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Postnatal relevance of HOXA5 transcription factor in cerebellum-associated behaviors and disorders.

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1. Abstract

Hoxa5 encodes a transcription factor essential for embryonic patterning and organogenesis, with sustained expression in hindbrain precerebellar nuclei during postnatal development. Given prior evidence implicating HOXA5 in synaptogenesis and early postnatal circuit maturation, we investigated whether its inactivation during this critical developmental window contributes to neurodevelopmental disorder (NDD)–related phenotypes. Using previously generated transcriptomic data, we identified multiple deregulated genes classified as autism spectrum disorder (ASD) risk genes in the SFARI database, several of which are associated with a cerebellar phenotype in mice. We then performed a comprehensive behavioral assessment across motor, social, stereotypical, anxiety-related, and attentional domains in a postnatal inactivation mouse model (*Hoxa5-cKO*). Motor coordination, learning, gait, and sensorimotor functions were preserved. Social behavior assays yielded no consistent genotype-dependent effects, although results were sensitive to analytical methods and cohort variability. In contrast, *Hoxa5-cKO* mice exhibited increased stereotypical behaviors, including elevated scratching and marble burying, in the absence of anxiety- or locomotion-related confounds. Importantly, interpretation of social and cognitive phenotypes was impacted by well-known constraints of behavioral neuroscience. We discuss these downfalls and propose additional guidelines. Altogether, our findings indicate that postnatal *Hoxa5* deficiency selectively enhances stereotyped behaviors without broadly affecting motor or social functions. The data support a model in which HOXA5 acts as a modulator of postnatal precerebellar circuit connectivity and/or function, with subtle behavioral consequences that require further research in specific genetic or environmental contexts.

2. Introduction

Hoxa5 is a member of the *Hox* gene family, which encodes transcription factors that play essential roles in embryo patterning and organogenesis [1,2]. During embryogenesis, *Hoxa5* expression is required for the proper development of multiple organs, including mammary and thyroid glands, and most critically the respiratory system, where loss of function results in tracheal dysmorphogenesis, impaired branching of the lungs and incomplete diaphragm innervation, leading to high rate of perinatal mortality [3–5]. In the central nervous system, *Hoxa5*, along with other members of paralog group (PG) 5 contributes to the specification of spinal motor neuron identities [6,7]. While initially restricted to the spinal cord at early embryonic stages, *Hoxa5* domain of expression reaches hindbrain territory by embryonic day (E) 13.5 where it participates in a *Hox-PG3-5* code regulating the orderly migration of neurons from the lower rhombic lip towards the pons [8–10]. These migrating neurons retain *Hoxa5* expression upon reaching their final destination. Indeed, in the postnatal brain, HOXA5 protein is detected in subsets of neurons within many precerebellar nuclei of the hindbrain, including the pontine nuclei, reticulotegmental nucleus, lateral reticular nucleus and external cuneate nucleus [10,11]. Noteworthy, HOXA5 was detected only in neurons, no labelling being observed in glial cells [10,11]. These structures are classically recognized as major precerebellar nuclei that send mossy fibers to the granule cell layer of the cerebellum [12,13]. Synaptogenesis, which includes establishment and maturation (i.e., refinement and pruning), of these excitatory cerebellar afferents occurs during postnatal development in mice, leading to structural and functional maturity of circuits around postnatal day (PN) 20 [12,13].

Regarding the molecular relevance of the early postnatal expression of *Hoxa5* in the hindbrain, our previous transcriptomic analysis of PN21 *Hoxa5* conditional mutants revealed that HOXA5 regulates genes involved in synaptic organization, including components of glutamatergic and GABAergic signaling as well as calcium-dependent pathways [14]. These findings suggest that HOXA5 contributes to synaptogenesis in neuronal circuits during critical periods of early postnatal life [15,16]. *Hoxa5* expression being enriched within precerebellar neurons, it could regulate precerebellar circuit connectivity, with potential relevance for cerebellar functions, which encompass motor coordination and learning as well as cognitive and affective processes [17].

This hypothesis is further supported by analogous roles of *Hoxa5* in the spinal cord. Indeed, *Hox-PG5* genes – and in some contexts *Hoxa5* alone – are required in phrenic motor neurons (MN) for proper diaphragm innervation and respiratory function, as well as typical dendritic arborization, and MN-specific inactivation is sufficient to reproduce lethality observed in the constitutive knockout [5,18,19]. Moreover, MN-specific deletion of *Hoxa5* in a *Hoxc5* heterozygous background disrupts the firing pattern of phrenic MN through disinhibition [19]. In the developing hindbrain, ectopic expression of *Hoxa5* in *Hoxa5*-negative pontine neurons induces a posterior positional shift and preferential innervation by somatosensory cortical inputs, consolidating its role in neuronal positioning and circuit assembly [20].

If HOXA5 is important for synaptogenesis and neuronal circuit connectivity, one could expect synaptic deficits or synaptopathies associated with *Hoxa5* mutations. Although no mutations of *Hoxa5* have been described in human neurodevelopmental disorders (NDD), the *Hoxa5* promoter was found to be hypermethylated in CHARGE and Kabuki patients, two NDDs that are caused by loss-of-function mutations in *CHD7* and *KMT2D*, respectively [21]. Hypermethylation of the promoter is expected to decrease *Hoxa5* expression [22]. Loss of function mutations in these genes lead to syndromes that share clinical features with each other and with autism spectrum disorder (ASD). Strikingly, emerging evidence points to synapse dysregulation during peri- and postnatal periods in the pathogenesis of ASD [23–25]. One hypothesis would place the deregulation of *Hoxa5* expression as a common element contributing to synapse dysregulation and NDD in these two syndromes.

In the present study, we aim to test these hypotheses in a mouse model using two strategies. First, we leveraged our former transcriptomic data [14] to search for deregulated genes that could be associated with NDD/ASD. Second, if HOXA5 contributes to synaptogenesis and/or function of hindbrain precerebellar circuits, its inactivation could result in a functional impact on cerebellar functions. Building upon recent data [17,26] and *Hoxa5* clinical association [21], we also hypothesize an impact on non-canonical cerebellar cognitive functions. To test this, we performed a comprehensive behavioral assessment following postnatal deletion of *Hoxa5,* starting with canonical cerebellar functions using established paradigms such as the beam walking and rotarod tests, followed by screening of ASD-related phenotypes. Our results revealed an impact of the postnatal deletion of *Hoxa5* on stereotypical behaviors. These results will be discussed focusing on the paradigms used and their analysis, with a broader perspective on behavioral analyses.

3. Material and methods

3.1. Animals

All mice used here were maintained in a conventional facility (ANCA platform – UCLouvain) and fed in standard conditions (mice maintenance and mice breeding diets, Carfil Quality, Turnhout, Belgium), on a 14 h light/10 h dark cycle. Food and water were available ad libitum. Experimental procedures on animals were approved by the animal ethics committee of the UCLouvain under number #122803 and performed in accordance with the European directive 2010/63/UE.

The transgenic CMV-CreER^{T2} mice have been described elsewhere [27,28]. *Hoxa5*$^{flox/flox}$ mice have already been described [29]. Double transgenic animals used in this project are bred in a >97% C57Bl/6J background (incipient congenic).

3.2. Tamoxifen administration

The administration protocol is adapted from previous work conducted by our team [27], which consisted of four consecutive administrations of 50 µg (PN1), 75 µg (PN2–PN3), and 100 µg (PN4) of Tamoxifen to newborns. Breeding of *Hoxa5*$^{flox/flox}$;CMV-CreER$^{T2\ +/-}$ males with *Hoxa5* $^{flox/flox}$ females led to a Mendelian ratio of control (no CreER^{T2} transgene) and *Hoxa5-cKO* (carrying the transgene) animals. The entire litter is thus treated with tamoxifen, without requiring prior genotyping. For induction treatments, tamoxifen (Sigma–Aldrich, T5648) was dissolved in corn oil (Sigma–Aldrich, C8267). In this study, intragastric injections of 50 µl of two different stock solutions were performed: we first injected a solution at 1 mg/ml at PN1 (50 µg), then a second solution at 1.5 mg/ml (75 µg) at PN2 and PN3. Pups stayed with their mother throughout the treatment period and until weaning.

3.3. RT-qPCR

Mice were euthanized by progressive CO_2 delivery in accordance with guidelines (European Directive 2010/63/EU — Annex IV). RNA from dissociated murine medulla was extracted using a Roche High Pure RNA Isolation Kit (11828665001, Roche, Bâle, Switzerland) according to the manufacturer's instructions. Reverse transcription was performed using a reverse transcription kit (1708891, BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Gene expression was assessed by qPCR on a StepOne+ apparatus (Applied Biosystems, Waltham, MA, USA) using SYBR Green (204143, QIAGEN, Hilden, Germany) as the detection method, and the geometric mean of two reference genes (*H2a*, *36b4*) was used as internal normalization reference [10]. Data were analyzed and presented using the ΔΔCt method. The primer pairs are described in [10] and in Table 1.

