

Exploring the Behavioral Traits of Male Mutant *Crup* Mice as an Experimental Neurodegenerative Disease Model

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The congenic BALB/c-crup ('*crup*' meaning '*cruza-pernas*' in Portuguese or cross legs in English), a homozygous recessive mutant mouse derived from *N*-ethyl-*N*-nitrosourea mutagenesis, exhibits a unique phenotype characterized by hindlimb crossing when the mouse is suspended by its tail, along with age-related neuromotor issues. The study aimed to identify the genetic mutation causing the BALB/c-crup phenotype and evaluate the behavioral responses of the mice. Open field test, elevated plus maze, and elevated beam experiments were conducted to evaluate general activity, motor function, as well as sensorimotor and autonomic nervous systems. Genetic mapping and exome analysis identified a nonsynonymous (missense mutation), a single nucleotide variant, in the *Taf15* gene. This mutation results in the p.G55S substitution, where glycine is replaced by serine at position 55 in the gene product. Longitudinal assessment by the open field test revealed altered locomotion, decreased mobility, and reduced rearing and grooming frequency in mutant mice. Sensorimotor function declines were observed through reduced surface righting reflex scores, grip strength, and increased hindquarter angle. In the elevated beam test, mutants exhibited tail hypotonia and aversion to traversing the beam. The elevated plus maze revealed altered behavior in closed arms, suggesting increased anxiety-like behavior or sensorimotor impairment. Our findings provide insights into neurologic and behavioral anomalies associated with a *Taf15* gene mutation. The altered locomotion, sensory impairments, and disorientation observed in the *crup* phenotype indicate a progressive neuromotor condition, potentially serving as a novel mouse model for neurodegenerative diseases.

Abbreviations and Acronyms: AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; *crup*, *cruza-pernas* or cross legs; EB, elevated beam; OFT, open field test; ENU, *N*-ethyl-*N*-nitrosourea; EPM, elevated plus maze; FTD, frontotemporal dementia; SNV, single nucleotide variant; TAF15, TATA-binding associated factor 15

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Introduction

The phenotypic-driven genetic approach, also known as forward genetics, begins with the observation of a phenotype, leading to the identification of the genetic basis of the mutation.⁴¹ Mice chemical mutagenesis, using ENU (*N*-ethyl-*N*-nitrosourea), allows the production of random point mutations all over the genome, revealing novel genes or new alleles of already known genes.^{1,16}

A study conducted in 2006 by our research team aimed to induce novel mutations in BALB/cJ mice using the chemical mutagenic agent ENU.²⁴ This effort resulted in generating 11 mutant mouse lines with the genetic background of BALB/cJ.²⁴ Among the recovered recessive lineages, one was named BALB/c-crup ('*crup*' meaning '*cruza-pernas*' in Portuguese or cross legs), signifying its distinct phenotype of hindlimbs crossing when a homozygous (*crup/crup*) mouse is suspended by its tail, along with age-related motor issues. These findings

led us to assume the presence of neuromotor impairment in mutant mice.

Various neurologic disorders lead to significant motor impairment and could elicit comparable alterations in a specific behavioral assessment.⁹ Disruption of RNA metabolism is emerging as a significant role in neurodegenerative diseases.¹³ Mutations in the genes responsible for neurologic disorders within the family of RNA-binding proteins (RBPs) known as FET can play a role in the initiation and progression of these conditions.³² The functions of FET proteins, including fused in liposarcoma (FUS), Ewing sarcoma (EWS), and TATA-binding associated factor 15 (TAF15), provide substantial support for the notion that abnormalities in RNA processing play a significant role in neurodegenerative diseases such as Alzheimer disease (AD), frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), Parkinson disease, Huntington disease, and multiple sclerosis.³²

A recent literature review highlighted several RBPs that exhibit strong associations with ALS.⁴⁴ These RBPs possess both structural and functional characteristics that contribute to the disease mechanisms. Despite the current lack of comprehensive understanding of the normal functions and pathologic implications of these RBPs, mutations in these proteins have the potential to impact gene expression, thereby influencing DNA

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repair responses, apoptosis, cell growth, and proliferation. Other authors have described genetic variations within the TAF15 gene in a limited group of ALS patients, emphasizing the wider role of FET proteins in the process of neurodegeneration.³²

This study aimed to identify the genetic mutation responsible for the observed phenotype, as well as to assess the responses to behavioral tests of the BALB/c-*crup* mutant mice. This evaluation comprised characterizing changes in general activity, locomotion, sensorimotor functions, and those linked to the autonomic nervous system (ANS). Our observations regarding the phenotype further supported the hypothesis that the *crup* mutant mouse could serve as a model for studying neurodegenerative diseases.

Materials and Methods

Ethics statement. All protocols in this study received approval from the IACUC (permit no. 3201280920) of the School of Veterinary Medicine and Animal Science at the University of São Paulo. The study followed the *Guide for the Care and Use of Laboratory Animals* published by NIH.

Genetic mapping, exome enrichment, and sequencing. The congenic *crup* mutation was obtained following the same protocol described for other mutants induced by ENU.²⁴ Briefly, 30 male BALB/cJ mice, aged 8 to 10 wk, were treated with intraperitoneal injections of 100 mg/kg ENU, administered at one-week intervals for 3 wk. After this, a 3-generation breeding protocol was employed to recover recessive mutations. In one of the pedigrees established, the homozygous mutation named ‘*cruza-pernas*’ (cross legs) (BALB/cJ-*crup*) was identified by the main phenotype of crossed hindlimbs when the mouse was held by its tail. A genome scan protocol with 23 polymorphic microsatellites distributed over the mouse genome was employed, and 9 extra markers on chromosome 11 were used to define better the candidate region carrying the mutation. Exome enrichment was performed using the SureSelect Mouse All-Exon kit (Agilent Technologies), and the libraries from *crup* and the isogenic C57BL/6J and BALB/cJ strains were sequenced using the SOLiD 5500xl platform (Thermo Fisher Scientific) in single end mode generating 75-bp reads.³⁹

Animals. A total of 44 SPF male mice, comprising 22 BALB/c-*crup* (*crup*) and 22 BALB/cJ (wild-type [WT] control), were bred in the animal facility of the Department of Immunology, Institute of Biomedical Science at the University of São Paulo, São Paulo, Brazil. Each group of 4 to 5 males was housed in IVC, 30 cm (length) × 20 cm (width) × 13 cm (height) with a floor area of 451 cm², provided by Alesco Indústria e Comércio in Monte Mor, Brazil. Every cage contained 150 g of flake bedding (Good Life™; Granja RG, São Paulo, Brazil) with a height of 2.5 cm. Two sheets of paper towels (Kleenex™; Kimberly-Clark, Irving, TX) weighing 6 g were also provided as nesting materials. Mice had unrestricted access to filtered, acidified, and autoclaved water and irradiated commercial pellets formulated according to the AIN-93 M rodent diet (Nuvilab CR-1™; Quimtia, Paraná, Brazil). The mice were kept under controlled environmental conditions, with room temperature maintained at 22 ± 5 °C and humidity at 55% ± 5%. The light-dark cycle followed a 12-h light period, with the lights turning on at 0700.

The absence of research on the phenotype of heterozygous (BALB/c-*crup*/+) animals led us to use WT BALB/cJ mice as controls. This decision was driven by several factors. First, since the *crup* mutation arose onto the BALB/c background, using BALB/cJ controls ensured a consistent genetic background for comparison with mutant mice. This minimizes confounding variables and allows for a clearer interpretation of results.

