Assessing the developmental trajectory of mouse models of neurodevelopmental disorders: Social and communication deficits in mice with Neurexin 1α deletion

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Abstract
Neurexin 1α mutations are strongly associated with neurodevelopmental disorders such as autism spectrum disorders and schizophrenia in humans. Studies using the Neurexin 1α knock-out mouse have showed behavioral abnormalities of relevance to these disorders and baseline deficits in excitatory synaptic function have been described. However, little is known about the effect of Neurexin 1α deletion on behavior during development. This study examined the effects of Neurexin 1α deletion on behavior across a range of developmental time points to determine whether potential abnormalities follow a developmental trajectory. Pups lacking Neurexin 1α emitted a reduced number of ultrasonic vocalizations early in development combined with a restricted repertoire of calls indicative of a loss in complexity in vocal production and showed delays in reaching certain developmental milestones. Behavioral testing showed that juvenile and adult male Neurexin 1α knock-out mice exhibited social deficits and increased levels of aggression, confirming previous findings. No increases in repetitive behaviors or deficits in motor learning or olfaction were seen. In conclusion, this research showed that Neurexin 1α deletion does result in social and communication deficits that follow a developmental trajectory. These are the first experimental data that associate a deletion of Neurexin 1α with alterations in behaviors relevant to autism spectrum disorder across development and highlight the importance of assessing the developmental trajectory in mouse models of neurodevelopmental disorders.

KEYWORDS
autism, communication, genetics, models, mouse, neurexin, neurodevelopment, schizophrenia, social behavior, vocalization

1 INTRODUCTION

Strong evidence exists for a role of synaptic genes in autism spectrum disorders (ASDs), including genes encoding the Neurexin family of genes.
proteins. Neurexins are a family of transmembrane proteins located at the presynaptic terminal. They play an important role in synapse function and development, which they perform via the formation of cell adhesion complexes with postsynaptic neuroligins and several other binding partners. Neurexin 1 (NRXN1), 2 (NRXN2) and 3 (NRXN3) have been implicated in a wide range of neurodevelopmental disorders including ASD and schizophrenia, for which the strongest evidence of an association appears to be in Neurexin 1α. Heterozygous deletions involving NRXN1 shows replicated association with schizophrenia. In addition, point mutations, disruption and genomic deletions of NRXN1 have also been found to confer a high risk of intellectual disability, as well as ASD, epilepsy and for homoygous loss-of-NRXN1 function, Pitt-Hopkins-like syndrome. Recently, the observed variable phenotypic spectrum associated with exonic NRXN1 deletions was investigated further in 25 previously un-described carriers, and some new related phenotypes were identified as well as reconfirming recurrent phenotypic features, including moderate to severe ID, severe language delay, ASD, seizures and hypotonia, confirming that NRXN1 deletions predispose individuals to a wide spectrum of neurodevelopmental disorders. Almost all of these deletions only affect the 5’ exons that encode the alpha isoform of NRXN1. It is estimated that a NRXN1 deletion is present in 0.32% of ASD cases, in comparison to only 0.057% in control cases. While this number may seem low, it is important to consider that the most highly associated genes are only present in 1% to 2% of individuals with ASD because of the high levels of genetic heterogeneity, meaning that NRXN1 deletions are in fact among the most common mutations in ASD.

Previous studies using a mouse model for the human NRXN1 copy number variation (CNV) mutation, the Neurexin 1α mouse knockout (Nrxn1α−/−/Nrxn1α−/−) maintained on a mixed genetic background (C57BL6/SV129), found significant deficits in prepulse inhibition, a measure of sensorimotor gating, increased repetitive behaviors (self-grooming) and impaired nest building behaviors in the Nrxn1tm1Sud/Nrxn1tm1Sud knockout (KO; −/−) mice, that have been associated with altered social behavior. In addition, increased responsiveness to novelty and accelerated habituation to novel environments were observed in male but not female Nrxn1tm1Sud/Nrxn1tm1Sud heterozygous (HET; +/−) mice and deficits in social memory have been seen in both male and female Nrxn1tm1Sud/Nrxn1tm1Sud HET (+/−) mice. Given the confounding effect of genetic background on observed phenotypes, Nrxn1α KO mice were generated on a pure genetic background (C57BL/6J) based on our previous study and may appear from an earlier age than previously reported, possibly showing an altered developmental trajectory for ASD-like behaviors in mice.