Table 1. Primer pairs used in qPCR analysis of Hoxa5 mRNA expression

Target	Gene ID	Sequence (5′-3′)
Hoxa5	NM_010453	gcgcaagctgcacattagt
		ggcatgagctatttcgatcc
H2A	NM_016750	gctggtggtggtgtcatcc

		tttcttcccgatcagcgatt
36B4	NM_007475	tgagattcgggatatgctgttg
		ttccaatggtgcctctggaga

3.4. Behavioral assays

Male mice from the same litter were used for behavioral testing. Most behavioral analyses were carried out at the Behavioral Platform (BEAP; UCLouvain) although a few tests were performed locally at the ANCA platform (social odor, ladder rung, self-grooming, scratching and burrowing). All mice had a week of acclimation at the BEAP platform before starting experiments. Investigators were blinded to experimental groups during tamoxifen treatment, behavioral assays and outcome assessment. The test sequence and the age ranges of the mice at the time of testing are presented in Table S1.

For the three chambers, one chamber, open field, light-dark and attention tests, Noldus Ethovision 6.1 software was used to track animals and extract data. The analog acquisition card was Euresys Picolo Diligent (sn/2146). Camera was an Ikegami Model ICD-49E with a resolution of 560 horizontal lines (CCIR) and the lens was a Computar 4.5-12.5mm 1:1:2. The detection settings were set on dynamic subtraction method with a video sample rate of 4.1667 sample/second and no smoothing. Detection of movement was based on the following thresholds: start velocity: 2 cm/s, stop velocity: 1.75cm/s, averaging sample: 1.

Unless commercial references are cited, all test devices were custom-made at the behavioral platform in compliance with standards and were thus thoroughly described in their respective sections.

3.4.1. Grip test

The grip strength test measures the muscular strength of either the forelimbs or the combined fore- and hindlimbs. Limb strength was recorded using a grid connected to a sensor (Panlab-Bioseb, Vitrolles, France). The mice were gently laid on the top of the grid so that their front paws (forelimb test) or both fore and hind paws (combined test) can grip the grid. Then, mice were pulled back steadily until they released their grip along the entire length of the grid. Each test was repeated three times at an interval of 20 min. Results are the mean of the two highest recorded force values, normalized to bodyweight [30]. Mice subjected to this test were 9 weeks old.

3.4.2. Food localization

The food localization test was performed to evaluate the olfactory function. Test mice were fasted for 16 hours with water supply ad libitum. They were then transferred into a clean standard housing cage (28 × 17 × 12 cm) with 3-cm-thick wood-chip bedding and lightly tamped down to make a flat surface. A 1.0 g amount of food pellet was placed at a specific location under the bedding and the mice were introduced into the cage at a constant position. The time interval between the introduction of the mouse and the moment it grasped the food pellet with its front paws was measured,

up to a maximum of 300 s [31]. Mice subjected to this test were between 11 and 15 weeks old.

3.4.3. Olfaction

Test animals were placed in a clean standard housing cage (28 × 17 × 12 cm). After a 5-min adaptation period, a dry disposable applicator with a cotton tip was placed at a depth of 2.5 cm through the grid of the cage. Every minute, the applicator was removed and immediately replaced with a new applicator. After the sixth minute, an applicator with a cotton tip saturated with an odorant solution was positioned through the hole and again replaced every minute by a fresh applicator containing the same odor. After the 12th minute, an applicator with a tip saturated with a different odorant was inserted and replaced six times at 1-min intervals. The two odors used were geraniol and lime. The number of times the animal headed toward each applicator introduced into the cage was recorded via a camera throughout the experiment and was counted by an experimenter blinded to the experimental conditions [32]. Mice subjected to this test were between 10 and 15 weeks old.

3.4.4. Acoustic startle

Mice were individually placed in a a clean standard housing cage (28 × 17 × 12 cm) for a 15-min session. The cage was located in a quiet room separated from the housing room. During the first 10 min (habituation period), mice were allowed to freely explore the cage. Next, during the 5 min of the test session, mice were exposed to sporadic 70-dB auditory stimuli delivered by a clicker. Startle responses were observed by a trained observer positioned approximately 1 m from the test cage. Mice subjected to this test were between 11 and 12 weeks old.

3.4.5. Beam walking

The beam walking test is used to evaluate motor coordination, balance, and sensorimotor integration. The key parameter assessed here is the ability of mice to go across an elevated narrow runway towards a dark box with bedding. The runway was 100 cm long and 1.2 cm wide, placed at 50 cm height. Mice realized 5 trials per day for 5 consecutive days. The crossing time (in seconds), the number of falls and the number of times the feet slipped off the beam were registered. Results are presented as the mean values of 5 trials [33]. Mice subjected to this test were between 9 and 12 weeks old.

3.4.6. Open field

During the open field test, mice were placed in a square arena (60 × 60 cm). The central area (44 x 44 cm) was more illuminated (200lux) than the periphery (150lux). Animals were first allowed to freely explore the arena without any influence of the examiner. During the test phase, the time spent in the center and the frequency of entering the central area were measured over a 20 min period, using video recording (Ethovision 6.1). The mobility was based on the variation of the complete detected area of an animal. Changes of more than 20% and more than 60% of the surface area were associated with mobile and highly mobile statuses, respectively. The test was

performed for two consecutive days [30]. Mice subjected to this test were between 8 and 10 weeks old.

3.4.7. Rotarod

Mice were tested for their ability to keep their balance and coordination on a rotating rod (Bioseb, Vitrolles, France). The time (latency) taken to fall off the rod rotating under continuous acceleration (e.g. from 4 to 40 rpm) was measured. Mice performed 3 trials per day, separated by 15 min inter-trial intervals, for 5 days, then after a five-week break, they were tested again in the same conditions for 3 days. Animals that remained on the rod for more than 300 s were removed and time was recorded as 300 s. Results are presented as the mean of the 3 trials [33]. Mice subjected to this test were between 10 and 13 weeks old.

3.4.8. Ladder rung

The apparatus is composed of 78 metal rungs spaced 1cm from each other across 1m long Plexiglas walls. The apparatus was set 50cm above the tabletop, and dark boxes containing food were placed at each end. The week before the test, during the habituation phase, mice were allowed to freely explore the apparatus on two separate occasions, and experimenters ensured that they crossed the whole ladder at least once in each session. During the test phase, each mouse is video recorded during four complete crossings of the ladder. Using the video, the number of complete foot placement misses is counted [34,35]. Mice housed in the same cage are tested consecutively. Mice subjected to this test were 7 weeks old.

3.4.9. Catwalk

The Catwalk test was used to assess the gait of voluntarily walking mice (Catwalk 7, Noldus; Wageningen, The Netherlands). Paw print recording allowed analysis of various aspects of the gait such as the base of support, the pressure of the paws on the floor, the duration of contact and the regularity of the step sequence. A minimum of three runs was conducted per animal, and analysis was performed on the fastest uninterrupted run [33]. Mice subjected to this test were 9 weeks old.

3.4.10. Social hierarchy – Tube test

Mice were tested for social hierarchy assessment. A 30 cm-long transparent plexiglass tube with a diameter of 3 cm was used. After training the animals to cross the tube on Day 1, two subjects of different genotype were released simultaneously on opposite sides of the tube for the test phase on Day 2. The encounter ended when one of the mice completely retreated from the tube. The subject remaining in the tube was the winner, scoring 1 point, and the retreated subject was scored with 0 points. If none retreated after 5 min, the encounter was scored as a draw and removed from the analysis. Each mouse was matched with different subjects of the opposite genotype (CTL vs. *Hoxa5-cKO*). Depending on cohort size, mice underwent the test 3 to 5 times with a different individual each time. The victory ratio is the number of points obtained divided by the number of iteration of the tests the mouse underwent [36]. Mice subjected to this test were between 11 and 17 weeks old.

3.4.11. Three-chamber social test

The three-chamber social test was used to assess sociability and interest in social novelty. It was performed as previously described [37]. Briefly, the apparatus consisted of three-chambered box where the central chamber has open doors allowing the mouse to move freely between chambers. The whole device is 60 x 40 x 22cm (L x l x h) and split lengthwise in three chambers of 20cm each. The two side chambers contained small wire cages composed of 3mm bars spaced by 7mm each, that can host stranger mice. A photo of the apparatus and wire cages is shown in Figure S1. The test consisted of three sessions of 10 min (habituation, sociability, and social novelty). First, the test mouse was placed in the center chamber and was habituated to the device containing the empty wire cages for 10 min. Then, to test the sociability, an age-matched stranger was placed into either of the side chambers under the wire cage while the other wire cage remained empty. The test mouse was allowed to freely explore the chambers for 10 min. In the third session, to test the social novelty, a second mouse (new mouse) was placed into the other side chamber and the test mouse was allowed to move freely within the device for another 10 min. The entire apparatus was wiped with 70% ethanol between mice to eliminate odor cues between animals. The time spent interacting with each mouse or empty cage (nose ≤ 1cm) was recorded by a video tracking system (Ethovision 6.1) for a period of 10 min in each test [31]. Mice subjected to this test were between 10 and 11 weeks old.

3.4.12. One-chamber social interaction test

For the one-chamber social interaction test, mice were familiarized with a square arena (40 x 40 cm) containing an empty wire cage (see description in section 3.4.11) during two 10-min trials on Day 1, with an inter-trial interval (ITI) of no less than 3 h. On Day 2, subjects were reintroduced into the arena for 10 min, briefly removed and then reintroduced for another 10 min while an age-matched stranger mouse was present. Time spent and number of visits in interaction (a 8 cm wide corridor surrounding the cage) were recorded, as well as avoidance (“corners” of the arena opposite to the location of the cage) zones, using a video tracking system (Ethovision 6.1) [38,39]. Mice subjected to this test were between 10 and 11 weeks old.