Second, the extensive research already conducted on BALB/c mice provides a robust baseline of behavioral data, facilitating the analysis of any differences observed in the *crup* mutant phenotype. Finally, focusing on male mice initially was a strategic decision due to the extensive number of experiments planned at each age.

Pathogen-free status. The mice used in this study were confirmed to be SPF for a wide range of pathogens, including ectromelia virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse hepatitis virus, mouse parvovirus, pneumonia virus of mice, reovirus, Sendai virus, Theiler murine encephalomyelitis virus, hantaviruses, cilia-associated respiratory bacillus, *Clostridium piliforme*, *Klebsiella pneumoniae*, *Mycoplasma pulmonis*, *Pasteurella multocida*, *Pasteurella pneumotropica*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Staphylococcus aureus*, *Streptobacillus moniliformis*, β-hemolytic *Streptococcus* spp., *Streptococcus pneumoniae*, endoparasites, and ectoparasites.

Inclusion and exclusion criteria and study endpoints. To ensure the reliability of our study and the well-being of the animals, we established strict inclusion and exclusion criteria for mice participating in the one-year experiment. This rigorous selection process aimed to ensure that only healthy mice with stable social behavior remained in the study, minimizing the impact of individual differences on our findings.

Inclusion criteria. Overall health. Mice were monitored daily for overall health status, including motor activity, posture, appearance, and social behavior.

Absence of aggression. Mice exhibiting no signs of aggression toward their cage mates were included in the study.

Exclusion criteria. Aggressive behavior. Mice displaying random aggression toward their cage mates were excluded from the experiment.

Injuries. Mice exhibiting skin or tail injuries resulting from fights were also excluded.

Study endpoints. Health deterioration. Any mouse exhibiting significant health deterioration, as determined by daily observations, was immediately removed from the study.

Uncontrolled aggression. If a mouse displayed persistent and uncontrolled aggression toward cage mates, despite initial exclusion criteria, it was removed from the study.

Study completion. The study was concluded after one year, at which point all remaining mice were euthanized humanely.

Phenotypic characterization. Phenotypic characterization included observations in the open field test (OFT), elevated beam (EB), and elevated plus maze (EPM). The animals were taken to the testing room 30 min before the start of the experiments. OFT and EPM were recorded for 5 min, and the EB test was recorded until the mouse crossed the bar or for a maximum of 5 min if the mouse did not cross. Testing was performed in a small room with dim lighting (50 lx at 50 cm from the floor). The devices and parameters related to the general activity, sensory nervous system, sensorimotor system, central nervous system, and ANS followed the protocol previously described.¹⁴ The apparatuses were cleaned with a 5% alcohol/water solution before placement of each animal to prevent possible bias caused by odor cues left by the previous mouse. All tests were taken between 1300 and 1600. The trials were recorded using a digital camera and analyzed with EthoVision™ XT 15 software (Noldus).²⁷

The sample size was determined using standardized phenotyping protocols as described in the International Mouse Phenotyping Resource of Standardized Screens (IMPreSS). Given the large number of experiments performed at each age, we initially prioritized males. The study design is illustrated in Figure 1. Sixteen males (8 *crup* and 8 controls) at 8 wk of age

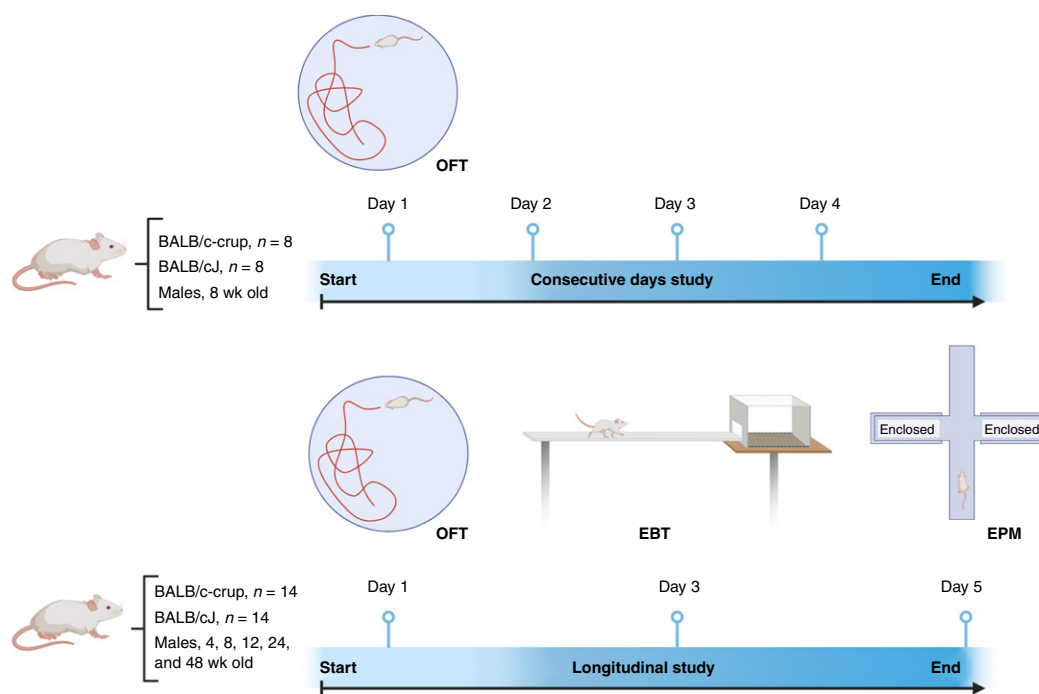


Figure 1. Experimental timeline and design for behavioral studies in BALB/cJ and BALB/c-crup male mice. (Top) 8-wk-old male BALB/cJ ($n = 8$) and BALB/c-crup ($n = 8$) mice were tested in the open field over 4 consecutive days. (Bottom) Male BALB/cJ ($n = 14$) and BALB/c-crup ($n = 14$) mice, aged 4, 8, 12, 24, and 48 wk, were assessed in a longitudinal study over 5 d, with behavioral assessments conducted at designated intervals as shown in the timeline. The study included open field, elevated beam, and elevated plus maze behavioral tests represented in the diagram by different icons. Created with BioRender.com.

were used to evaluate locomotor activity in the OFT. During a 5-min session, for 4 consecutive days, the animals were allowed to move freely, and their horizontal ambulatory activity was recorded.²¹ The following parameters were measured: total distance moved (in centimeters), average speed (in centimeters per second), and mean mobility (the movement of the animal's body was calculated independently of the movement of the coordinates identified as the center-point). Following, a longitudinal study involving 28 male mice (14 *crup* and 14 controls) was made. The variables of interest were observed at the ages of 4, 8, 12, 24, and 48 wk. At each time point, OFT, EB, and EPM were performed with a 48-h gap between them. OFT was performed as described elsewhere.¹⁴ Distance traveled (in centimeters), average speed (in centimeters per second), frequency of rearing, and frequency and duration (in seconds) of grooming were recorded. Considering the parameters related to the sensorimotor, autonomic, and sensory nervous systems, it was not possible to conduct a blinded analysis because the phenotype was observable, and the scores were assigned during the test. Each parameter was scored from 1 to 5, except for urine spots and defecation, which were quantified by the number of urine spots and fecal pellets, respectively. At the end of the observations, the scores were summed and used for the statistical analysis. EPM was performed as described previously.² The time spent in the open arms, closed arms, and central platform, as well as the number of entries into each arm, were recorded.

