval steel tub (103 cm × 55.5 cm) and required to swim the length of the tub to a visible platform (8.5 cm diameter) resting 2 cm above the water level, providing a dry escape area for the animals. Latency in seconds to reach the platform was recorded for four trials and occurred on 1 day. Across these four trials, two random trials required the mice to swim left to the remaining to the right to control for side bias.

2.10 | 4/8 Radial arm water maze (P82)

Next, a 4/8 radial arm maze was employed to assess spatial reference and working memory ability. The 8-arm stainless steel configuration is placed in a large circular tub filled with water, with 8 open "arms" stemming from a circular central area. Four of these arms are "baited" at the end (i.e., contain a hidden escape platform), whereas four arms are never baited with a platform. Configuration of goal arms were counterbalanced between subjects but remained fixed per subject across all test sessions. Extra-maze room cues were provided, and the locations of these cues remained consistent for all subjects. Subjects were given a training session (consisting of four training trials) where all non-baited arms were blocked, leaving only the four baited arms open. Upon finding a platform, the arm would be closed off and the mice would have to find all platforms before the training trial was complete. Subjects were given 120 s to find each arm, wherein an experimenter would guide the mouse to the platform if not reached in time. Once on the platform, mice remained there for 20 s and then were removed to their home cage for a 30 s inter-trial interval (ITI). Testing began with all eight arms open, but only four baited. Mice were placed in the center and allowed to freely visit any arm, with the discovery of a platform ending a trial (or 120 s, whichever came first). Each of four trials were separated by an ITI. However, instead of blocking the goal arm of the most recently located platform, the platform was removed during the ITI but the arm left open. This allowed us to measure working memory errors (erroneous visits to previously baited but visited arms), as well as reference memory errors (erroneous visits to never-baited arms). Testing sessions were recorded using a Sony camera integrated with the SMART video-tracking program (Panlab, Barcelona, Spain). Latency in seconds and arm visiting sequence were recorded during the trial and errors were assessed at a later date. Testing occurred for eight consecutive days.

2.11 | Auditory testing (P116–142)

Mice were then assessed for auditory processing deficits using a startle-reduction paradigm as previously described.[26] This modified pre-pulse inhibition task is used to assess whether mice are able to detect a cue presented prior to a startle-eliciting stimulus (SES), as measured through a gross motor startle reflex. During testing, mice were placed on a load cell platform (Med Associates, Georgia, VT) to record movement (Figure 1). Auditory stimuli were generated using a Penguin 4 Dell PC with custom programmed sound files and a Tucker Davis Technologies (RP2) real-time processor. Sound files were played through a Marantz integrated amplifier connected to nine Cambridge Sound Works speakers, whose sound levels were calibrated before

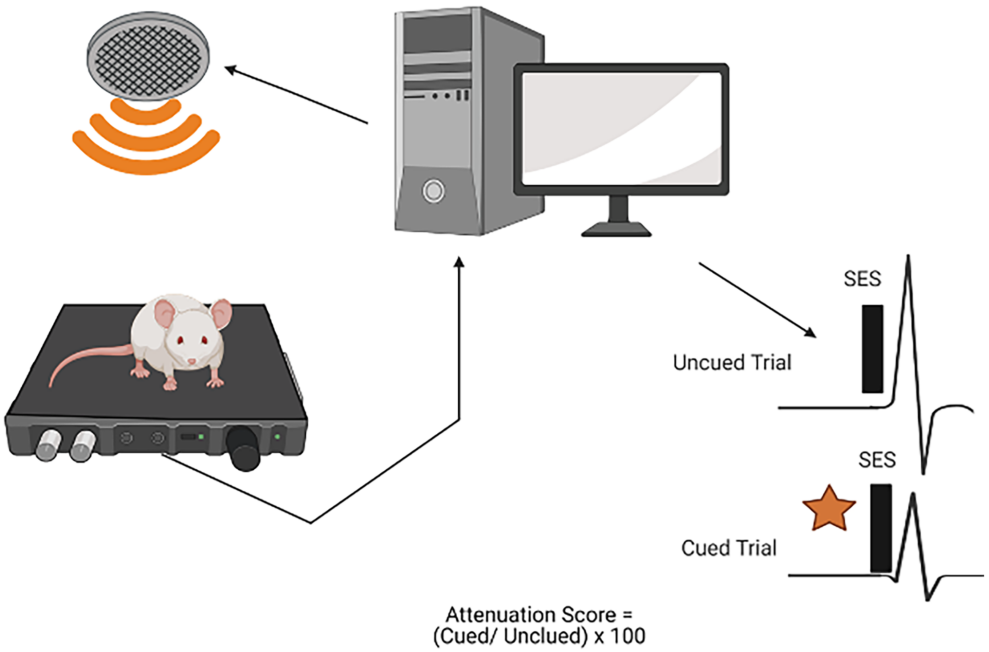


FIGURE 1 Auditory experimental set-up. This is a graphic representation of the modified pre-pulse inhibition task, detailing mouse placement upon spring-loaded, pressure-sensitive platform as well as SES and cue presentation during testing. There is also a formula for calculating attenuation score, the main result measured during this task, included.

testing with a decibel meter. Each pair of platforms had one speaker centered 30 cm above it. The voltage of each platform was passed through a load cell amplifier (PHM-250-60) into a BioPac MP100WS acquisition system (BioPac Systems, Santa Barbara, CA). The BioPac system was connected to a Macintosh computer which recorded the amplitude of the motor startle reflex in millivolts following each presentation of the SES using the Acknowledge v.4.0 program. The maximum peak value of each subject's startle reflex was extracted for the 200 ms time period following the onset of the SES. Attenuated response scores (ATT Score) for each subject were calculated using the following formula, which represented a ratio of the amplitude of each animal's displacement of the load cell during a cued trial as compared with that of an uncued trial: ([mean cued startle response]/[mean uncued startle response]) × 100. This score was represented by a percentage value with lower scores indicating better cue detection. Attenuation scores higher than 100 indicate a higher startle response at the cue than the startle-eliciting stimulus, known as pre-pulse facilitation. This indicates that the pre-pulse circuit requires more time to process the cue, leading the cue and the SES to merge, causing an additive effect and increasing startle response as compared with SES alone.

2.11.1 | Normal single tone

Normal single tone (NST) was used to assess subjects' baseline pre-pulse inhibition and general hearing ability. Against a silent background, subjects were required to detect a 50 ms auditory cue consisting of an 8000 Hz pure tone (70 dB). One-hundred and four trials were conducted during the testing session and both cued and uncued trials were presented 52 times, pseudorandomized. Attenuation scores calculated from this task were used as a covariate on subsequent auditory processing tasks to control for individual differences in hearing ability.