3.4.13. Social odor

The social odor test was conducted in the same manner as the olfaction test described above. The mice were exposed to a total of twelve cotton buds, for 1 min each. The three first buds were soaked in water, the following three were infused in soiled litter from a cage occupied by a group of males, the next three in soiled litter from another male-occupied cage, and the final three were exposed to urine from females in the oestral phase. Number of rearing events were counted for each cotton bud as an index of interest. Mice subjected to this test were 7 weeks old.

3.4.14. Grooming, burrowing and scratching

These tests were conducted in empty standard housing cages filled with wood-chip bedding. After a period of 10 min habituation, mice were video recorded for a period

of 10 min. The duration of grooming and burrowing bouts and the number of scratching events were manually scored using BORIS, an open-source video annotation software [40]. Mice subjected to this test were 7 weeks old.

Grooming behavior was defined as proposed by Berridge and colleagues [41]. Burrowing was defined as the mouse using its front paws to displace litter, whether by pushing it forward or backward between the hindlimbs. Scratching events were counted only when using the hindlimbs to scratch any parts of the body [31]. Given scratching bouts were very short (<1s), only the number of events was recorded and not the total duration.

A supplemental figure is provided to illustrate the behaviors assessed (Fig.S2).

3.4.15. Marble burying

For the marble burying test, large housing cages (38 x 22cm) were filled with wood-chip bedding and lightly tamped down to make a flat surface. Twenty 15mm glass marbles, evenly spaced in 4 rows, 5 marbles per row, were placed on the surface of the bedding. A photo of the experimental setup and marbles disposition is shown in Figure S1. The mouse was then placed in the cage for 30min. At the end of this period, moved marbles and marbles that were completely buried in bedding were counted [31]. Mice subjected to this test were between 10 and 11 weeks old.

3.4.16. Light-dark test

Unconditioned anxiety was assessed using the light–dark test (LDT) consisting of a cage divided into two sections of equal size by a partition with a door, with one side illuminated while the other side remained dark. At the beginning of the test phase, mice were placed into the dark chamber (considered to be a safe area) and were allowed to move freely between the chambers for 10 min. Time spent as well as distance travelled in the illuminated chamber was recorded by a video tracking system (Ethovision 6.1) [42]. Mice subjected to this test were between 9 and 10 weeks old.

3.4.17. Attention test

Mice were placed in a square arena (40 × 40 cm) on the floor of which four different items were placed. The test was split into two sessions of 15 min each, separated by a 30-min interval. During the first session of the experiment, the animals were allowed to explore the objects freely in a silent environment. During the second session, the mice were introduced in the arena in which the four items were placed differently to create a novel environment, and 5 min later an intermittent noise (rock music at about 80 dB) was delivered at random. The total durations of the noisy and silent periods were 3 and 7 min, respectively. The number of items visited during the two sessions was recorded by a video tracking system (Ethovision 6.1) [43]. Mice subjected to this test were between 12 and 15 weeks old.

3.5. Statistical analyzes

The software R [44] and RStudio IDE [45] were used for statistical analyses and graphical representation. The ggplot2, ggpubr and ggbeeswarm packages [46–48]

were used for graphical representation, while car, performance, emmeans and lme4 [49–52] packages were used for statistical modelling. For version details, R session info can be found in supplemental data (Table S2).

Appropriate statistical modeling was applied based on the nature of the data. Continuous data was analyzed using (mixed) linear modeling. A random effect term for the cohort was included when the experimental design permitted and the number of cohorts included was equal or greater than 4. A random effect for individual mice was included in the analysis of tests that involved repeated measures over time. Count data was analyzed using (mixed) generalized linear modeling, considering the finite or infinite possible range of values through binomial or Poisson distribution, respectively. Underlying modeling hypotheses were verified. When hypotheses could not be met, the experimental groups were compared using Wilcoxon rank exact sum test. The list of statistical models, model structure, post-hoc tests and the exact values of p-value represented in the graph can be found in Supplemental Table S2.

4. Results

4.1. *Hoxa5* and ASD

To assess the role of HOXA5 during the critical postnatal period of circuit connectivity, we previously used a conditional postnatal *Hoxa5* loss-of-function mouse model (*Hoxa5-cKO*). In this model, both *Hoxa5* alleles carry loxP sites flanking exon 2 [29]. Introducing a tamoxifen-inducible $CreER^{T2}$ transgene in this background enables *Hoxa5* exon 2 excision – and thus gene inactivation – following 4 consecutive intragastric tamoxifen injections in newborn pups (PN1 to PN4) [27]. Using this *Hoxa5* cKO model, a comparative transcriptomic analysis, conducted 3 weeks after birth (PN21), reveals a set of differentially expressed genes among which many genes associated with synaptic function [14]. Briefly, this set was obtained through CuffDiff analysis, setting a threshold of $p<0.05$ on the p-value adjusted using Benjamini-Hochberg correction. Building upon the increasing knowledge of risk factors for NDD/ASD during the last 10 years, we reconsider here the potential association between *Hoxa5* and ASD.

We therefore crossed the list of differentially expressed genes with the Simons Foundation Autism Research Initiative (SFARI) database, which classifies ASD-associated genes, and obtained 8 genes in common (Fig.1) [53,54]. Among these genes, downregulation of *Cadps2*, *Adcy1* and *En2* in *Hoxa5* cKO samples has already been tested and confirmed by RT-qPCR in our previous analysis [14]. Summary of the protein functions encoded by these genes is reported in Table S3. Among these genes, two human homologs have a score of 1 in SFARI, pointing to strong candidates (*Chd7* and *Reln*). In humans, *CHD7* mutations, as previously mentioned, result in the CHARGE syndrome while *RELN* mutations lead to Norman-Roberts syndrome, a form of microlissencephaly [55]. Additionally, with the exception of *Pde1c* and *Adcy1,* all of those genes have been associated with some sort of cerebellar abnormalities in mice, e.g. morphology, size, Purkinje cells or granule cells defects, as evidenced by a systematic search of the mammalian phenotypes annotation database provided by the Mouse Genome Informatics initiative (Table S3) [55]. It should be noted that the

mechanism through which HOXA5 regulates those genes has yet to be determined, and it remains to be established whether these are direct regulatory targets or if they act downstream in the regulatory cascades. Overall, this analysis thus highlights the regulation of several ASD-associated genes by HOXA5.

Gene	Adjusted p-value	Genetic category	Gene score	# reports
Reln	0.022	Rare Single Gene Mutation, Syndromic, Genetic Association, Functional	1	65
Chd7	0.012	Rare Single Gene Mutation, Syndromic, Functional	1	53
En2	0.012	Rare Single Gene Mutation, Genetic Association, Functional	2	27
Itpr1	0.012	Rare Single Gene Mutation	2	20
Cadps2	0.012	Rare Single Gene Mutation, Functional	2	13
Camk4	0.012	Rare Single Gene Mutation, Genetic Association	2	6
Pde1c	0.012	Rare Single Gene Mutation, Genetic Association	2	3
Adcy1	0.012	Rare Single Gene Mutation	3	4

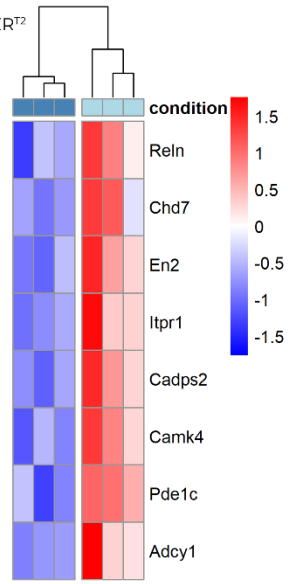

Figure 1: ASD-associated genes downregulated in *Hoxa5* postnatal knockout transcriptomic analysis. Table data are from SFARI and relate to human genes (https://gene.sfari.org/). Score goes from 3, suggestive evidence of link with ASD, to 1, strong candidate. Heatmap represents z score normalized expression values across RNA-seq samples from [14].

Given the expression of *Hoxa5* in precerebellar nuclei, the increasing evidence linking ASD to the cerebellum further supports the potential implication of HOXA5 in this NDD. Indeed, the cerebellum is one of the most consistently impacted brain regions in ASD patients, and animal studies have shown that Purkinje cell (PC)-specific gene knockouts or PC-specific inhibition are sufficient to induce ASD-related phenotypes [56–59].

In light of these data and the growing body of evidence showing a role of the cerebellum in non-motor functions, we decided to include assessments of both motor and cognitive (ASD-related) cerebellar functions in our behavioral analyses.

4.2. Behavioral phenotyping of *Hoxa5-cKO* mice

To specifically target the impact of HOXA5 during the critical periods of precerebellar circuits connectivity, we used our conditional postnatal *Hoxa5* loss-of-function mouse model. At first, we optimized the protocol to reduce the number of tamoxifen injections from 4 to 3 (PN1 to PN3 only), reducing stress for pups and mothers (Fig.2a). The breeding strategy described in section 3.2 allows tamoxifen treatment of entire litter without prior genotyping, as the CreER^{T2} transgene segregates in the

expected 1:1 Mendelian ratio, ensuring littermate controls within each treated group. Our two experimental groups, both treated with tamoxifen, differ by the presence (cKO) or absence (CTL) of the CreER^{T2} transgene. RT-qPCR analyses confirmed that this shortened regimen achieved inactivation efficiency comparable to the original four-dose protocol in *Hoxa5-cKO* by PN10 (Fig.2b). Recombination was verified in all animals of the first four cohorts after behavioral phenotyping and showed proper inactivation in *Hoxa5-cKO* animals (Fig.2c).