Statistical analysis. Statistical analysis was performed on the data collected from each behavioral test. As the experimental design predicts factors such as strain, time, and age, 2-way ANOVA followed by the Tukey post hoc test was adopted, sphericity was considered if data were quantitative, and no missing values were observed. Continuous qualitative data or data containing missing values by chance mixed-effect analyses were performed, followed by the Tukey post hoc test. In that case, no sphericity was considered. Some continuous qualitative, given as scores

1 to 5, data were analyzed by the Kruskal-Wallis followed by Dunn post hoc tests after the identification and elimination of outliers by the ROUT method ($Q = 5\%$). The statistical analyses were conducted by using R for Windows (version 2022.07.1) and GraphPad Prism version 10.0.2(232) for Windows, GraphPad Software (Boston, MA). The significance level of $\alpha < 0.05$ was established to determine the presence of statistical significance.

Results

Animals. BALB/c-crup mice were homozygous for the mutation, and phenotypic alterations were observed exclusively in homozygous individuals. In our breeding pairs of mice, we observed a longer interval between mating and the birth of the first litter, which occurred on average 50.5 d after mating, compared with 36.85 d for BALB/cJ mice. These findings were based on 29 pairs of each strain. When considering the fertility of BALB/c-crup pairs, only 58.62% were fertile, while 100% of the BALB/cJ pairs were fertile. In addition, zootechnical data indicated that only 37.33% of BALB/c-crup pairs remained fertile after 4 litters, whereas 79.31% of BALB/cJ pairs continued reproducing. The average number of pups was similar in both strains, with 4.31 for BALB/c-crup and 3.78 for BALB/cJ. The interval between births, starting from the second litter, was 36.85 d for BALB/c-crup and 35.67 d for BALB/cJ. The preweaning mortality rate was 8% for both strains.

Genetic mapping, exome enrichment, and sequencing. A thorough genome scan using polymorphic microsatellites that cover the whole mouse genome was employed to map the *crup* mutation. Our data indicated that a linkage on chromosome 11 might be discovered with this procedure. Nine more markers were examined to improve the resolution of the linkage map and find prospective candidate genes. As a result, a candidate interval on chromosome 11 between 27 and 58 cM was identified. An exome sequencing analysis yielded the following insights: 4,851 variants were identified within the mapped

region, out of which 4,386 were homozygous mutations. Among these homozygous mutations, 313 were exclusively found in the control group. In addition, 76 mutations were not present in the SNP database, signifying their novelty. Fifteen of these mutations were identified as novel exonic nonsynonymous or splice site mutations, with 6 of them being unique. After a thorough examination of pertinent literature sources,^{8,12,20,22,32,44} only one potential candidate, *Taf15*, remained relevant for further investigation. Comprehensive Sanger validation protocols were subsequently implemented, using C57BL/6J, BALB/cJ, and A/J inbred mice, along with unrelated ENU-mutant controls whose exomes had not undergone prior sequencing. This rigorous validation process confirmed the presence of the mutation.

The mutation was characterized as a nonsynonymous (missense mutation) single nucleotide variant (SNV) located at position c.G163A within exon 4 of the *Taf15* gene (NM_027427), resulting in the substitution of glycine with serine at position 55 in the gene product (p.G55S). Importantly, this SNV has not been detected in any of the analyzed mouse strains. This information is corroborated by both Mouse Genome Informatics (MGI; <https://www.informatics.jax.org/snp/marker/MGI:1917689#myDataTable=results%3D100%26startIndex%3D0%26sort%3Daccid%26dir%3Dasc>) and Ensembl (10.1093/nar/gkac958, 8/9/20230).

Inclusion and exclusion criteria. Implementing exclusion criteria in the longitudinal evaluation resulted in removing some mice from the experiment due to aggressive behavior, as the manifestation was randomic. Consequently, there was a decrease in the sample size at specific time points, outlined as follows: 4 wk (14 BALB/cJ and 14 BALB/c-*crup*), 8 wk (14 BALB/cJ and 13 BALB/c-*crup*), 12 wk (14 BALB/cJ and 13 BALB/c-*crup*), 24 wk (13 BALB/cJ and 12 BALB/c-*crup*), and 48 wk (5 BALB/cJ and 12 BALB/c-*crup*).

Phenotypic characterization. The results derived from the 4-day locomotion assessment in the OFT for both the 8-wk-old

mutant and control groups revealed distinct patterns. Notably, the mutant group exhibited reduced distance traveled (Figure 2A; $P = 0.0121$; $P = 0.0145$; $P = 0.0007$, respectively, for the second, third, and fourth observational days) and decreased speed (Figure 2B; $P = 0.0047$; $P = 0.0046$; $P = 0.0017$, respectively, for the second, third, and fourth observational days) during the second, third, and fourth tests across all observed days, comparing to the control group. In addition, the mutant mice consistently demonstrated a diminished level of mobility throughout the 4-day evaluation (Figure 2C; $P = 0.0478$; $P = 0.0008$; $P = 0.0007$; $P = 0.0024$, respectively, for the first, second, third, and fourth observational days) compared with the control group. For the distance traveled, the strain accounted for 37.19% of the variance and was considered extremely significant ($F_{[1,28]} = 29.69$; $P < 0.0001$), time accounted for 3.023% of the variance and was not considered significant ($F_{[3,28]} = 1.27$; $P = 0.3041$), and the interaction accounted for 2.48% of the variance was also considered not significant ($F_{[3,28]} = 0.60$; $P = 0.5836$). For the mean velocity, the strain accounted for 38.41% of the variance and was considered extremely significant ($F_{[1,14]} = 17.67$; $P = 0.0009$), time accounted for 3.551% of the variance and was not considered significant ($F_{[3,42]} = 1.96$; $P = 0.1540$), and the interaction accounted for 2.189% of the variance was also considered not significant ($F_{[3,42]} = 1.21$; $P = 0.3194$). For the mobility mean, the strain accounted for 44.39% of the variance and was considered extremely significant ($F_{[1,14]} = 25.16$; $P = 0.0002$), time accounted for 6.911% of the variance and was considered significant ($F_{[3,42]} = 4.54$; $P = 0.0126$), and the interaction accounted for 2.692% of the variance was considered not significant ($F_{[3,42]} = 1.77$; $P = 0.1679$).

In the context of the longitudinal OFT investigation, the *crup* mutant mice exhibited distinct behavioral patterns compared with the controls. The mutant mice covered a considerably shorter distance up to the age of 24 wk and recovered it at 48 wk (Figure 3A; $P = 0.0114$; $P = 0.1123$; $P < 0.0001$; $P = 0.0007$;