2.11.2 | Embedded tone 100

Following NST, an embedded tone (EBT) task was conducted to test subjects' ability to detect a change in frequency within a constant, pure tone background (75 dB; 10.5 kHz). All subjects were exposed to 300 pseudorandomized trials with a variable ITI between 16 and 24 s. Cued trials contained a 5.6 kHz cue occurring 100 ms before the 105 dB SES in variable duration ranging from 2 to 100 ms. Uncued trials presented the SES with the absence of a cue.

2.11.3 | Embedded tone 10

Following EBT 100, another embedded tone task was conducted to test subjects' ability to detect a shorter change in frequency within a constant, pure tone background (75 dB; 10.5 kHz). Cued trials contained a 5.6 kHz cue occurring 100 ms before the 105 dB SES in variable duration ranging from 2 to 10 ms.

2.11.4 | Silent gap 300

Next, a silent gap (SG) task was employed to measure subjects' ability to detect temporal changes in auditory information. On all trials, subjects were presented with a continuous white noise background (1–10 kHz; 75 dB). On cued trials, a gap of sound variable in duration (50–300 ms) occurred within the white noise background noise prior to the SES. On uncued trials, no silent gap was present.

2.11.5 | Silent gap 100

This task occurred as above but required subjects to detect a silent gap in the white noise background varying from 2 to 100 ms on cued trials.

2.12 | Euthanasia

Following behavioral testing, subjects were euthanized via ketamine (100 mg/kg) and xylazine (15 mg/kg) and transcardially perfused using a 0.9% saline solution followed by a 10% formalin solution. After extraction from the skull, the brains were post-fixed in 10% formalin.

2.13 | Statistical analyses

All statistical analyses were performed in accordance with APA guidelines, and were run using *SPSS v29* as supported by the University of Connecticut. Datasets were further imported into *GraphPad Prism v6* for graphic visualization. For all tasks, sex was used as a covariate in any analyses between groups. If not reported, sex differences were non-significant and sexes were combined for analysis.

3 | RESULTS

3.1 | Pup vocalizations

Following analysis using the MUPET program, a repeated measures ANOVA was performed showing a main effect of Genotype. Specifically, cKO animals showed fewer overall total vocalizations than WTs across postnatal day 5, 7, 9 and 15 ($F[1, 55] = 10.413$, $p = 0.002$; covariate of sex $F[1, 55] = 0.759$, $p = 0.387$; Figure 2). cKO subjects also had significantly shorter calls ($F[1, 55] = 4.226$, $p = 0.045$). We did not see an effect of Genotype on types of vocalizations, nor mean duration for each category of vocalization, which was expected since pup distress calls are essentially white noise bursts. We did find a main effect of Sex, with more vocalizations overall among female pups, as well as a more robust reduction in the number and duration of calls for female cKOs although cKO male pups also showed few calls ($F[1, 55] = 6.668$, $p = 0.013$; Figure 2). This pattern is consistent with findings from *Foxp2* KO mice, wherein the wild-type sex difference of more distress calls among female pups was reduced in *Foxp2* KO mice due mainly to reductions among KO females.[27]

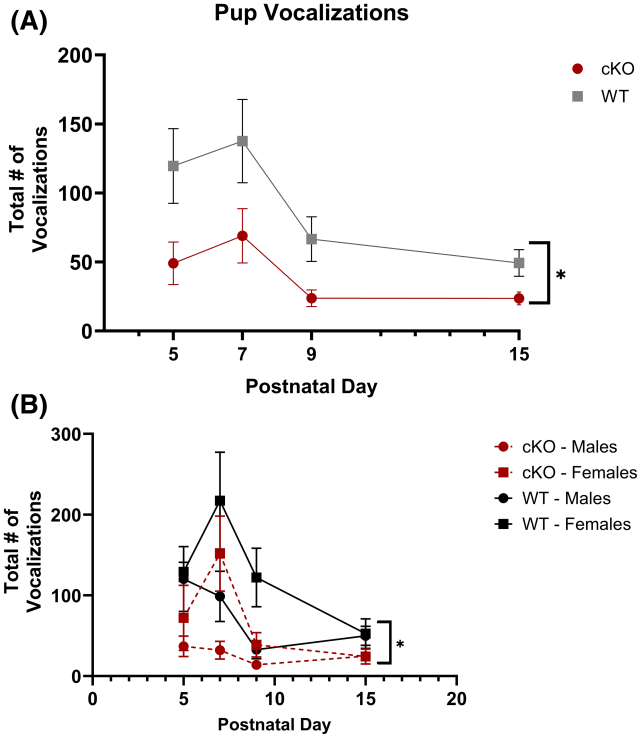


FIGURE 2 Pup Vocalizations: (A) An average of the total number of vocalizations uttered by mice pups at postnatal day (P) 5, 7, 9 and 15 (grouped by genotyped ±SEM). (B) An average of the total number of vocalizations uttered by mice pups at P5, 7, 9 and 15 (grouped by genotype and sex ±SEM).

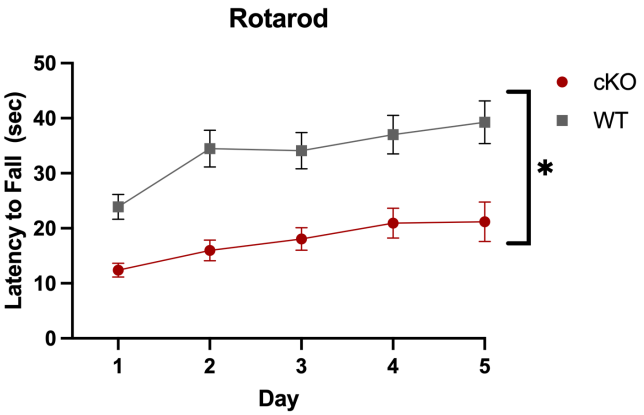


FIGURE 3 Rotarod. The average latency to fall from a spinning drum as its speed increases from 4 rotations per minute (RPM) to 40 RPM over 2 min. Five days of testing were completed, with four trials being completed per day, averages per day shown (grouped by genotype ±SEM). *$p < 0.05$.

3.2 | Rotarod

Again using a repeated measure ANOVA, we found that cKO animals performed significantly worse ($F[1, 59] = 21.271$, $p < 0.001$; Figure 3) on the rotarod task, falling off the rolling barrel significantly faster than their WT littermates. There was no main effect of Sex nor interaction between Genotype × Sex. There was no main effect of Day ($F[4, 52] = 1.489$, $p = 0.219$) for the cKO group, however, there was a main effect of Day ($F[4, 52] = 7.669$, $p < 0.001$) for the WT group (indicating improvement over days). This difference reflects a motor learning deficit in the cKO group relative to WT littermates. Using covariate analysis, we excluded any contribution from weight (F

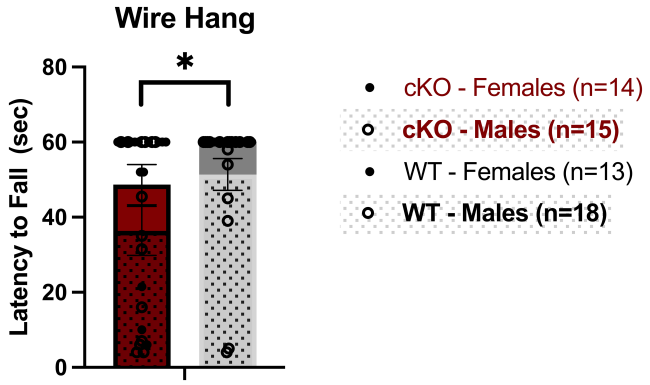


FIGURE 4 Wire hang. The average latency to fall from a wire grate suspended 25 cm from the top of a table (grouped by genotype ±SEM). *$p < 0.05$.