2 MATERIALS AND METHODS

2.1 Mouse generation, breeding and husbandry

Male and female Nrxn1α KO mice were generated as described and were genotyped by PCR using genomic mouse DNA from ear punches (WT primer: CGA GCC TCC CAA CAG CGG TGG CGG GA, KO primer: GAG CGC GCG AGT TGT TGA C, common primer: CTG ATG GTA CAG GCC AGT AGA GCA CCA). These mice had previously been maintained on a C57BL6/SV129 mixed genetic background. In order to transfer the KO allele onto a pure C57BL/6J genetic background, mice were subjected to 8 generations of backcrossing to C57BL/6J mice. From the offspring of the F8 pairing, Nrxn1α HET mice were crossed together to generate the test mice (Nrxn1α WT (+/+), Nrxn1α HET (+/−) and Nrxn1α KO (−/−) mice).

All mice were housed in Techniplast cages (32 x 16 x 14 cm) with sawdust (Litaspens premium, Datesand Ltd, Manchester, UK) and basic cage enrichment, consisting of sizzleneat (Datesand Ltd) and a cardboard shelter (LBS Biotech, Horley, UK). Cages were never cleaned the day before, or on the day of testing in order to minimize the potential effects of cage disturbance on the behavior of the mice. All mice had ad libitum access to water and food (Rat and Mouse No. 3 Diet (RM3) for breeders and No. 1 (RM1) for test mice; Special...
Diet Services, Essex, UK). The housing room was maintained at constant room temperature (~21°C) and humidity (~45%) and kept under a regular light/dark schedule with lights on from 08:00 to 20:00 hours (light = 270 lx). Test mice were singly housed when weaned. This has been shown previously to have minimal effects on C57BL/6J mice and eliminates the potential confounds of group housing, such as the establishment of social hierarchies.26,27 The oestrous phase of the female mice was not checked in this study, but it is unlikely that this affected the results as there were no major differences in the variance observed in the behavioral measures between males and females. C57BL/6J male and female conspecifics for social tests (juvenile play, social investigation, three-chamber social approach) were purchased from Charles River (Margate, UK) one week before testing to allow for a habituation period. These conspecific mice were always pair-housed by sex and kept in different holding rooms to prevent any exposure to the test animals before social testing.

All housing and experimental procedures were performed in accordance with the U.K. Home Office Animals Scientific Procedures Act 1986, and the work was carried out under license. All efforts were made to keep animal suffering to a minimum and to reduce the number of animals used.

2.2 Ultrasonic vocalizations (USVs) and spontaneous motor behaviors

Ultrasonic vocalizations (USVs) and spontaneous movements were recorded in pups across 3-minute sessions in response to social separation from the mother and siblings at postnatal day (PND) 2, 4, 6, 8 and 12, in a dimly lit (< 10 lx) soundproof chamber. The tattooing for early identification of the mice was carried out on PND 2. Tattooing (colored tattoo ink, Vet Tech Solutions Ltd, Congleton, Cheshire, UK) for identification was carried out at PND 2 by inserting the ink subcutaneously through a 0.3 mm hypodermic needle into the center of the paw. Ear marks were collected at PND 10 for later identification since as animals grow older, the pigment of their skin darkens, and the tattoo is harder to see. USV testing was performed on a batch of 112 mice (Males; 15 WT, 26 HET and 11 KO and Females; 18 WT, 32 HET and 10 KO).

An Ultrasound Microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany), sensitive to frequencies of 10 to 180 kHz, was placed through a hole in the middle of the cover of the sound-attenuating box, about 20 cm above the pup in its glass’s container. Vocalizations were recorded using the Avisoft Recorder software (Version 3.2). For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.40) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated at a frequency resolution of 488 Hz and a time resolution of 1 ms. Acoustical parameters that were analyzed for each test day included: number of calls, duration of calls, frequency and amplitude of the sound at the maximum of the spectrum, as described previously.24 Moreover, every USV emitted was classified in distinct categories based on internal pitch changes, lengths and shapes, using our previously published categorization.23