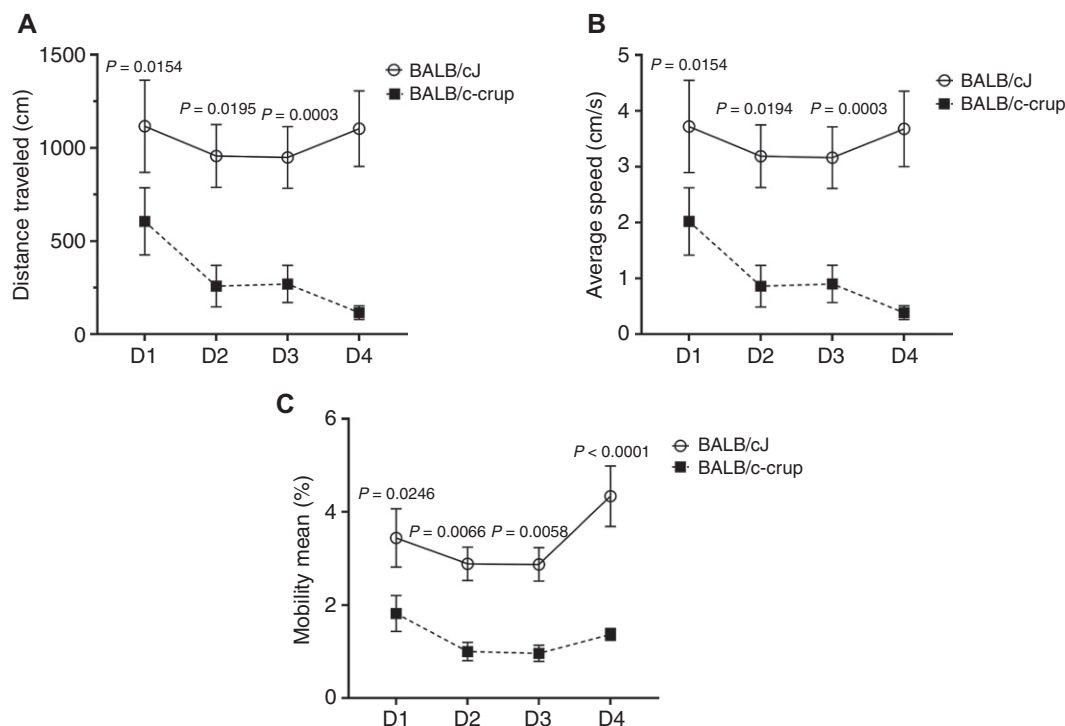


Figure 2. Comparative performance analysis of BALB/cJ and BALB/c-*crup* mice ($n = 8/\text{group}$) in the open field test over 4 d: (A) distance traveled (cm); (B) average speed (cm/s); and (C) mobility mean (%). Results are presented as mean and SE. Significance at $P < 0.05$.

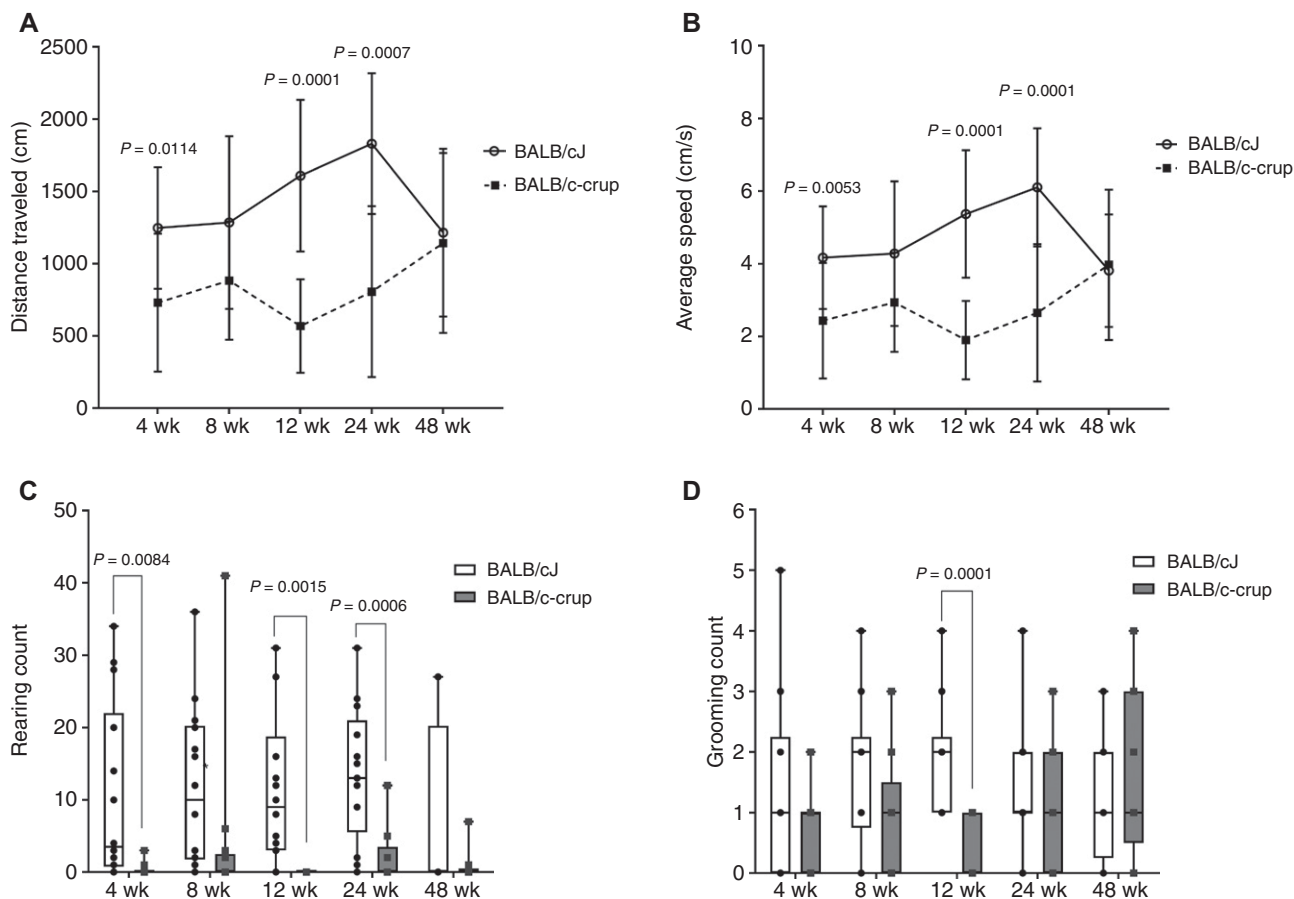


Figure 3. Behavior analysis in the open field test: (A) distance traveled (cm); (B) average speed (cm/s); (C) rearing frequency; and (D) grooming frequency in BALB/cJ and BALB/c-crup mice at 4, 8, 12, 24, and 48 wk of age ($n = 5$ to 14/group). Results are presented as mean, SE, and ranges (minimum to maximum) with individual data points. Significance at $P < 0.05$.

$P = 0.6191$, consecutively at 4, 8, 12, 24, and 48 wk of age) and maintained a significantly lower speed accompanying the previous parameter (Figure 3B; $P = 0.0053$; $P = 0.0513$; $P < 0.0001$; $P < 0.0001$; $P = 0.8421$, consecutively at 4, 8, 12, 24, and 48 wk of age) when compared with the control group.

The rearing frequency consistently decreased in the mutant mice across 4, 8, 12, 24, and 48 wk of age (Figure 3C; $P = 0.0084$; $P = 0.0841$; $P = 0.0015$; $P = 0.0006$; $P = 0.2146$, consecutively at 4, 8, 12, 24, and 48 wk of age), in comparison to the control group. Moreover, a significant reduction in grooming frequency was observed at 12 wk of age in comparison with the control group (Figure 3D; $P < 0.0001$; 12 wk of age). The fixed effects obtained for the traveled distance were $P = 0.1025$ ($F_{[1,26]} = 17.63$); $P = 0.0008$ ($F_{[1,26]} = 17.63$); and $P = 0.0067$ ($F_{[1,26]} = 17.63$) for time, strain, and the interaction between them, after correction by the Geisser-Greenhouse epsilon of 0.6735, 1.000, and 0.6174, respectively. The fixed effects obtained for the mean velocity were $P = 0.07035$ ($F_{[3,161,73,49]} = 2.4214$); $P = 0.0003$ ($F_{[1,26]} = 17.63$); and $P < 0.0001$ ($F_{[4,93]} = 6.954$) for time, strain, and the interaction between them, after correction by the Geisser-Greenhouse epsilon of 0.7902 (time). The fixed effects obtained for the grooming frequency were $P = 0.7478$ ($F_{[3,159,73,46]} = 0.4219$); $P = 0.0315$ ($F_{[1,26]} = 5.166$); and $P = 0.0098$ ($F_{[4,93]} = 3.542$), for time, strain, and the interaction between them, after correction by the Geisser-Greenhouse epsilon of 0.7898 (time). Results obtained for grooming duration were obtained, but an error type II occurred and requires further analysis.