[1, 54] = 1.376, $p = 0.246$) or wire hang latency (i.e., strength [see below]; F[1, 54] = 0.032, $p = 0.860$) on rotarod performance. Collective results indicate both a gross motor deficit, and motor *learning* deficits, in cKO subjects.

3.3 | Wire Hang

When analyzing grip strength, we found that cKO animals performed worse than wildtype counter-parts (F[1, 62] = 9.396, $p = 0.003$; Figure 4), losing their grip on the wire grating significantly faster than their WT littermates. Again, we saw no main effect of Sex, nor Genotype × Sex interaction. These results indicate a robust fine motor strength deficit in cKO animals.

3.4 | Lickometer

Using multivariate ANOVAs, several indices of licking and orofacial movement were analyzed including: number of licking bouts; duration of bouts; and total volume consumed. Results showed that cKO animals drank significantly less (F[1, 60] = 4.445, $p = 0.039$; Figure 5) sugar water than their WT littermates despite comparable time spent licking (F[1, 60] = 0.863, $p = 0.857$; Figure 5) and comparable bouts of licking in the 5-min test period (F[1, 60] = 1.312, $p = 0.257$; Figure 5). The fact that cKO mice obtained less fluid with a comparable number and duration of orofacial motor movement indicates that they drank less effectively. We saw no main effect of Sex nor interaction of Sex × Genotype on these measures. These results indicate a fine motor deficit *specific to oral-motor control* in cKO animals.

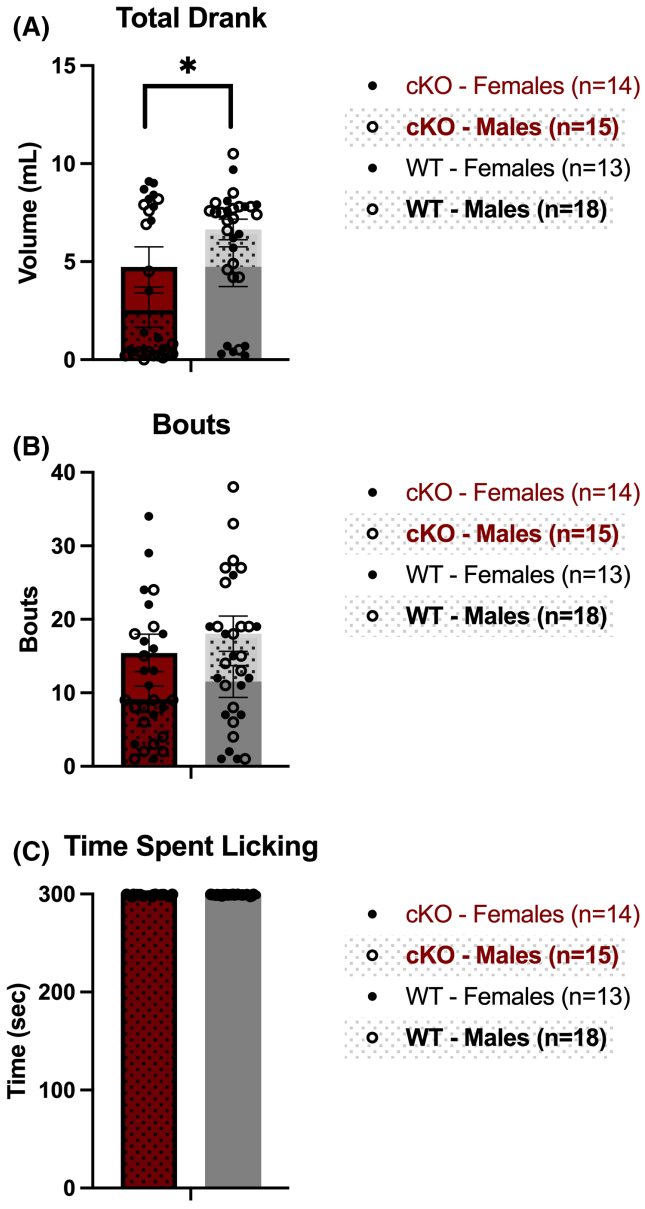


FIGURE 5 Lickometer: (A) The average amount of water consumed by mice, separated by genotype ±SEM, during the testing session. *$p < 0.05$. (B) The number of times the mouse touched the water spout throughout a 10 min testing period (grouped by genotype ±SEM). (C) The average amount of time spent, in seconds, licking throughout the testing period (grouped by genotype ±SEM). A bout was defined as a period of constant licking followed by at least a 100 ms gap in activity at the water spout.

3.5 | Open field

Locomotor and anxiety behaviors were measured in an open field task, which assessed total movement and speed of movement over a session, as well as the tendency to stay near walls versus explore an open center. No significant differences were seen in time spent per region, speed of movement, nor millimeters traveled within each region for Genotype, Sex, or Genotype × Sex (Figure 6).

3.6 | Three-chamber social task

Social preference was assessed using a three-chamber social task. ANOVAs showed that cKO animals spent marginally less time in the center chamber (F[1, 59] = 3.689, $p = 0.06$; Figure 7) than WT