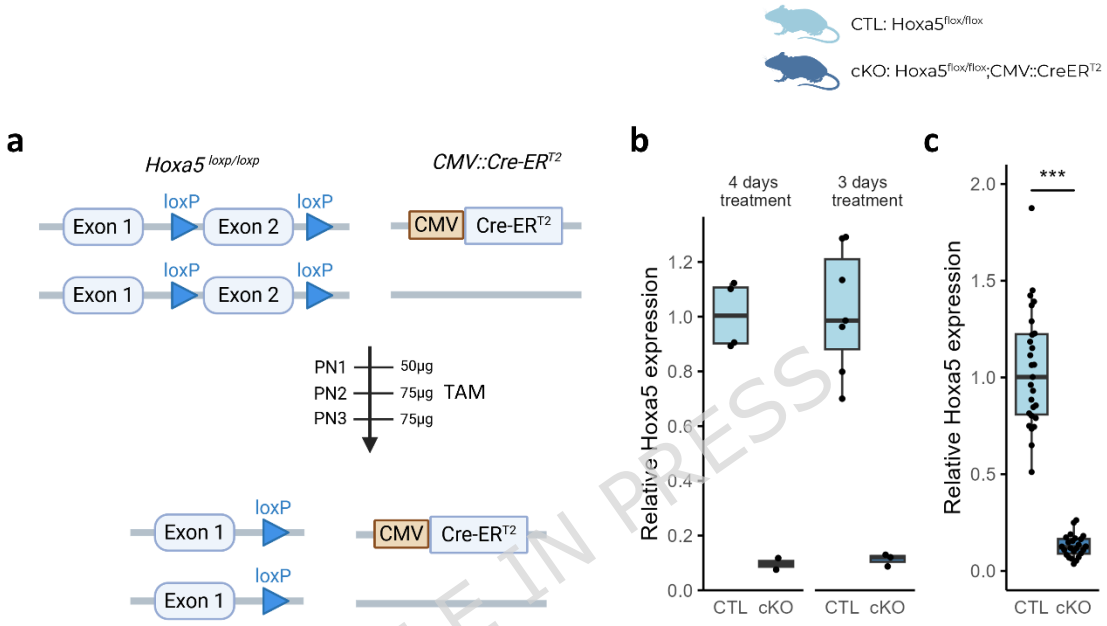

Figure 2: Protocol for the postnatal inactivation of *Hoxa5* and validation. a. Schematic of the genotype and protocol leading to tamoxifen (TAM)- induced Cre recombination. Protocol modified from Lizen et al., 2015, effectively reducing TAM intragastric administration from 4 to 3 postnatal days. The scheme was created in BioRender. Gofflot, F. (2026) https://BioRender.com/s7n26ux. **b.** RT-qPCR analysis of *Hoxa5* mRNA in animals treated with the short 3-days TAM treatment (right) or the original 4 days treatment (left). **c.** RT-qPCR analysis of *Hoxa5* mRNA in animals from cohorts G1 to G4. Brainstem medullary samples were collected at adult stage after behavioral phenotyping. (N_{CTL}= 26/N_{cKO}=24). ***: $p < 0.001$.

For the needs of this work, a total of 98 male mice were evaluated in 6 consecutive cohorts (G1 to G6). A summary of the cohorts, the corresponding number of mice of each genotype and the tests they were subjected to is presented in Table 2.

Table 2. Summary of the tests performed, their main purpose and the number of mice of each genotype for each, along with the corresponding cohorts (G). cKO: *Hoxa5-cKO* mice, CTL: control mice

Behavioral function	**Name**	**G1**	**G2**	**G3**	**G4**	**G5**	**G6**	**#CTL/#cKO**
Control	Food localization	x	x	x				15/14
	Olfaction	x	x	x				15/14
	Audition					x		11/13
	Grip test				x			12/10
Motor	Beam walking	x	x	x				15/14
	Rotarod				x	x	x	31/38
	Catwalk				x			12/10
	Open field	x	x	x				15/14
	Ladder					x	x	18/26
Social	Social hierarchy	x	x	x	x		x	30/31
	One chamber					x	x	19/28
	Social odor					x	x	13/27
	Three chambers	x	x	x	x			26/24
Stereotypy	Grooming					x	x	17/28
	Scratching					x	x	17/28
	Spontaneous burying					x	x	17/28
	Marble burying	x	x	x	x			27/24
Anxiety	Light-dark					x	x	19/28
	Open field	x	x	x				15/14
Attention	Attention test				x	x	x	31/38

4.2.1. Baseline assessment of sensory motor functions

To ensure an accurate interpretation of the behavioral data, a series of control assays was conducted to verify that all mice exhibited intact olfactory, auditory, and motor capacities. Because olfaction plays a central role in numerous mouse behaviors — particularly social interactions [60] — tests assessing odor detection and food localization were performed (Fig.S3). These assays revealed no significant differences

between genotypes, and the same result was obtained for the auditory startle–based assessment of hearing function. A grip strength test was additionally carried out to confirm that postnatal *Hoxa5* deletion did not impair forelimb or hindlimb strength, which could otherwise bias the interpretation of motor performance in subsequent tasks (Fig.S3).

4.2.2. Motor functions

Motor functions were thoroughly evaluated using five assays designed to assess motor performance, learning, memory, coordination, and fine motor control. First, the open field test was used to examine basic locomotor function and activity. We monitored the total distance traveled as an index of general activity, as well as the proportion of time spent being immobile, mobile and highly mobile (Fig.3a). None of those parameters differed between CTL and *Hoxa5-cKO* mice.

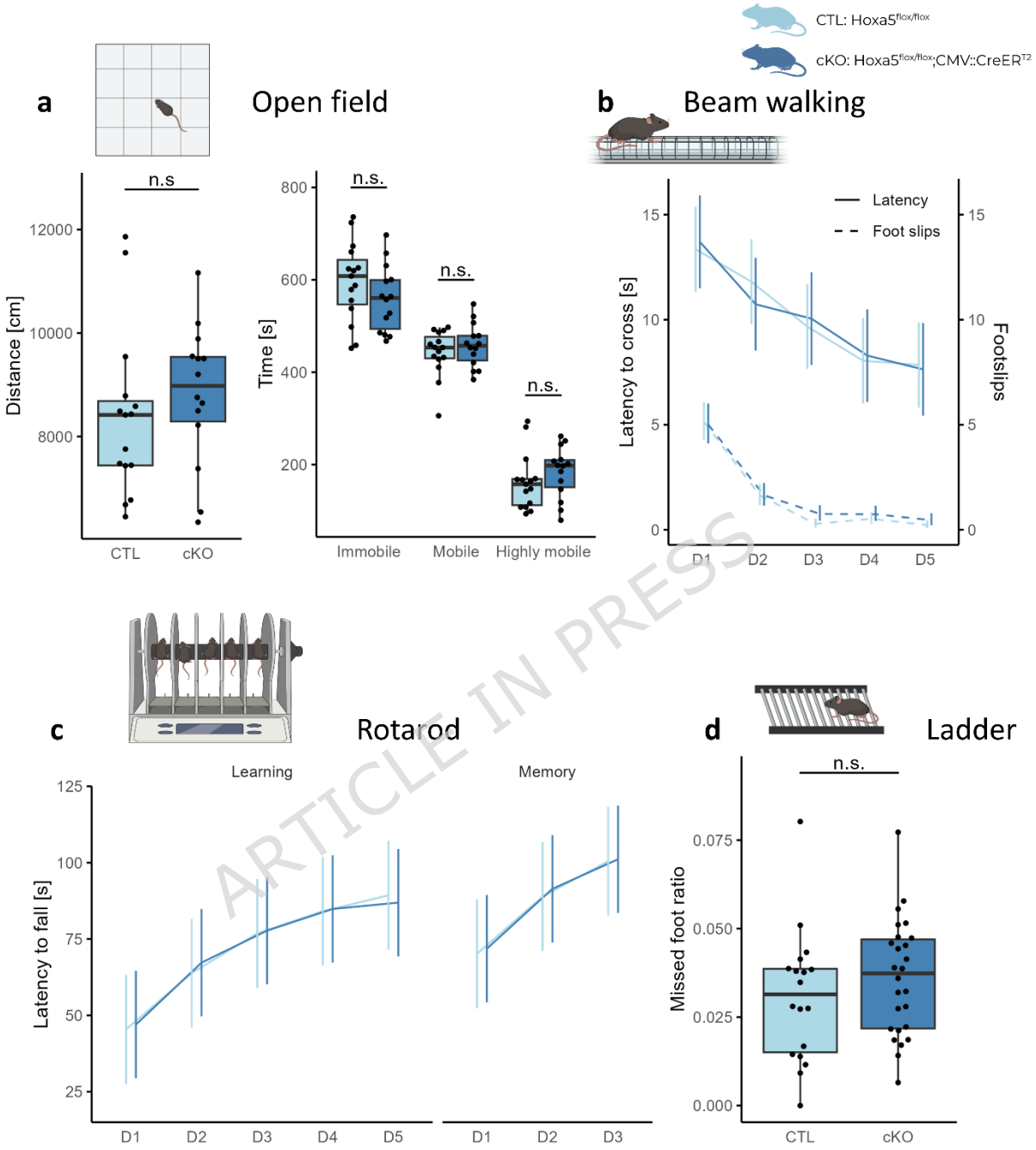

Figure 3: Motor functions in *Hoxa5-cKO* mice. a. Open field test. Results show the total distance moved and the total time spent in three activity profiles from immobile to highly mobile. (N_{CTL}= 15/N_{cKO}=14). **b.** Beam walking test shows latency to cross (upper curve, full line) and number of foot slips (lower curve, dotted line). (N_{CTL}= 15/N_{cKO}=14). **c.** Rotarod test shows latency to fall from the apparatus plotted for each day of testing. The first five days are labelled learning, and memory was tested for 3 additional days after a five-weeks break. (N_{CTL}= 31/N_{cKO}=38). **d.** Ladder rung test. Number of completely missed food placements are represented. (N_{CTL}= 18/N_{cKO}=26). CTL are in light blue and cKO are in dark blue. n.s.: non significant. The illustrations of the tests were created in BioRender. Gofflot, F. (2026) https://BioRender.com/hcr7v0b.