Evaluation of parameters associated with the sensorimotor system demonstrated an extremely significant decline in

the surface-righting reflex scores (Figure 4A; $\chi^2_{[10,118]} = 103.2$; $P < 0.0001$) and grip strength (Figure 4B; $\chi^2_{[10,124]} = 112.0$; $P < 0.0001$), accompanied by an elevation in the hindquarter angle (Figure 4C; $\chi^2_{[10,121]} = 114.5$; $P < 0.0001$) within the *crup* mutant group when compared with the control BALB/cJ mice throughout the examined time points of 4 to 48 wk.

Analysis of ANS parameters demonstrated a reduction in the count of urine spots in *crup* mutant mice at 8 and 12 wk (Figure 5A), along with a decrease in the number of fecal boli at 8, 12, and 24 wk of age (Figure 5B) when compared with the control group BALB/cJ. The fixed effects obtained for the amount of urine spots were $P = 0.4545$ ($F_{[3,341,77,68]} = 0.8990$); $P = 0.0895$ ($F_{[1,26]} = 3.110$); and $P = 0.0011$ ($F_{[4,93]} = 4.977$), for time, strain, and the interaction between them, after correction by the Geisser-Greenhouse epsilon of 0.8353 (time). The fixed effects obtained for fecal boli amount were $P = 0.0866$ ($F_{[3,088,71,79]} = 2.264$); $P < 0.0001$ ($F_{[1,26]} = 51.32$); and $P = 0.1672$ ($F_{[4,93]} = 1.655$), for time, strain, and the interaction between them, after correction by the Geisser-Greenhouse epsilon of 0.7719 (time).

During the EB test, it was observed that all *crup* mutant mice across all ages examined were unable to successfully traverse the beam. Instead, they consistently remained positioned within the initial third of the beam, maintaining approximately 40 to 50 cm away from the aversive stimulus (Figure 6A). In this position, the mutant mice relied on their forelimbs and hindlimbs to support themselves on the bar, while their tails hung down laterally, displaying a state of tail hypotonia (Figure 6B).

In the EPM test, *crup* mutant mice displayed distinct behavioral patterns compared with the BALB/cJ mice. The mutants

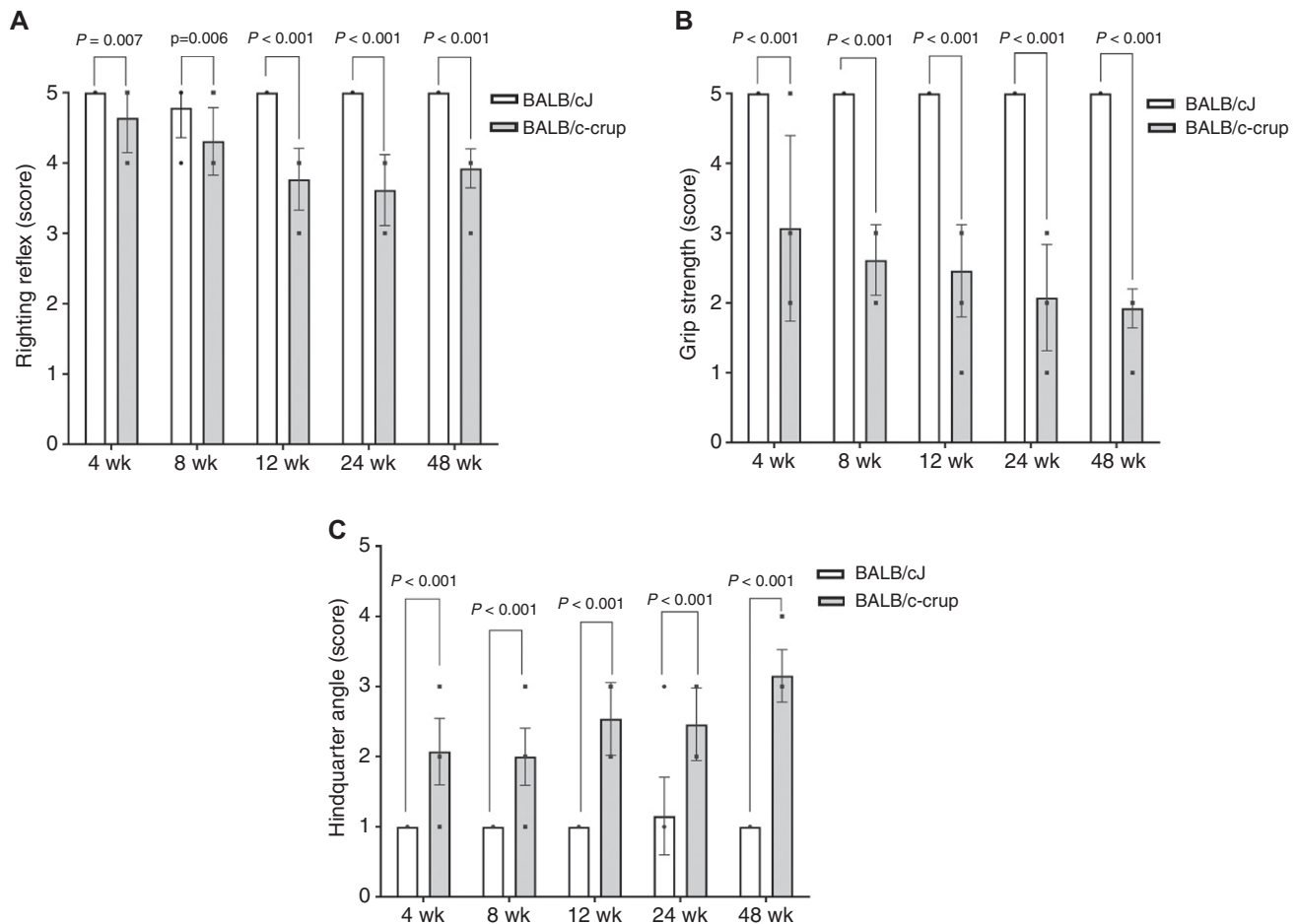


Figure 4. Evaluation of sensorimotor system parameters: (A) surface-righting reflex; (B) grip strength; and (C) hindquarter angle in BALB/cJ and BALB/c-crup mice at 4, 8, 12, 24, and 48 wk of age ($n = 5$ to 14/group). Results are presented as mean and SE. Significance at $P < 0.05$.

exhibited a lower frequency of entering in the closed arms at 4, 12, and 24 wk (Figure 7A; $P = 0.0030$; $P < 0.0001$; $P = 0.0227$, respectively). In addition, *crup* mice spent less time in the central platform in the fourth week (Figure 7B; $P < 0.0001$) but recovered over time ($P = 0.1517$; $P = 0.1988$; $P = 0.5847$, and $P = 0.0713$, respectively, at 4, 8, 12, 24, and 48 wk of age). Also, the *crup* mice crossed less than the control mice at 4, 8, and 12 wk of age (Figure 7C; $P = 0.0075$; $P = 0.0233$; $P = 0.0044$, respectively), then crossed as the animals from the control group at 24 wk ($P = 0.1772$), and finally crossed more than the *crup* animals from