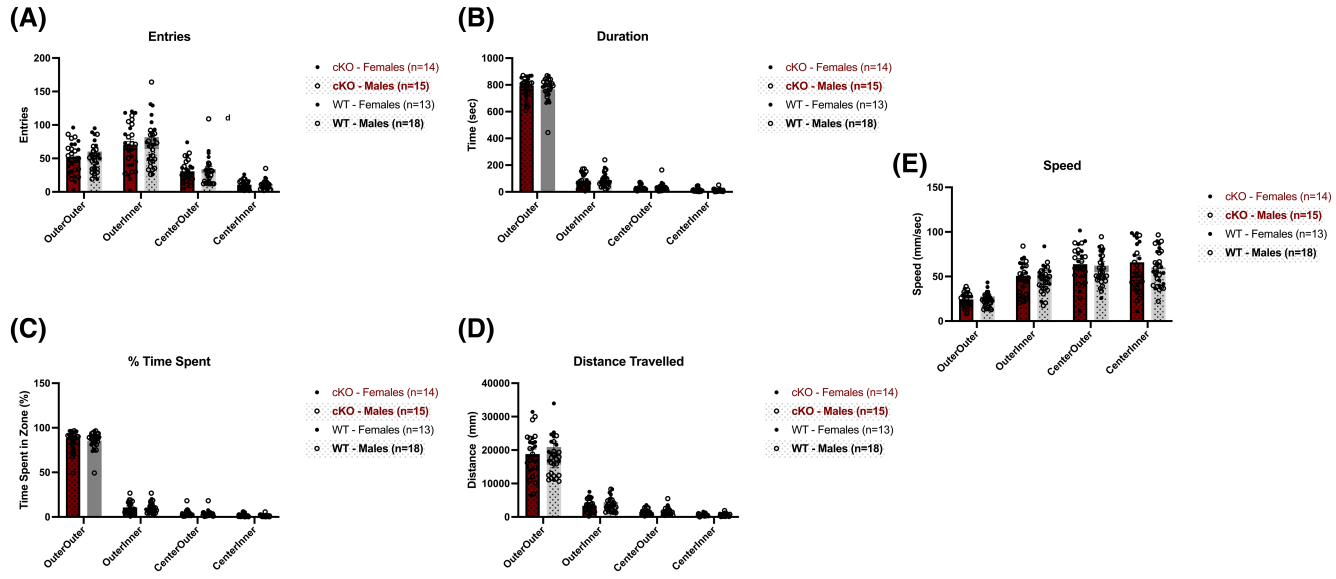
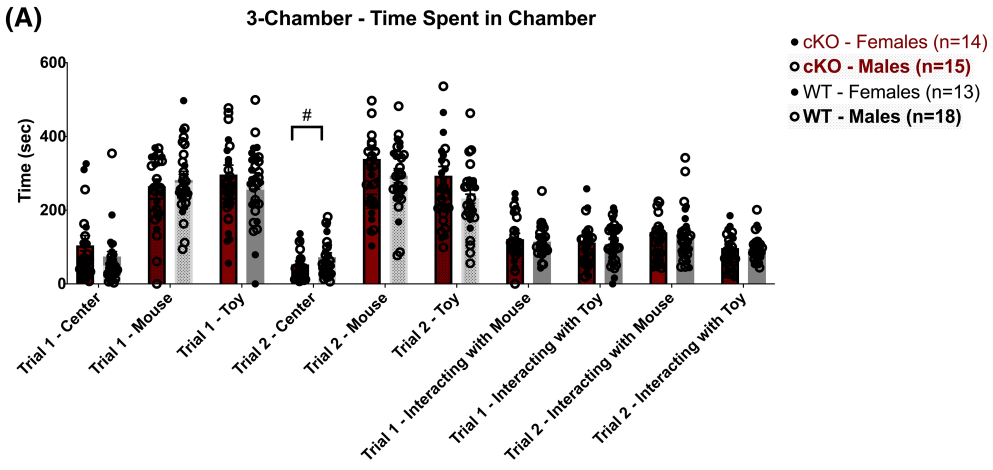


FIGURE 6 Open field: (A) The average amount of entries into each zone of the open field (grouped by genotype ±SEM). (B) The average duration of time spent in each zone (grouped by genotype ±SEM). (C) The average percentage of time spent in each zone (grouped by genotype ±SEM). (D) The average distance traveled in each zone (grouped by genotype ±SEM). (E) The average speed at which mice traveled when in each zone (grouped by genotype ±SEM).



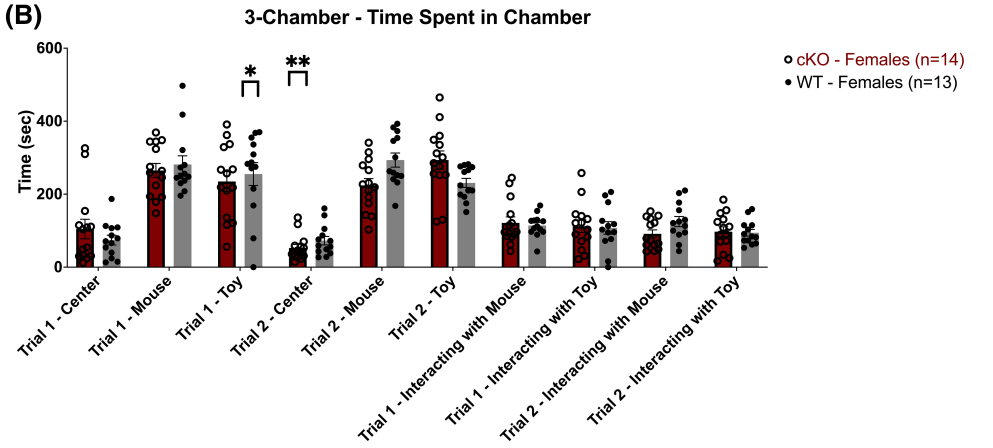


FIGURE 7 Three-chamber social task: (A) The average amount of time spent in each chamber for all subjects, as well as the time spent interacting with each object, separated by trial (grouped by genotype and sex ±SEM). #$p < 0.07$. (B) The average amount of time female mice spent in each chamber, as well as time spent interacting with each object, separated by trial (grouped by genotype ±SEM). *$p < 0.02$, **$p < 0.05$, #$p < 0.07$.