Next, motor coordination and learning were investigated in the beam walking test (Fig.3b). Both genotypes displayed similar baseline coordination abilities on the first day of testing. Learning trajectories across the five sessions were also similar, with no significant differences in foot slips or crossing latency. The rotarod assay, which evaluates similar capabilities, confirmed these results, showing equivalent baseline coordination and learning rates in both groups. A second test phase, conducted after a five-week break to assess motor memory, also revealed no genotype-dependent differences (Fig.3c). To assess fine motor skills, we then used the ladder rung walking test. Mice were recorded when walking a horizontal ladder with evenly spaced rungs, and each paw placement was scored either as correct foot placement or complete misstep (Fig.3d). No significant difference in the ratio of missed foot placements were highlighted. Finally, gait and locomotion were analyzed using the Noldus Catwalk apparatus. Among the numerous parameters providing detailed information regarding gait pattern and paw placement, none showed significant differences between CTL and *Hoxa5-cKO* mice (Table S4).

Taken together, these results consistently indicate that postnatal inactivation of *Hoxa5* does not affect motor function, coordination, learning or memory.

4.2.3. Social behaviors

Impaired social behaviors are a hallmark symptom of ASD. We first examined whether *Hoxa5* postnatal inactivation influenced social hierarchy. The tube test – a validated method to assess social dominance and subordination between mice [61] – revealed no significant difference in the victory ratio between groups (Fig.4a). Because this experiment involved 61 animals from several cohorts transferred independently to the behavioral platform, we tested whether the genotype effect varied across cohorts. The interaction term between genotype and cohort yielded a significant p-value and can be visualized by analyzing cohorts separately (Fig.4b). Although *Hoxa5-cKO* mice tended to lose more frequently in the G1, G2, G3 and G6 cohorts, this trend reached statistical significance only in G2. The victory ratio was completely reversed in G4, supporting the absence of a consistent genotype effect overall.

Based on the olfaction test (Fig.S3), we applied a similar strategy to evaluate responsiveness to social cues, i.e. the social odor test. Mice were exposed to cotton swabs infused with male-soiled bedding or female estrous urine, and rearing events were quantified as an index of interest (Fig.4c). No differences were detected between CTL and *Hoxa5-cKO* mice, indicating preserved social odor reactivity.

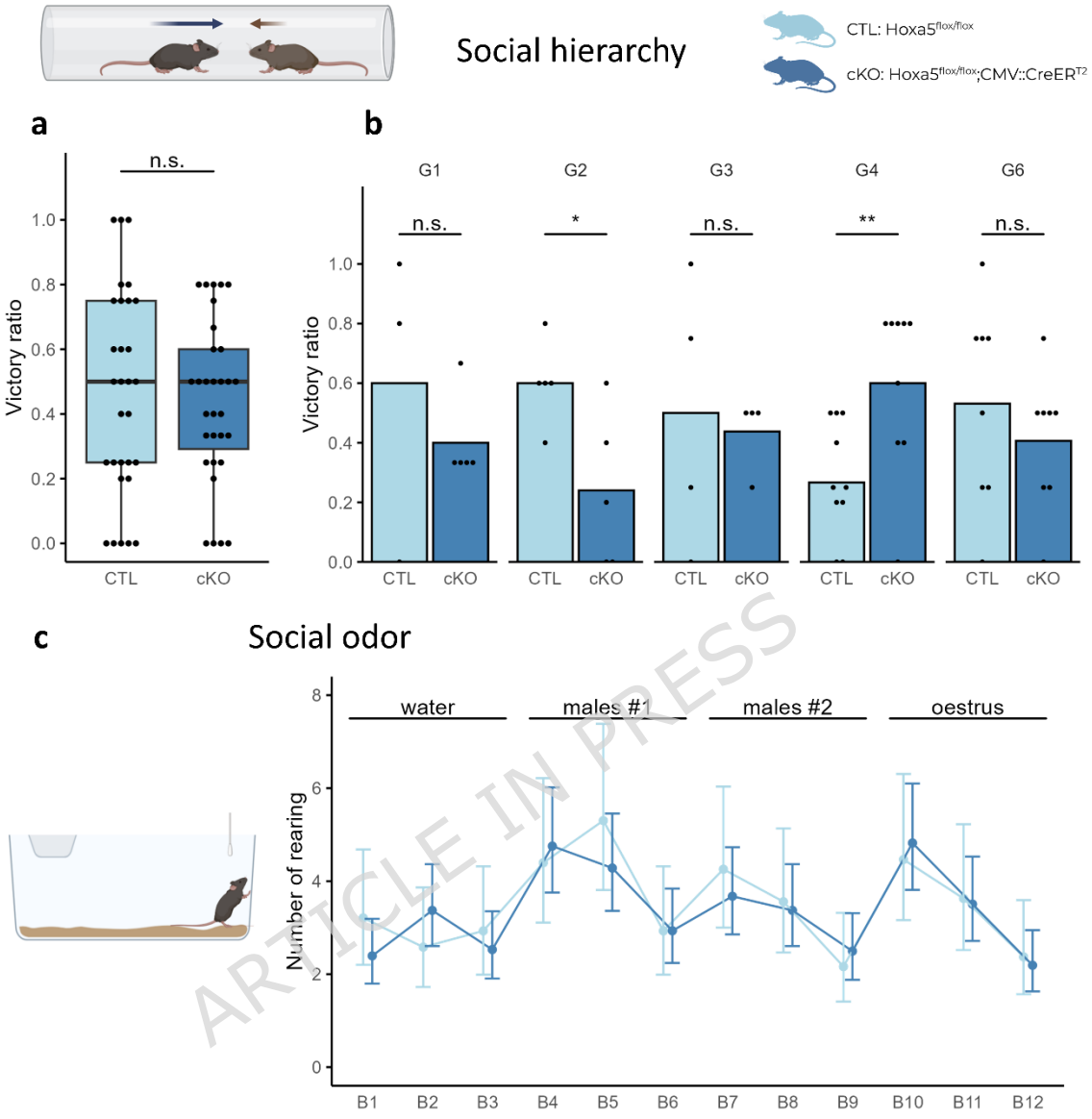

Figure 4: Social hierarchy and interest in social olfactive cues. a. Social hierarchy/tube test. Victory ratio is the number of times a mouse managed to push its opponent to the other end of the tube, divided by the number of trials. (N_{CTL}= 30/N_{cKO}=31). **b.** Illustration of the variability in the results of the tube test according to the cohort (G). (N_{CTL}/N_{cKO} per cohort: 3/5 – 5/5 – 4/4 – 10/9 – 8/8). **c.** Social odor test. Mice are presented with cotton buds (B) infused with water or socially related odors (refer to material and methods for specific details) for one minute each. Number of rearing are counted as a measure of interest. (N_{CTL}= 13/N_{cKO}=27). CTL are in light blue and cKO are in dark blue. n.s.: non significant; *: $p < 0.05$; **: $p < 0.01$. The illustrations of the tests were created in BioRender. Gofflot, F. (2026) https://BioRender.com/hcr7v0b.

The three chambers test is a classic sociability assessment test. In the first phase of the test, called social preference, an unknown mouse is used as social stimulus. In the second phase, called social novelty, a new unknown conspecific is added, which is then considered as the social stimulus. Two standard analytical strategies were applied. The first method quantifies the time spent in each chamber, associating

entire chambers with social or non-social contexts. This analysis highlighted reduced sociability in *Hoxa5-cKO* mutants during the social novelty phase (Fig.5b), as illustrated by the shorter amount of time spent in the 2nd stranger chamber by the *Hoxa5-cKO* mice. It also revealed a preference for the non-social stimulus during the social preference phase (Fig.5a). Although atypical, this preference was not significant in *Hoxa5-cKO* individuals, suggesting different social behaviors according to the genotype. To refine the analysis, we implemented a higher-resolution analysis limited to the time during which the mouse's nose remained within 1 cm of each cup (Fig.5c-d) [62]. This method revealed a preference of both genotypes for the empty cup in the social preference phase, and increased interest for the second stranger in the social novelty phase. This second analysis therefore eliminated any statistical differences between CTL and *Hoxa5-cKO* mice and confirmed atypical social preference but expected novelty preference. Alternative representations of the data were also considered and are proposed in Supplemental Figure S4. Those include the preference (or sociability index) and the ratio between the time in the interaction zone and the total time in the chamber (Fig.S4).