8 and 12 wk ($P = 0.0239$; $P = 0.0153$, respectively), in an attempt to recover the regular number of crossings according to the control group. The fixed effects obtained for closed arms entries were $P = 0.6045$ ($F_{[2,963,62.22]} = 0.6173$); $P < 0.0001$ ($F_{[1,26]} = 21.28$); and $P = 0.1063$ ($F_{[4,84]} = 1.972$), for time, strain, and the interaction between them, after correction by the Geisser-Greenhouse epsilon of 0.7408 (time). The fixed effects obtained for time in the central platform were $P = 0.0936$ ($F_{[2,783,58.44]} = 2.277$); $P = 0.0015$ ($F_{[1,26]} = 12.59$); and $P = 0.0153$ ($F_{[4,84]} = 3.270$), for time, strain, and the interaction between them, after correction by

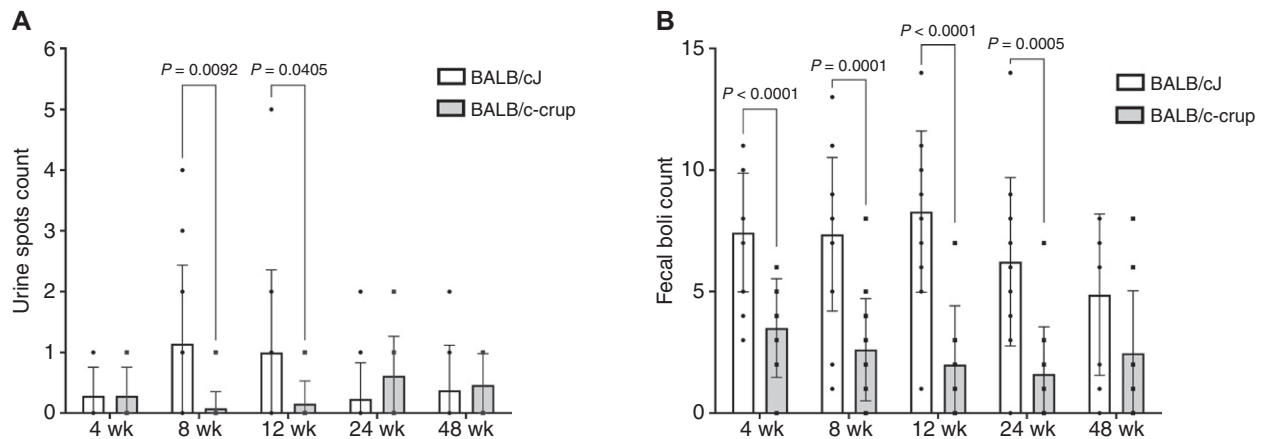


Figure 5. Autonomic nervous system parameters: (A) micturition (number of urine spots) and (B) defecation (number of fecal boli) in BALB/cJ and BALB/c-crup mice at 4, 8, 12, 24, and 48 wk of age ($n = 5$ to 14/group). Results are presented as mean and SE. Significance at $P < 0.05$.

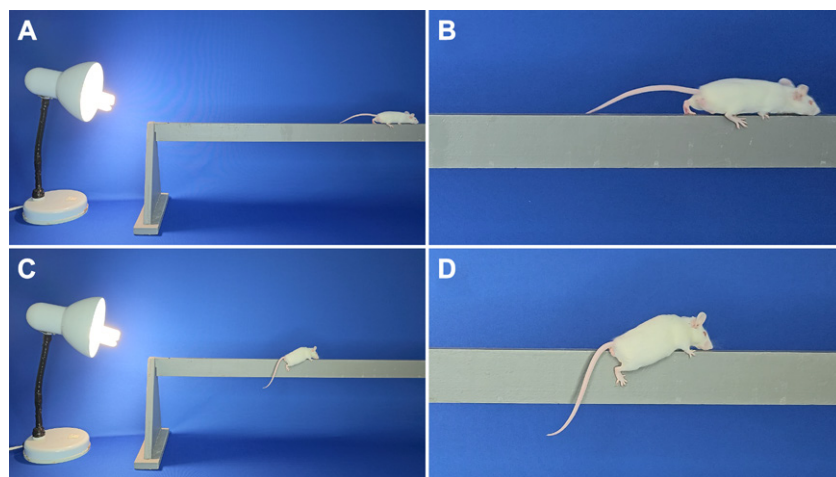


Figure 6. Elevated beam test performance in BALB/cJ and BALB/c-crup mutant mice. (A and B) BALB/cJ mouse exhibiting normal performance on the beam, demonstrating good balance and coordination while successfully traversing the beam. (C and D) BALB/c-crup mutant mouse displaying impaired performance, including hesitation, difficulty navigating the beam, and tail hypotonia, characterized by a limp tail that droops downward, a distinctive feature of the BALB/c-crup phenotype.

the Geisser-Greenhouse epsilon of 0.6957 (time). The fixed effects obtained for the number of crosses in the central platform were $P < 0.0001$ ($F_{[2,458,51.61]} = 11.50$); $P = 0.0017$ ($F_{[1,26]} = 12.28$); and $P = 0.1151$ ($F_{[4,84]} = 1.917$), for time, strain, and the interaction between them, after correction by the Geisser-Greenhouse epsilon of 0.6144 (time).

Discussion

Neurodegenerative disorders, which can lead to severe disability and death, often lack an evident genetic cause. Nonetheless, animal models have proven valuable in this research area. While genetic models of neurodegenerative disorders may not perfectly replicate human diseases, they have offered