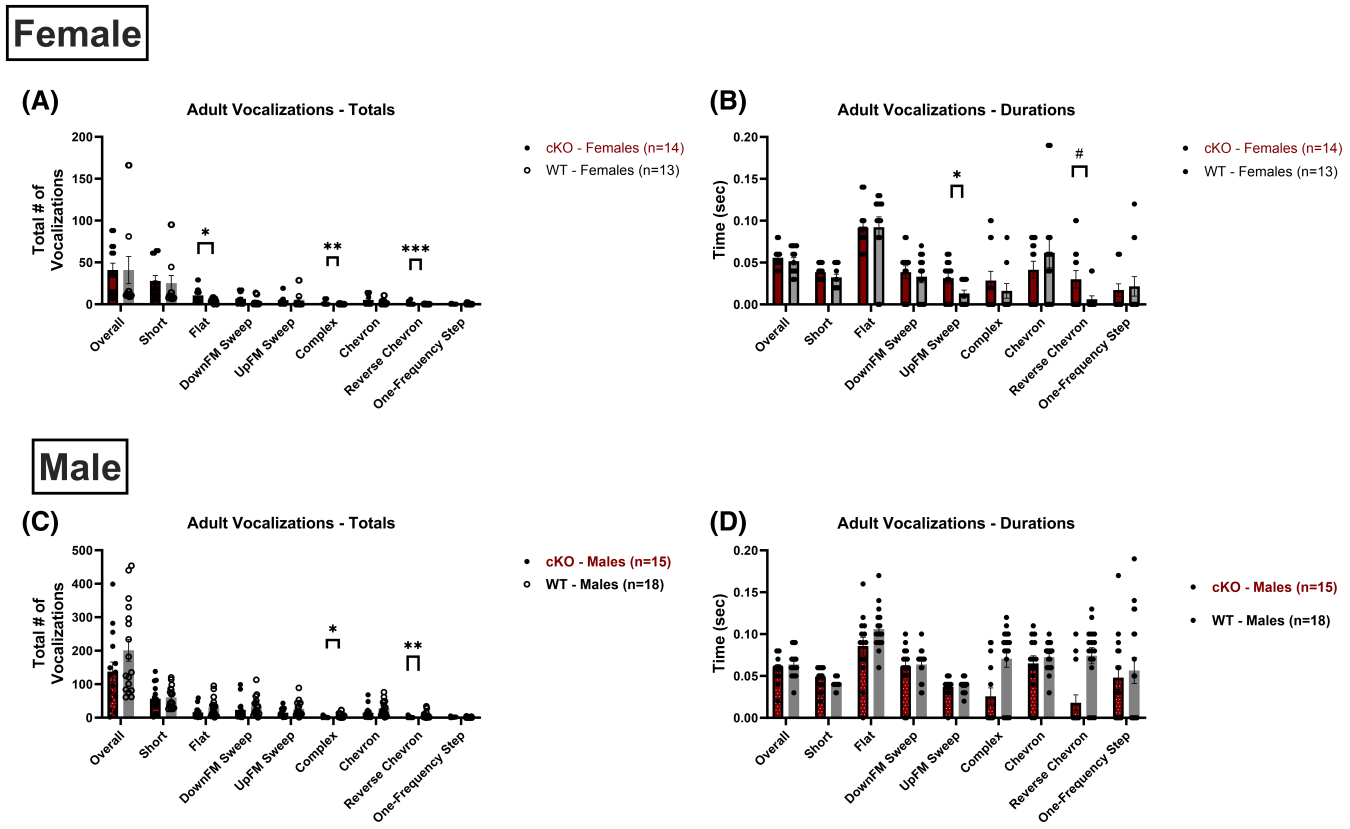


FIGURE 8 Adult vocalizations: (A) The total number of vocalizations emitted by female mice, categorized into the eight main categories of murine vocalization adapted from Heckman et al. (2016)[24] (grouped by genotype ±SEM). $^{*}p < 0.05$, $^{**}p = 0.05$, $^{***}p < 0.04$. (B) The average duration of vocalizations emitted by female mice, categorized into the eight main categories of murine vocalization adapted from Heckman et al. (2016)[24] (grouped by genotype ±SEM). $^{*}p < 0.05$, $\#p < 0.1$. (C) The total number of vocalizations emitted by male mice, categorized into the same eight categories as female mice (see Figure 7A,B) (grouped by genotype ±SEM). $^{*}p < 0.02$, $^{**}p < 0.03$. (D) The average duration of vocalizations emitted by male mice, categorized into the same 8 categories as female mice (see Figure 7A,B) (grouped by genotype ±SEM). $^{*}p = 0.005$, $^{**}p < 0.001$.

littermates, but no other significant Genotype effects were seen. We did see Sex × Genotype interactions for several measures, however, ($F[1, 59] = 4.447$, $p = 0.039$; Figure 7). Among female subjects, the amount of time spent in the chamber containing the mouse during the second trial was significantly less in cKO subjects ($F[1, 25] = 6.609$, $p = 0.016$; Figure 7) and the amount of time spent in the chamber containing the toy during the second trial was significantly higher in cKOs ($F[1, 25] = 4.838$, $p = 0.037$; Figure 7). The amount of time spent interacting with the other mouse was also marginally less in cKO females ($F[1, 25] = 3.648$, $p = 0.068$; Figure 7) as compared with WT littermates.

3.7 | Adult vocalizations

In order to analyze adult vocalizations, it was necessary to split the data by sex because of the varying types of vocalizations (primarily mating calls in males and social calls in females). Male-to-female and female-to-female calls differ in both quantity and classification of vocalizations, and the number of female calls are typically fewer than males per unit of time by a magnitude of about 1 to 10. For females, a multivariate ANOVA was used to analyze both total number and duration of various types of calls/syllables. Female cKOs showed greater numbers of flat vocalizations ($F[1, 25] = 4.651$, $p = 0.041$; Figure 8), complex vocalizations ($F[1, 25] = 4.228$, $p = 0.050$; Figure 8) and reverse chevron vocalizations ($F[1, 25] = 5.062$, $p = 0.034$; Figure 8). Female cKOs also showed longer durations of upFM sweep individual syllables ($F[1, 25] = 5.921$, $p = 0.022$; Figure 8) and, marginally, reverse chevron syllables ($F[1, 25] = 3.969$, $p = 0.057$; Figure 8). Male vocalizations showed the opposite trend, with cKO male subjects using significantly fewer complex vocalizations ($F[1, 32] = 7.214$, $p = 0.012$; Figure 8) and reverse chevron vocalizations ($F[1, 32] = 5.834$, $p = 0.022$; Figure 8). Similarly, cKO males showed significantly shorter complex ($F[1, 32] = 9.172$, $p = 0.005$; Figure 8) and reverse chevron ($F[1, 32] = 13.242$, $p < 0.001$; Figure 8) syllables.

3.8 | Water escape

We saw no significant group main effect of Genotype on the time required to swim to a visible platform ($F[1, 57] = 0.522$, $p = 0.473$; Figure 9), nor did we see main effects or interactions with sex.