A one-chamber variant of the assay – reported to be more sensitive to social interaction [38,39] – was also performed. In this paradigm, non-social time is measured during the habituation phase when the cup is empty. Consistent with the results of the social novelty phase, both CTL and *Hoxa5-cKO* mice spent more time investigating the cup when it contained an unfamiliar conspecific, and the two genotypes were not statistically different (Fig.5e).

Overall, collected data did not reveal robust genotype-dependent alterations in social behavior. However, the substantial variability—particularly across cohorts—hinders interpretation of the results and underscores the need for cautious conclusions.

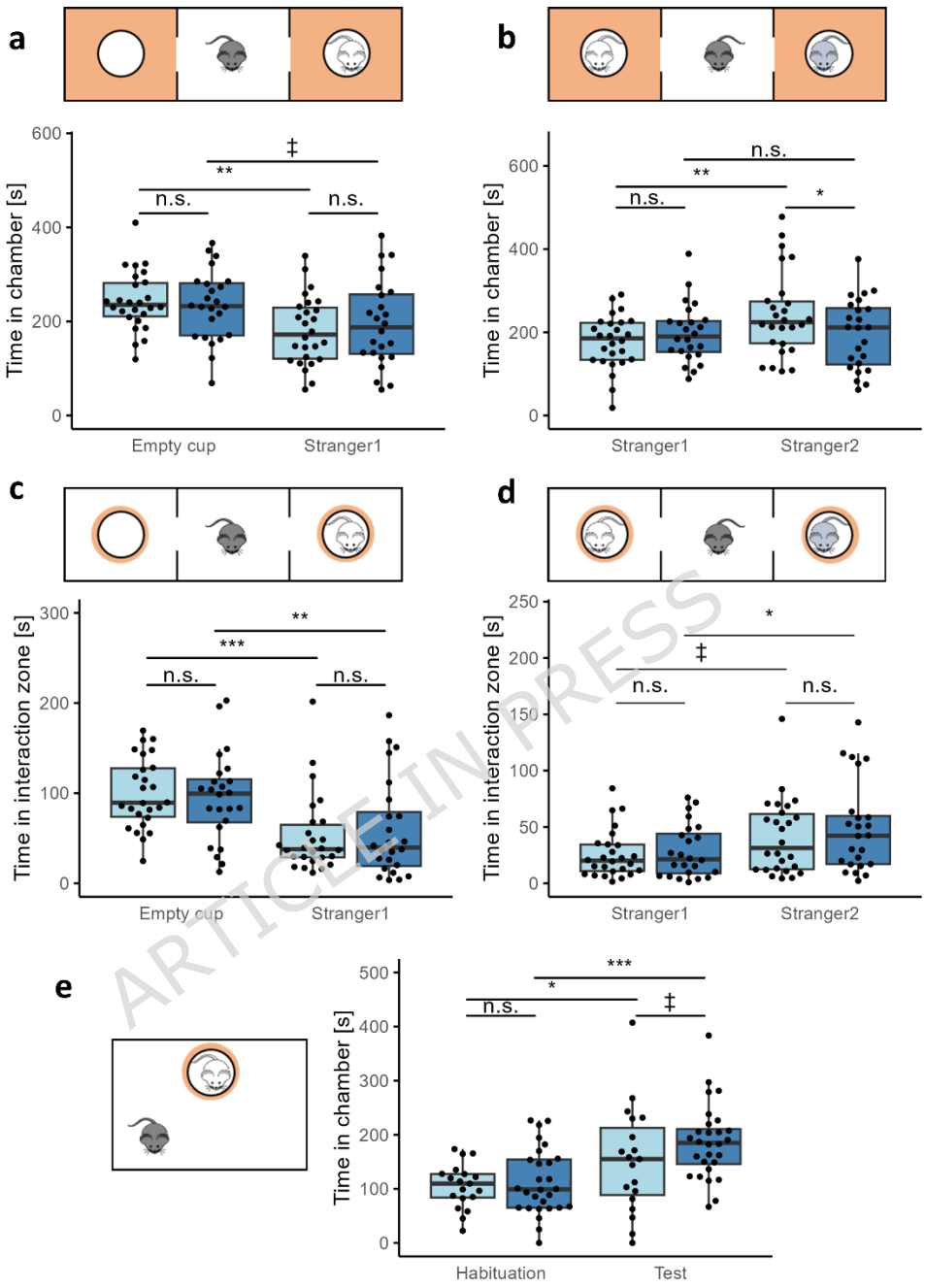

Figure 5: **Sociability evaluation. a - b.** Three chambers test, social preference and social novelty phases, respectively. Time spent in each chamber is monitored. **c - d.** Three chambers test using the 1-cm zone analysis, the social preference and social novelty phases, respectively. Time that the mice spend with the nose within 1 cm of the cage is measured (N_{CTL}= 26/N_{cKO}=24). **e.** One chamber test. Variant of the three chambers, the time spent in a 1-cm zone around the cage during habituation and during the test (with a mouse inside) are measured. (N_{CTL}= 19/N_{cKO}=28). CTL are in light blue and cKO are in dark blue. n.s.: non significant; ‡: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. The illustrations of the tests were created in BioRender. Gofflot, F. (2026) https://BioRender.com/hcr7v0b.

4.2.4. Stereotypical behaviors

The other core diagnostic dimension of ASD consists of stereotyped and repetitive behaviors, defined as recurrent actions lacking an apparent adaptive purpose. In mice, such behaviors commonly manifest as increased self-grooming, burrowing, rearing, circling, or other highly repetitive motor patterns.

To evaluate those motor stereotypies, mice were placed in an empty cage containing only bedding, and multiple behaviors were recorded and quantified. The time spent on self-grooming and burrowing did not differ between genotypes. In addition, scratching events were recorded. Although scratching is not in itself a stereotyped behavior, an elevated frequency in the absence of a clear trigger—such as skin irritation or parasitic infection—can indicate a shift toward repetitive, nonfunctional behaviors. *Hoxa5-cKO* mice displayed significantly higher scratching counts than the CTL (Fig.6a). Finally, the marble burying test revealed a highly significant increase in the number of marbles buried or displaced by *Hoxa5-cKO* mice relative to CTL mice (Fig.6b), consistent with increased repetitive or compulsive-like behaviors.

Taken together, these findings indicate that postnatal deletion of *Hoxa5* contributes to an increase in some motor behaviors classified as stereotyped.