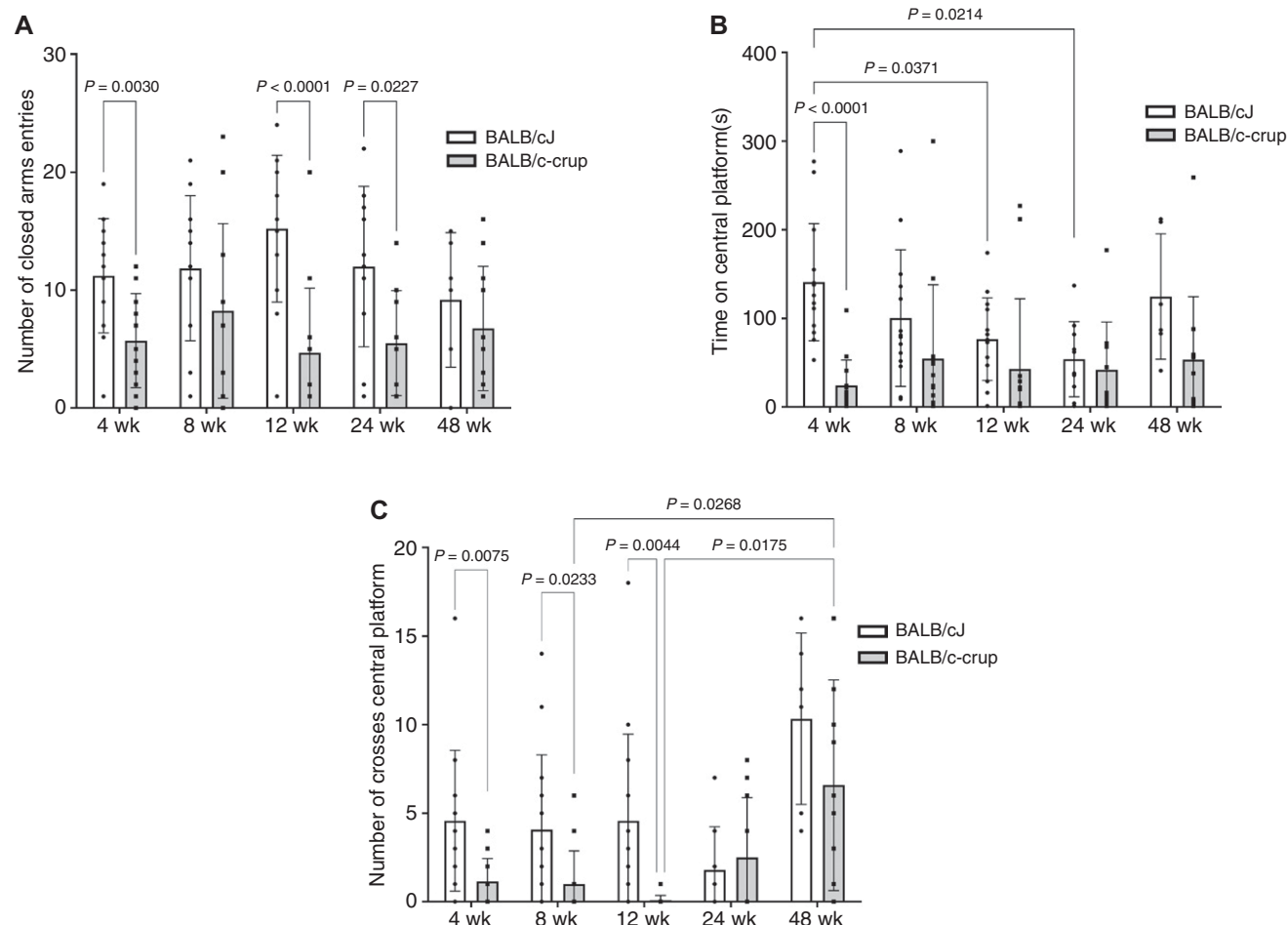


Figure 7. Elevated plus maze parameters: (A) closed arms entries; (B) time spent in the central platform; and (C) number of central platform crosses of BALB/cJ and BALB/c-crup mice at 4, 8, 12, 24, and 48 wk of age ($n = 5$ to 14/group). Results are presented as mean and SE. Significance at $P < 0.05$.

critical insights, identifying potential candidates for effective therapies.¹¹ Given the limitations of current animal models, investigating a novel model could help unravel the mechanisms underlying the pathology of these diseases.

As mentioned above, our work included many experiments at each age studied, necessitating a focused approach. Therefore, we initially used male mice as subjects. This decision was made in light of the substantial differences between male and female neurobiology. A complex interplay of factors including genetics, epigenetics, environmental influences, reproductive hormones, and neurotransmitters interact to model the brain in a sexually dimorphic pattern during early development and throughout life.³⁵ Furthermore, sex-dependent differences in the immune system can lead to significant variations in the clinical presentation of neurodegenerative diseases.⁴ For example, AD is more prevalent in women, while Parkinson disease is more common in men.⁴² Increasing evidence from both preclinical and clinical studies points to significant differences in brain structure and function between men and women, in both healthy and diseased states.⁴⁰

Therefore, due to the complexity of these sex-specific differences, we chose to initially focus on male mice to minimize potential confounding variables and to allow for a more controlled analysis of the specific effects of the *crup* mutation in a specific sex. Future studies will investigate the effects of the *crup* mutation in females to fully understand sex-dependent variations in the phenotype.

Longitudinal analyses are essential for validating models used to study neurodegenerative diseases such as AD.¹⁷ Considering this, BALB/c-*crup* mice were included in a longitudinal investigation protocol that evaluated the same group of animals from 4 to 48 wk of age. In addition, mutants at 8 wk of age were exposed to the OFT for 4 consecutive days. Over 4 d in the OFT, the *crup* mutants traveled a shorter distance, at a lower speed, when compared with controls of the same genetic background. Similarly, another study observed a decrease in locomotion among 3xTg-AD mutant mice compared with the control group.²¹ According to the author, this reduction was attributed to the animal's immobility behavior, commonly called 'freezing.' This result could also be explained by a possible decrease in the exploratory impulse in the *crup* mutant mice as it occurs in the 3xTg-AD mice.³⁶

Also, in the OFT conducted over 4 d, the mutants exhibited a consistent behavior of stopping right after the test began, remaining stationary until its conclusion. They showed no head movement, grooming, or rearing while standing still. Among the observed mutants, 2 out of 8 mice rotated in circles and remained at the center of the arena throughout the test. On the contrary, the control mice explored the entire arena, occasionally pausing for self-grooming or navigation. Studies indicate that repeated OFTs result in a decrease in locomotion, rearing, and central area activity in C57BL/6J mice.⁷ The authors proposed that this decrease could be attributed to the consolidation of environmental memories in the OFT, leading the mice to perceive no danger and consequently lose interest in further exploration. Although this behavior was noticeable in controls, it was attenuated in mutants. To validate or better interpret this behavior, additional research is required.

Likewise, the current study demonstrated that the *crup* mutants exhibited poorer performance than the control group in the longitudinal investigation of the OFT. Similar results were observed analyzing the behavior of TgCRND8, a model overexpressing a mutant human APP with features resembling familial. AD TgCRND8 mice presented reduced distance and

speed compared with controls from 4 to 24 wk.⁵ Furthermore, 3xTg-AD female mice presented increased immobility time than WT mice at 48 wk-old.⁴³

Neurodegenerative disorders often lead to issues with balance, spatial orientation, and dizziness. To maintain balance, posture, and stabilize gaze, the brain integrates sensory inputs from the vestibular, visual, and proprioceptive systems, which then modify the outgoing motor response.¹¹ Furthermore, the processing of self-movement cues appears crucial for organizing rodent exploratory activity.⁶ Navigation hints are provided through sequential analysis of open field behavior, which can help us comprehend spatial orientation.⁵ The study demonstrated that TgCRND8 mice exhibited spatial disorientation characteristic of AD, as indicated by their disordered movements with progressions and pauses, at 2 and 4 mo of age, in the OFT under both light and dark conditions. Similarly, the *crup* mutant shows disorientation in the open field. Their progression in the arena was characterized by short distance traveled and extended stops without changing in head direction. On the other hand, some of the mutants also showed circling behavior, and particularly the mice that stood still in the center of the arena remained stationary for almost the entire test time.