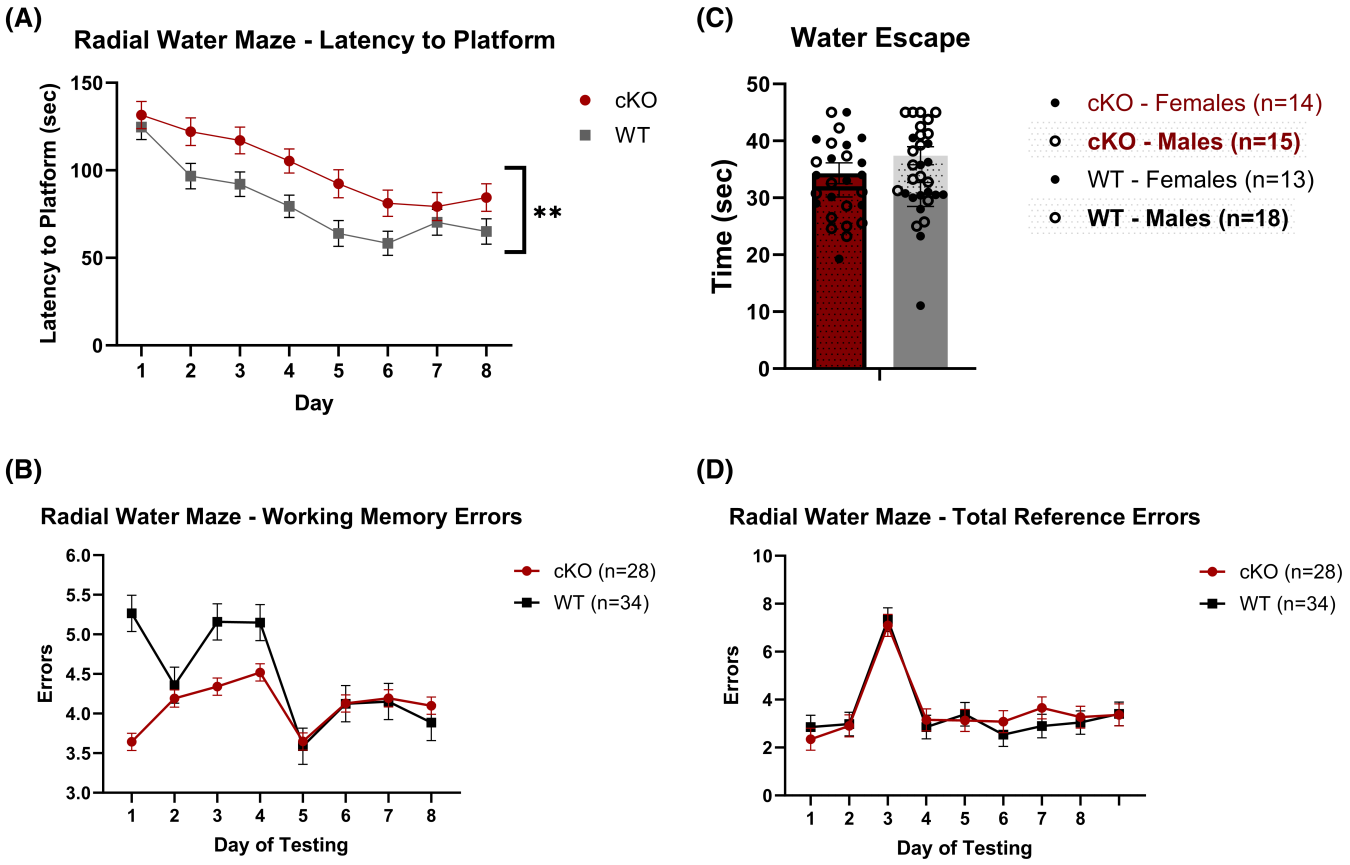


FIGURE 9 Radial water maze: (A) The average latency to reach a hidden platform across 8 days of testing (grouped by genotype ±SEM). *$p < 0.005$. (B) The average number of working memory errors made across 8 days of testing (grouped by genotype ±SEM). (C) The average latency to reach a visible platform (grouped by genotype ±SEM). (D) The average number of reference errors made across 8 days of testing (grouped by genotype ±SEM).

3.9 | 4/8 Radial water maze

When analyzing numbers of working memory and reference memory errors on the 4/8 radial arm maze task we saw no significant differences between WT and cKO groups, nor main effects of Sex or interactions with Sex. There were also no effects of Day on working memory or reference errors. However, we did find that cKOs on average took longer to swim to the hidden escape platforms ($F[1, 57] = 9.260$, $p = 0.004$; Figure 9), even when using water escape latency on the visible platform task as a covariate. Given the comparability in memory errors and learning across the WT and cKO groups, this increased latency could reflect subtle motor deficits elicited by the longer and more complex swimming requirements, which may not be evident in a short swim to a visible platform. Alternately, the longer latency could reflect hesitation or difficulty in selecting the correct arm. We saw no main effect of sex, nor interactions between Sex × Genotype. These results suggest a deficit in gross motor ability over an extended period of physical demand, but do not offer evidence of cognitive impairment based on comparable errors made.

3.10 | Auditory testing

For all auditory tasks, lower attenuation scores indicate better auditory processing functioning. This is because better attenuation of startle indicates better detection of the pre-pulse cue (with scores at 100% or chance indicating no detection). Attenuation scores *over* 100 indicate auditory hypersensitivity in the form of pre-pulse facilitation, meaning the pre-pulse cue merges with the startle-eliciting stimulus because of insufficient processing time.

3.10.1 | Normal single tone

The NST is a simple task that requires detecting a simple-frequency tone in silence. Here, we found that cKO subjects performed worse than WTs ($F[1, 60] = 11.015$, $p = 0.002$; Figure 10), suggesting a mild hearing or processing impairment. As a result, subsequent tasks were analyzed using NST as a covariate to control for the baseline differences between groups. We did not see a Sex effect or Sex × Genotype interaction for NST scores.