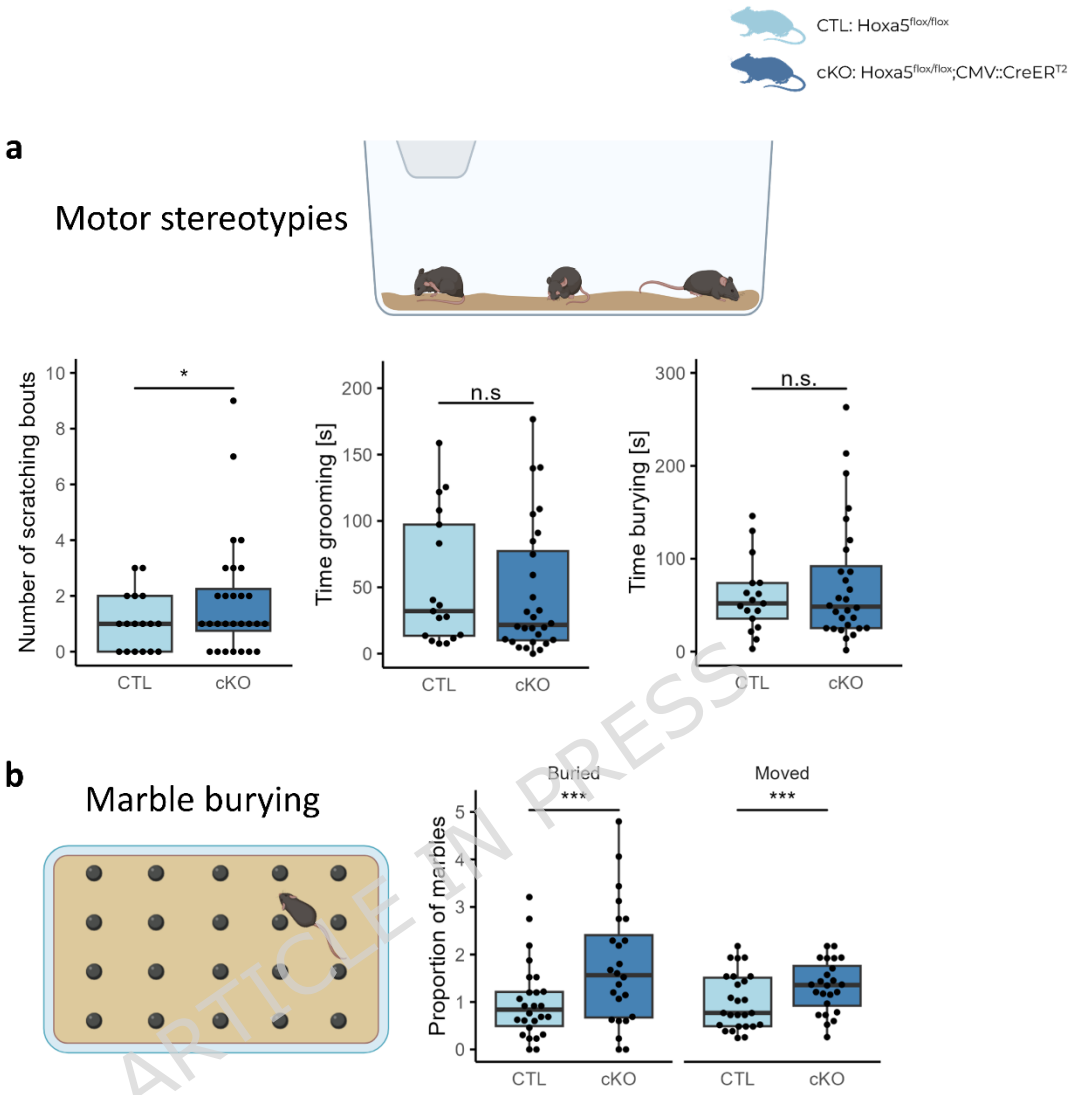

Figure 6: **Stereotypical behaviors. a.** Motor stereotypies assessed in a single test, from left to right, number of scratching bouts, time spent self-grooming and time spent burrowing (N_{CTL}= 17/N_{cKO}=28). **b.** Marble burying. Displayed is the proportion of marbles (for a total of 20) buried relative to the mean of the control by cohort, in order to better illustrate the results of the mixed model, which included a random effect for the cohort. (N_{CTL}= 26/N_{cKO}=24). CTL are in light blue and cKO are in dark blue. n.s.: non significant; *: $p < 0.05$; ***: $p < 0.001$. The illustrations of the tests were created in BioRender. Gofflot, F. (2026) https://BioRender.com/hcr7v0b.

4.2.5. Common ASD comorbidities

In addition to the core symptoms of ASD, attention deficits and anxiety disorder are frequently reported comorbidities in patients [63,64]. In the standard open field test, the time spent in the brightly lit center of the arena is commonly interpreted as an inverse correlate of anxiety [65]. *Hoxa5-cKO* mice displayed a greater tendency to explore the center than CTL mice (Fig.7a), suggesting the absence of anxiety and/or an increased exploratory interest. Another test was thus applied, the light-dark test (Fig.7b), that is considered a stronger anxiogenic challenge, as mice choose between a fully dark compartment and a brightly illuminated one. In this test, although the

median value for *Hoxa5-cKO* mice was higher than that of CTL mice, the high individual variability prevented to highlight a significant difference. In addition, the latency to first entry in the illuminated area was also analyzed but did not reveal significant differences across genotypes (Fig.S4).

To evaluate attention, we adapted an exploratory paradigm designed to detect changes in exploratory behavior under unpredictable sensory distraction [43]. Mice were allowed to freely explore a novel, object-enriched environment, during which 1-minute music excerpts were played at random intervals. Time spent moving was then compared across genotypes and between silent versus sound sessions. Both CTL and *Hoxa5-cKO* mice showed reduced locomotion during music playback, and no genotype effect was observed (Fig.7c).

Overall, these assays did not reveal evidence of anxiety- or attention-related impairments following postnatal *Hoxa5* deletion.

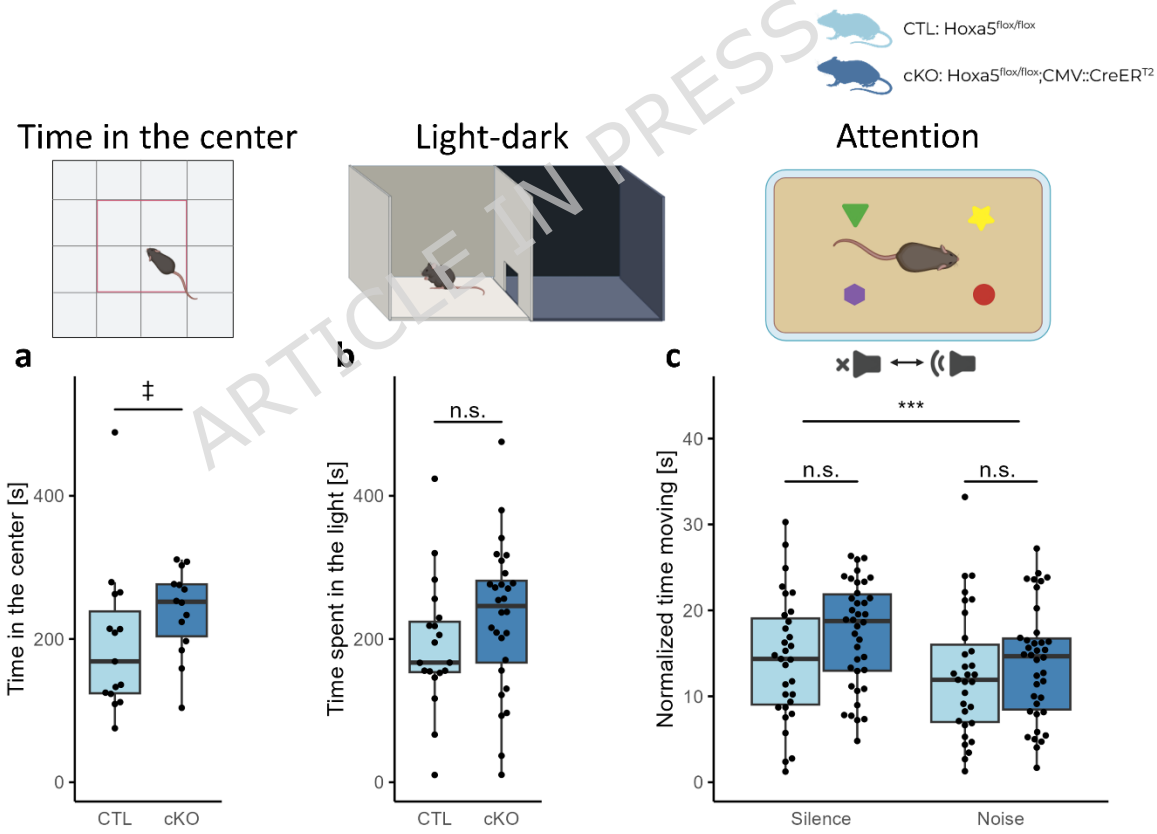

Figure 7: **Anxiety and attention-related behaviors. a.** Open field. Represented here is the time spent in the center. (N_{CTL}= 15/N_{cKO}=14). **b.** Light-dark. Mice have access to a brightly lit and a dark room. The time spent in both is monitored. Displayed here is the time spent in the brightly lit room. (N_{CTL}= 19/N_{cKO}=28). **c.** Attention test. Mice are left to explore a cage with various objects. During exploration, loud music is played at random. Time in movement normalized by the duration of silent/noisy periods is displayed. (N_{CTL}= 31/N_{cKO}=38). CTL are in light blue and cKO are in dark blue.

n.s.: non significant; ‡: $p < 0.1$; ***: $p < 0.001$. The illustrations of the tests were created in BioRender. Gofflot, F. (2026) https://BioRender.com/hcr7v0b.

5. Discussion

Based on our previous data, we hypothesized that HOXA5 could contribute to the establishment and maturation of precerebellar neuronal circuits during critical periods of early postnatal life through its regulation of synapse-related players. As a corollary to this hypothesis, its inactivation could result in deficits in these processes, leading to synaptopathies and cerebellar dysfunction. Here, we confirmed that the early postnatal *Hoxa5* inactivation leads to deregulation of several genes showing strong evidence of association with ASD, one NDD classified as synaptopathy. Using this conditional postnatal *Hoxa5* loss-of-function mouse model, we further evaluate the role of HOXA5 through a comprehensive characterization of the behavioral consequences of its postnatal deletion. As assessed by selected tests, the motor functions of *Hoxa5-cKO* animals remained unimpacted by the deletion, while results from investigating non-canonical cerebellar functions and ASD-related phenotypes appeared more challenging to interpret. Indeed, results of the social behavior tests were sensitive to analytical methods and stereotypical manifestations increased in some testing paradigms. Anxiety and attention-related behaviors showed no difference between CTL and *Hoxa5-cKO* mice. These contrasting results are discussed below.

5.1. *Hoxa5* and motor behaviors

Although *Hoxa5* expression in the hindbrain is predominantly detected in precerebellar nuclei, its inactivation has consistently no impact on motor coordination and learning, a finding that may be explained by several non-mutually exclusive hypotheses. First, while tamoxifen administration was initiated early after birth (PN1-PN3), recombined cell numbers progressively increased during – and potentially shortly after – the treatment window [27]. Depending on the stability of HOXA5 protein, the depletion of HOXA5 might occur too late to fully disrupt early postnatal HOXA5-dependant processes. Second, *Hoxa5* expression is only detected in restricted populations within each precerebellar nuclei, which may limit the functional impact on global motor phenotype. Finally, evidence shows some level of functional redundancy within *Hox-PG5* genes, as functional impacts of *Hoxa5* deletion in phrenic motor neurons require a *Hoxc5* deficient background to be observed [18,19]. Consistently, significant spatial overlap of *Hox-PG5* gene expression domains has been reported at both perinatal and postnatal stages [10,66], suggesting co-expression of the *PG5* genes and subsequently reinforcing the likelihood of functional redundancy. Of note, neither *Hoxb5* nor *Hoxc5* were significantly deregulated in the RNA-seq conducted on PN21 Hoxa5-*cKO* animals [14].