The poor performance of the *crup* mutant in the OFT can be related to the number of stops observed during the activity. This could be seen as a possible spatial disorientation, which often results from vestibular dysfunction.^{3,11} Furthermore, *crup* mutants also run in circles, both in the home cage and in the open field arena. Likewise, circling behavior has already been described in TgCRND8 mice⁵ and has been associated with vestibular impairment such as in the circling mutant *Pcdh15^{roda34}* and tilted mice observed in open field under dark conditions.⁶

Considering aging, after the differences found from 4 to 24 wk, our results show that 48-wk-old mutant mice performed likewise their controls. This might suggest that younger mutants, unlike controls, have the same level of difficulties as older ones. Nonetheless, the outcome could have been influenced by the limited and uneven sample size, resulting from a shortage of control subjects. In addition, one cannot forget that open field activity can also be influenced by other factors, such as exploratory impulse, anxiety, illness, and environmental factors.³⁸ In the OFT, the animal must choose between exploring its surroundings out of curiosity and either keeping close to a wall for protection or becoming paralyzed with fear. The lack of active movement and increased immobility observed during OFT in the 3xTg-AD mouse may therefore constitute a sign of anxiety.³⁶

The reduction in the frequency of rearing observed in the *crup* mutants compared with the control group suggests an impairment of motor function due to the mutation or a possible impact on exploratory or cognitive function.^{7,31} In 12-wk-old *crup* mutants, the distance traveled in OFT, the speed, and the frequency of grooming behavior all decreased, indicating that these behaviors cannot only be due to the strain differences in their sensory abilities and general activity levels but rather reflect a complex interplay between anxiety, motor, and displacement activity.¹⁹ The alterations observed in the sensorimotor system of *crup* mutants, including the righting reflex, grip strength, and increased hindquarter angle, worsened with age, indicating a degenerative condition consistent with other longitudinal studies.¹² A comparable outcome was demonstrated in 3xTg-AD mice, as they displayed lower grip strength compared with WT mice in the grid suspension test, irrespective of their body weight. These findings indicated that at 6 mo of age, 3xTg-AD

mice had a deficiency in grip strength that was not observed at earlier ages.³⁰

During the EB test, *crup* mutant mice displayed an inability to traverse the bar, with their movement confined to the initial third of the beam. Consequently, they remained stationary on the bar throughout the test. The mutants used the bar to uphold their bodies, leaning on their forelimbs and hindlimbs, while their tails hung by the side of their bodies, suggesting hypotonia. This observed tail hypotonia, in line with sensorimotor disability, provided additional support for the presence of neurologic dysfunction. Consistent with our findings, 16-mo-old 3xTg-AD mice performed poorly on the balance beam, traveling a shorter distance, and displaying slower locomotor speed compared with WT mice.¹⁵ Furthermore, TgCRND8 mice exhibited more foot slips than WT mice while crossing the narrow beam.²⁸

Currently, establishing a direct correlation between the identified abnormalities in *crup* mutants and specific neurologic disorders presents a challenge. Likewise, while numerous well-established murine models can replicate the initial proteinopathy or other pathologic features associated with diseases, there remains an urgent need for models that faithfully mimic human diseases. Some models replicate a more complex sequence of neurodegenerative events; however, their accuracy in representing the complete chain of pathophysiological events that occur in human diseases remains uncertain.¹²

The behavior observed in the *crup* mutant mice within the EPM, where they entered the closed arms less frequently and crossed the central platform fewer times while spending more time in the closed arms, further supported the findings of reduced locomotion observed in the OFT. This can be partially attributed to the apparatus' design. A recent study highlighted inconsistencies when measuring anxiety-like behavior across various transgenic mouse models employed to replicate preclinical changes in AD-related behavior. This variation is attributed to factors like different developmental phases during the progression of the disease and disparities in neural circuit involvement.²⁶ Overall, as observed in the results of other behavioral tests, the BALB/c-*crup* mutant mice explore the maze less and remain stationary for longer periods in the EPM. Some potential reasons for this behavior include a lack of interest, sensory changes, or a freezing state.

Mutations of RBPs have been implicated in RNA metabolism in animal models of ALS and FTD.²⁹ Furthermore, TAF15 mutations have been identified in a few cases of familial ALS^{10,33} After a thorough analysis of TAF15 proteins, it was shown that this RBP may contribute to the pathogenesis of ALS.¹⁰ Human ALS-linked mutations are concentrated in the C-terminal region of the gene, with 3 specific missense variants (c.1258G > A, p.G391E; c.1308C > T, p.R408C; and c.1504G > A, p.G473E) found in individuals with varying ages of onset. These mutations all occur within highly conserved regions, highlighting their potential significance in normal TAF15 function.³³ In contrast, the BALB/c-*crup* mutation is a single nucleotide variant (c.G163A) located in exon 4, resulting in a glycine-to-serine substitution at position 55 (p.G55S).

Since missense or nonsense mutations account for 75% of ENU-induced changes,¹⁸ we decided to sequence the exome of the ENU-induced *crup* mutant. After filtering SNVs from the exome analysis and considering the results of a prior microsatellite assay, we identified 6 variations within the region of interest. We then conducted a literature review for each of the 6 genes containing these unique SNVs to determine their functions. The *Taf15* gene stood out as a strong candidate. *Taf15* encodes an RBP crucial for motor control in animals.³² Therefore,

a mutation in this gene could potentially explain the phenotypic changes observed in the mutant *crup*. To test this hypothesis, we performed behavioral tests that supported the idea that *Taf15* is the most likely gene responsible for the observed phenotype. While this approach provides strong evidence, it is important to acknowledge its limitations. Establishing definitive causality requires further investigation, such as creating a gene knock-in model. Future studies are planning to use CRISPR/Cas9 technology to generate a *Taf15* knock-in model, which will allow us to directly assess the role of this mutation in the *crup* phenotype.

Hence, a mutation in the *Taf15* gene could be linked to the sensorimotor changes seen in the *crup* mouse, including diminished righting reflex, weakened grip strength, and tail hypotony. These changes could potentially contribute to the reduced mobility and impaired performance demonstrated in the behavior of the *crup* mice. Further assessments, such as the Barnes or Morris maze, could provide validation for the hypothesis of spatial disorientation.

The main candidate protein TAF15 can shuttle between the nucleus and the cytoplasm, interacting with each other, and forming protein complexes.²³ Once bound to RNA, these proteins contribute to the control of transcription, RNA processing, and the cytoplasmic fates of messenger RNAs in metazoans.²⁹ The positioning of protein complexes resulting from *Taf15* mutations remains uncertain due to the absence of mouse models for this gene.³²

Several studies have failed to find a consensus RNA-binding sequence, hypothesizing that TAF15 binds with stem-loop structures located within intronic areas.⁴⁴ Furthermore, numerous other investigations have proposed that TAF15 could interact with GGUA or CUG motifs. Despite the *Taf15* gene mutation in *crup* not aligning with the previously identified region, specific glycine-to-serine mutations have been associated with genetic issues.³⁷ Since this mutation can lead to a structural impact of the protein, depending on its location, a glycine-to-serine substitution could potentially disrupt the local structure due to the increased size and potential hydrogen bonding capability of serine. On the other hand, if the glycine residue is part of an active site, binding pocket, or other functionally important region, a substitution to serine might impact the protein's ability to interact with its ligands, substrates, or other molecules.²⁵ Therefore, further investigations will be required to assess the structure and functionality of the TAF15 protein in *crup* mutant mice.

Conclusion

In conclusion, our study on the *crup* mutant mice provides valuable insights into potential neurologic alterations and behavioral anomalies associated with the mutation. The observed behavioral changes in the *crup* phenotype, spanning altered locomotion, sensory impairments, and disorientation, highlight a progressive neuromotor condition, and its characterization through behavioral tests may give rise to a novel animal model. These findings offer a basis for understanding the potential implications of the *Taf15* gene mutation and its effects on the sensorimotor system. While the underlying genetic basis remains complex, the presence of a *Taf15* mutation suggests a potential link to the observed phenotypic alterations. However, further investigations into the structure and functionality of the TAF15 protein are necessary to understand the full implications of this mutation. The study underscores the importance of animal models in understanding neurodegenerative disorders and calls for continued research to unravel the underlying mechanisms and potential therapeutic pathways.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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