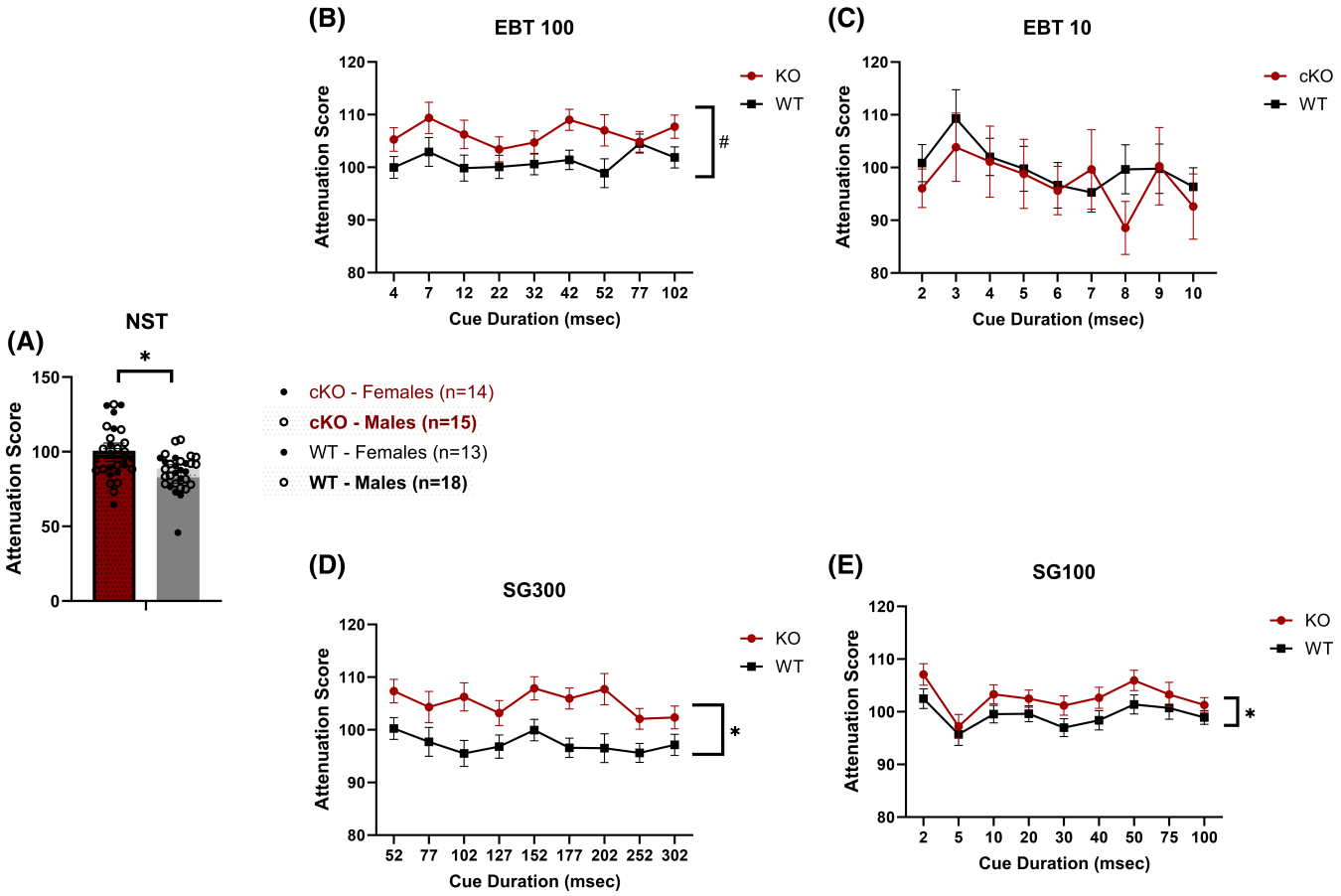


FIGURE 10 Auditory: (A) The average attenuation score achieved by all subjects (grouped by genotype ±SEM) on the Normal Single Tone task. *$p < 0.003$. (B) The average attenuation score achieved by all subjects (grouped by genotype ±SEM) on the Embedded Tone 0–100 task, separated by cue duration. #$p < 0.07$. (C) The average attenuation score achieved by all subjects (grouped by genotype ±SEM) on the Embedded Tone 0–10 task, separated by cue duration. (D) The average attenuation score achieved by all subjects (grouped by genotype ±SEM) on the Silent Gap 0–300 task, separated by cue duration. #$p < 0.08$. (E) The average attenuation score achieved by all subjects (grouped by genotype ±SEM) on the Silent Gap 0–100 task, separated by cue duration.

3.10.2 | Embedded tone 100

For all cue lengths, cKO animals had marginally higher attenuation scores ($F[1, 60] = 3.627$, $p = 0.065$; Figure 10) as compared with WT littermates, indicating startle facilitation (i.e., insufficient processing time, or alternately, acoustic processing deficits).

3.10.3 | Embedded tone 10

On the harder version of this task (using shorter embedded frequencies of 1–10 ms duration), we saw no significant group differences ($F[1, 60] = 0.257$, $p = 0.614$; Figure 10).

3.10.4 | Silent gap 300

This task requires subjects to detect relatively long (50–300 ms) gaps in sound. Here, cKOs again showed marginally higher attenuation scores ($F[1, 60] = 3.190$, $p = 0.079$; Figure 10) compared WT littermates, also indicating facilitaton.

3.10.5 | Silent gap 100

On a discrimination task using shorter gaps in sound (10–100 ms), we saw no group differences ($F[1, 60] = 0.365$, $p = 0.548$; Figure 10).

3.11 | Weight at perfusion

Using an independent samples *t*-test, cKO male animals were found to weigh marginally less than WT counterparts at the conclusion of testing ($t[34] = 0.964$, $p = 0.059$) (Figure 11). There were no significant differences between cKO and WT female subjects ($t[24] = 1.953$, $p = 0.344$), although the cKOs did tend to weigh less than WT littermates. Individual weight was used as a covariate in motor tasks, to eliminate the possible skewing of results because of an

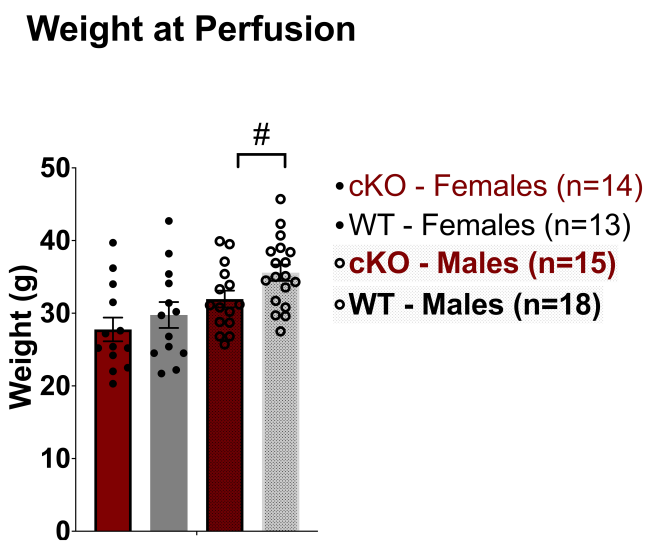


FIGURE 11 Weight at perfusion. The average body weight at perfusion of all subjects (grouped by genotype ±SEM). *$p < 0.003$.

advantage of a lower body weight. The well-known sex difference of lighter weights for females was expected ($F[1, 59] = 13.607$, $p = <0.001$).

4 | DISCUSSION

The current study aimed to distinguish the behavioral effects of a regional *Foxp1* knock-out predominately affecting the cerebellum through use of an *En1*-cre (although we acknowledge that this cKO also affected other hind- and midbrain structures with developmental co-expression of *Foxp1* and *En1*such as superior colliculus, inferior colliculus, tegmentum, periaqueductal gray and reticular formation [Allen Developing Mouse Brain Atlas, Seattle, WA 98109]). By applying a behavioral battery modeled on the human FOXP1 syndromic phenotype to mice with this regionally specific knockout of *Foxp1*, we show early cerebellar-specific loss of Foxp1 protein appears to be associated with a motor-specific deficit profile that includes altered vocalizations. This result suggests that oral-motor anomalies could account for a substantive portion of the communicative FOXP1 phenotype in humans. We also used cognitive, social and acoustic processing tasks, which showed anomalous social behaviors in female cKOs, as well as some hearing and mild sound processing impairments that could be linked to cerebellar or other hindbrain/midbrain Foxp1 loss. Implications of these results are discussed further below (Figure 11).

With respect to the pattern of specific motor deficits in cKO animals, rotarod and wire-hang results together show a clear pattern of physical impairment. On the rotarod, cKO animals fell off significantly faster, suggesting an issue with motor coordination. Additionally, the cKO animals did not exhibit a learning curve for rotarod latency over days 1–5 although WT littermates did, suggesting a motor learning deficit. On the wire hang, cKO animals slipped from the wire grate significantly faster, showing a lack of paws strength with fine motor deficits. These effects cannot be explained by weight differences, given that cKO weighed significantly less than their WT littermates at sacrifice (and lower weight is typically associated with better performance on these tasks). Moreover, weight was used as a covariate in motor tasks analyses. Next, the lickometer test showed that cKO mice received less sugar water than WT littermates while touching and manipulating the spout the same number of times and for the same duration, again showing a fine motor deficit in the oral-facial motor domain. Finally, open field results displayed a comparable anxiety/spontaneous movement level between genotypes indicating no lack in motivation to move.