5.2. *Hoxa5* and social behaviors

In the social hierarchy test, high inter-cohort variability resulted in an overall inconclusive outcome. While some degree of inter-individual and inter-cohort variabilities is inevitable when testing large groups of animals, it can generally be controlled in statistical analyses if the variability remains consistent across cohorts.

In this case, however, the genotype effect differed between cohorts, preventing robust conclusions from being drawn. Such variability may arise from uncontrolled environmental factors, including differences in housing, transport, handling, or subtle variations in social composition within cohorts [67]. In contrast, for all the other behavioral assays, the cohort term in our statistical models was often significant, indicating that although inter-cohort differences existed, their influence was stable across genotypes.

In the social preference test, an unexpected preference of control mice for the empty cup chamber prompted us to reanalyze the videos using a 1-cm interaction zone around each cup. This refined analysis confirmed a consistent preference for the empty cup over the unfamiliar mouse across both genotypes but also suppressed the genotype-dependent difference in sociability observed during the social novelty phase. Nonetheless, the 1-cm zone analysis is considered more sensitive for detecting genuine social interest and reduces the confounding influence of general exploratory activity [62]. Such atypical behavior may arise from the genetic background used. Indeed, it has been shown that some social behaviors vary between strains, and that the specific lack of interest for the novel individual is common for C57BL/6J mice, which is the background of mice used in this study [68,69].

Finally, considering all social behavior paradigms, including the one-chamber and social odor tests, the collective results do not provide evidence for a significant effect of postnatal *Hoxa5* deletion on social behaviors.

5.3. *Hoxa5* and stereotypical behaviors

Specific stereotyped behaviors appear to emerge following postnatal inactivation of *Hoxa5.* In the marble burying test, results highlighted a marked increase in the number of marbles moved and buried by *Hoxa5-cKO* mice (Fig.6b). Although this finding could potentially be confounded by increased locomotor activity of *Hoxa5-cKO* mice [70], mobility data from the open field test indicate that the overall activity levels did not differ significantly between genotypes. While elevated marble burying behavior has occasionally been interpreted as a manifestation of novelty-induced anxiety, evidence supports a stronger association between this measure and stereotypical or perseverative behaviors [71,72]. Consistent with this interpretation, performance in the light–dark box and the proportion of time spent in the center of the open field (Fig.7a) did not indicate heightened anxiety in *Hoxa5-cKO* mice. Together, these findings support the view that the increase in marbles buried reflects repetitive behavioral patterns rather than anxiety-related responses.

The lack of genotype difference in burrowing behaviors could suggest that the burying response was specifically triggered by the presence of marbles. Nevertheless, as discussed above, this reaction does not appear to be linked to anxiety induced by the environment. In the same experimental context, *Hoxa5-cKO* mice also displayed a higher number of scratching bouts. Given the shared housing conditions, regular veterinary inspections, and absence of dermatological or parasitic signs in the colony, skin condition can probably be ruled out of the equation.

Consequently, this repetitive scratching behavior may also represent a form of stereotypy.

5.4. Considerations about behavioral analyses

Our data highlighted that tests evaluating cognitive behaviors, such as social and stereotyped behaviors, are more sensitive than motor functions to parameters independent of the targeted genotype, such as genetic background, environmental factors or methodological practices, which may hinder the ability to obtain statistically valid results.

Indeed, behavioral assessment in the context of complex NDD such as ASD is subject to several important limitations. First, the etiology is highly heterogeneous, and the clinical manifestations are variable. The phenotypic penetrance of ASD-related traits can differ dramatically between individuals, which undermines the power of standard behavioral assays and statistical tests that typically assume large, consistent effect sizes across subjects. A well-established factor influencing ASD penetrance and/or diagnosis is sex [73,74]. Given the strong sex bias observed in ASD diagnosis, preclinical research has often focused on male mice only. In addition to further increasing existing sex bias, this may have hindered our understanding of ASD etiology [75]. While it is particularly true when establishing a mouse model of ASD that reaches face, construct and predictive validity, we argue that focusing on male mice only is relevant in achieving our goal of identifying potential new players of the disorders, as it requires to maximize statistical power. However, by ignoring females, this method prevents from drawing conclusions on the whole population.

Second, methodological practices in behavioral testing can significantly influence outcomes. Our own experience with three-chamber social interaction assay illustrates how analytic choices can influence the interpretation of results, highlighting the need for more rigorous standardization of behavioral protocols across laboratories. This aligns with recent recommendations advocating improved reporting and harmonization of behavioral phenotyping practices in neurodevelopmental disorder research [76].

Third, statistical analysis in behavioral neuroscience often requires more sophistication than is sometimes applied. Data from behavioral experiments are frequently non-normally distributed, and may have nested or hierarchical structure, therefore requiring generalized linear modeling and/or mixed modeling [77]. In such cases, use of parametric statistics may inflate error rates or miss real effects. Finally, significant variation across inbred strains is observed in standard assays used for ASD-associated behaviors [68]. Consistently, in our own preliminary tests, mice of a mixed background with a strong 129Sv component showed little to no motivation in participating in the tests, e.g. falling off immediately from the rotarod apparatus. Great care should be taken in selecting the appropriate genetic background [78–80].

Finally, another caveat of our system is associated to the CMV-CreER^{T2} mouse transgenic line that was generated using pronuclei injection of the transgene leading to random insertion in the genome, that could be associated to confounding effects. Indeed, the random insertion of the transgene could disrupt the expression of one or

several genes. However, this mouse line was first published in 2005, used in several studies, and no effect of the insertion site was reported since [28]. In the present study, we reasoned that the tamoxifen was more likely to impact behaviors of mice and thus favored using mice treated with tamoxifen and negative for the CMV-CreER^{T2} as control.

5.5. *Hoxa5* and NDD

ASD is known for its strong, yet highly heterogenous, genetic component. Genetic variation plays a major role, and it is thought that small effects of multiple common variants act additively [81]. Due to its crucial role in prenatal development, *Hoxa5* loss-of-function variants are highly unlikely to lead to viable individuals in humans [3]. However, a deregulation of *Hoxa5* expression through promoter methylation, similar to that uncovered in CHARGE and Kabuki syndromes, could contribute to an ASD-conducive genetic context [21]. Additionally, regulatory elements of ASD-risk genes have been found to exhibit higher rates of *de novo* mutations in ASD patients [82,83]. These mechanisms provide additional potential modes of modulation of *Hoxa5* expression that have not been systematically studied.

5.6. Conclusion & perspectives

Although many genes associated with synaptic functions and risk factors for ASD are regulated by HOXA5 at the early postnatal period, its postnatal loss-of-function as implemented here did not result in motor or neurological phenotypes, at the exception of increased motor stereotypies. Of interest, stereotypical and repetitive behaviors have been induced in mice with cerebellum-specific neuronal activity modulation or PC-specific gene knockout [58,59]. The observed phenotype could thus result from an alteration of precerebellar circuit connectivity and/or function. While the absence of a motor phenotype is strongly supported by robust and reproducible data, the lack of a significant effect on social behavior has been interpreted considering methodological limitations. Altogether, our results are consistent with a potential role of HOXA5 as a regulator of precerebellar circuits connectivity and/or function, although the low expressivity of the phenotypes suggests a rather minor contribution, a redundancy within the system, or possibly both.

According to these results and the identified limitations, new hypotheses and analyses can be proposed. First, postnatal deletion of *Hoxa5* in a *Hox*-PG5 deficient background might be required for quantifiable effects, as highlighted by research on *Hoxa5* activity in the developing spinal cord [18,19].

Second, deleting the *Hoxa5* gene without Tamoxifen injection of the pups would also likely benefit the study of complex cognitive behaviors such as social behaviors. Indeed, tamoxifen injection and pup handling induce stress in both pups and the mother, and tamoxifen has been shown to impact mice microbiota [84], which could in turn impact complex and ASD-related behaviors [85]. Deletion using specific promoter-driven Cre expression would therefore resolve those issues. Our investigation highlighted *Gabra6-Cre* transgenic line as a candidate that provides good spatial coverage of *Hoxa5* expression pattern, although its temporal expression might be too late for the purpose of this work [86].

Finally, one would expect that testing hypotheses in a more natural setup for the mice would bring better results. For example, analysis of social interactions in freely moving mice is now possible thanks to advanced deep learning tools, circumventing the use of some of the paradigms used in this study, thus enabling the identification of more subtle behavioral changes [87].

6. Competing interests

The authors declare that they have no competing interests

7. Funding

8. Authors' contributions

FG conceived the project, supervised the research and designed the experiments with HG, LB and CM. HG and LB collected the experimental data, with the contribution of OS, performed the statistical analysis and created the figures and tables. HG and LB performed the interpretations and discussion of the results and wrote the paper with FG. LJ, CM and FC discussed the data and revised the manuscript. LJ provided the Hoxa5$^{flox/flox}$ mouse line.

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Figure 2’s representation of tamoxifen treatment was created in BioRender. Gofflot, F. (2026) https://BioRender.com/s7n26ux. Illustrations of tests in figures 3 to 7 were created in BioRender. Gofflot, F. (2026) https://BioRender.com/hcr7v0b.

10. Data availability statement

Data gathered during this study and presented here can be shared upon reasonable request to the corresponding author.

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