Findings from the radial water maze were also consistent with this motor impairment. Although cKO mice showed learning (error) curves similar to WT mice on this task, they took longer to reach the hidden platforms on each trial as compared with WT littermates, suggesting a motor but not cognitive deficit. These systems are dissociable since motor learning relies primarily on extra-pyramidal and cerebellar circuitry,[28] whereas the radial arm maze relies on hippocampal and cortical learning.[29] Although this Genotype difference in latency to reach the platform in the radial water maze was not seen on the water escape task, the radial arm maze requires sustained swimming activity, which could indicate a motor deficit that becomes pronounced under higher demand. Additionally, there was no influence of wire hang results on rotarod performance across and within days, as shown by covariate analysis, which would indicate that motor learning, as well as general coordination, is worse in cKO subjects. Our combined results suggest a distinct motor deficit in cKO animals as compared with their WT littermate controls on motor learning and general motor ability, but not in cognitive learning.

Another interesting result came from the three-chamber social task, designed to assess social interest. While there were few sex differences on motor tasks (a slightly more robust deficit on the paw grip task in males), there were significant sex differences in the social preference of cKO animals. Female cKO animals showed a more classic ASD phenotype, with significantly less time spent in the chamber with the conspecific mouse as well as significantly less time interacting with the mouse. They also showed less time interacting with the toy as compared with the WT littermates, despite spending more time in the toy chamber. This could indicate both a lack of social interest and an aversion toward novel stimuli. Males did not show this pattern (behaving more similar to WTs), which points to a sex difference in the effects of gene mutations primarily affecting the cerebellum and hindbrain.

With regards to communication differences, both male and female cKO pups showed a reduction in distress calls, even although these effects were larger in females (possibly because females made more distress calls to begin with). Findings were comparable to similar distress call reductions observed in *Foxp2* KO mice, which also showed a larger deficit in KO females.[30] In adulthood, cKO males showed a significant reduction in complex vocalizations and a reduction in the duration of syllables. Based on the fine motor deficits seen in the wire hang, it is possible that cKO male mice struggle with complex vocalizations because of difficulty with necessary oral motor

movements, consistent with deficits seen on the lickometer. Interestingly, females also showed a significant *increase* in simple vocalizations as well as a heightened number of complex vocalizations. Although cKO females showed a different vocalization pattern than cKO males, there are several reasons this could be true. First, male and female vocalizations cannot be compared directly because of the nature of our paradigm. When testing, we pair a male with a novel WT female, given that females do not vocalize with males (and therefore most all calls can be attributed to the male). Also, it has been assumed that most of the male calls made in this context are mating calls directed at the females. Female vocalizations, on the other hand, are recorded with two females of the same genotype in the cage, attributing half of all vocalizations to each mouse. This is not as accurate as male recordings, and also means that the female vocalizations are made in a very different social (conspecific, not mating) context. The implication of this difference for observed results is not clear. Nonetheless, the fact that cKO females showed a pattern of increased vocalizations in our paradigm could also reflect the social anomalies observed in the three-chamber task. Whereas motor deficits would be expected only to limit oral-motor vocalizations, socially-based anomalies could lead to either too few *or* too many calls.

Inconsistencies between the sex differences reported in this study and human epidemiologic trends for ASD and FOXP1 Syndrome populations warrant discussion given evidence that both conditions are far more prevalent among males.[30,31] It is worth noting many ASD studies do not analyze symptom characterization separately as a function of sex,[32,33] and many studies include previously-diagnosed patients thought to reflect historically inflated male–female ratios.[32,34] Nonetheless, accumulating evidence does indicate that females may present different and more subtle ASD symptoms as compared with male counterparts, particularly in the social domain.[34,35] Such differences could inform the results reported here, specifically the different effects of Foxp1 manipulation on male and female vocalizations. We do note that our assessments of mouse vocalizations are limited by the fact that male pairs tend to fight, and that females paired with a male tend not to vocalize, such that male vocalizations were measured in male–female pairings while female vocalizations were obtained in female–female pairings. These different contexts complicate interpretation of the observed sex differences. Overall, further studies are needed to specifically investigate sex differences in the social and communicative effects of Foxp1 deletion in animal models in order to inform sex differences in affected human populations.

Further, lickometer testing, which is a reliable indicator of fine oral motor ability,[36] showed a decrease in the ability of cKO subjects to drink as much per lick as WT littermates. Clinically, the effect of a cerebellar lesion upon language is more complex than other language-related disorders. Therefore, it is important to include potential indicators of confounds in analyses when looking at cerebellar/language connections. Since no other measures of the test (bouts of drinking, time spent licking, etc.) were different between genotypes, it is logical to assume that the throat/tongue structure involved specifically in the action of drinking water and controlling how much water enters the mouth may have contributed to this finding. And while the presence of fine motor deficits does not equate to an overall gross motor deficit, results of the rotarod and swim latency support the hypothesis that a gross motor deficit is present as well.

In summary, our results show a constellation of behavioral anomalies in cerebellar cKO *Foxp1* mice including gross and fine motor deficits, as well as oral-motor deficits, reduced pup vocalizations, and reduced adult male vocalizations. These motor deficits are very likely related to cerebellar-specific loss of *Foxp1* expression. Moreover, the motor disruptions may have a particular effect on vocalizations, given the specific evidence of impact on oral-motor dexterity. It is important to note that loss in other regions involved in the knock-out could contribute to some phenotypes observed, particularly loss of *Foxp1* in inferior colliculus which may have impacted the auditory hypersensitivity observed as pre-pulse facilitation.[26] cKO females further show some autistic-like social behaviors, and an unexpected increase in several types of vocalizations to other females (which could be a reflection of social impairment). The cKO subjects did not show apparent changes in locomotion, anxiety, or working memory and spatial learning. Overall cKO mice did also show a mild hearing or acoustic processing impairment, and subtle disruptions in some complex acoustic processing, with unknown implications. It is possible that auditory processing deficits could play a role in reduced vocalizations, assuming cKO mice are less able to process maternal and littermate vocalizations or to receive feedback on their own calls. This could conceivably disrupt communicative development, much the same way birdsong learning is disrupted in deafened birds.[19]

Overall, our findings are important to an improved understanding of the *FOXP1* gene in neurodevelopmental disorders, as well as the specific role of the cerebellum—a topic of growing interest in the past decade. Results show that additional research is needed into the effects of cerebellar anomalies on neurodevelopmental communicative disorders.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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