For analysis of spontaneous movements, frequency and duration of behavioral items were analyzed by an observer blind to mouse genotype using the NOLDUS OBSERVER software V 10XT (Noldus Information Technology, Wageningen, the Netherlands). Consistent with previous studies focused on neonatal rodent behavior23 the following behavioral patterns were scored: locomotion (general translocation of the body of at least 1 cm in the glass container), immobility (no visible movement of the animal when placed with all the four paws on the floor, side (no visible movement of the animal when laying on the side), head rising (a single rising of the head up and forward), head shaking (a single lateral displacement of the head), face washing (forepaws moving back and forth from the ears to the snout and mouth), wall climbing (alternating forelimb placing movements on the wall of the container), nose probing (pushing the snout against floor or walls of the apparatus), pivoting (locomotor activity involving the front legs alone and resulting in laterally directed movements), circling (circular locomotor activity involving all the legs and resulting in laterally directed movements) and curling (roll, vigorous side-to-side rolling movements while on the back). Body temperature, body weight and righting reflex of pups were measured after vocal and motor recording on PND 2, 4, 6, 8 and 12. Body temperature of the pup was measured by gentle insertion of the thermal probe in the skin pocket between upper foreleg and chest of the animal for about 30 seconds (Microprobe digital thermometer with mouse probe, Stoelting Co., Illinois). The influence of body weight and body temperature on neonatal vocal emission was investigated using a linear mixed-effect model.

2.3 Developmental milestones

Before weaning, several developmental milestones can be observed in order to assess physical development along with the development of motor and sensory abilities.22 These were performed on a separate cohort of mice (Males; 8 WT, 24 HET and 7 KO and females; 16 WT, 21 HET and 6 KO). Separate batches of mice were used as it has been shown that excessive handling and maternal separation at an early stage can affect subsequent behavioral development in mice.28,29 Developmental milestones were assessed at PND 5, 7, 9, 11, 13 and 15, see Table 1 and References 30-34. Beyond the age of 15 days mice become very jumpy and certain tests become too difficult to accurately assess. Therefore, this was chosen as the final point. To avoid inter-observer variability, the same experimenter recorded all developmental milestone behaviors. The experimenter was blind to the genotype throughout testing. Before handling each litter, a clean pair of gloves were put on and rubbed with sawdust from the home cage. This was done to minimize unfamiliar smells of the pup and avoid the mother rejecting the offspring.

2.4 Juvenile and adult behavioral testing in Nrxn1α mice

All behavioral tests were performed in the light phase between 09:00 and 18:00 hours. For all experiments, the experimenter was blind to
TABLE 1 Items assessed in the developmental milestone screen battery

| Test/Observation                                      | Typical emergence (PND range) | PNDs scored |
|------------------------------------------------------|-------------------------------|-------------|
| Body weight (g)                                      | -                             | 3.5,7,9,11,13,15 |
| Body length (cm)                                     | -                             | 3.7,11      |
| Tail length (cm)                                     | -                             | 3.7,11      |
| Fur appearance: (0—absent, 1—present)                | 9 (3-15)                      | 3.5,7,9,11  |
| Eye opening: complete opening of both eyelids (0—absent, 1—present) | 12 (7-17)                    | 7.11,13,15  |
| Ear canal opening: complete permeation of the auditory conduct (0—absent, 1—present) | 15 (10-20)                   | 7.11,13,15  |
| Incisor eruption: scuff the mouse looking for the teeth appearance (0—absent, 1—present) | 7 (5-10)                     | 7.9,11,13,15 |
| Head elevation: (0—absent, 1—present)                | 12 (9-21)                     | 7.9,11,13,15 |
| Forelimbs and shoulder elevation: (0—absent, 1—present) | 7 (5-15)                     | 5.7,9,11,13,15 |
| Surface righting reflex: The pup is placed on its back and its ability to right itself is scored, with a 60-second cut-off (0—absence of response; 1—vigor; but unsuccessful attempts to right; 2—almost complete response, with one paw still underneath the body; 3—full response) | 5 (1-10)                    | 3.5,7,9,11  |
| Tactile startle reflex: An air puff is directed toward the pup, whose startle response is recorded (0—absent, 1—present) | 11 (3-20)                    | 9.11,13,15  |
| Auditory startle reflex: A tone is presented directly at the pup and its startle response is recorded (0—absent, 1—present) | 15 (11-21)                   | 9.11,13,15  |
| Grasp reflex: The pup’s ability to grasp a blunt metal dissecting rod that is stroked against its forepaw (0—absent, 1—present) | 7 (3-15)                     | 3.5,7,9      |
| Horizontal screen test: The pup is pulled along a wire mesh that is held horizontally; its ability to grasp it is recorded (0—no response; 1—animal grasps the mesh) | 10 (3-18)                    | 5.7,9,11,13,15 |
| Vertical screen test: The pup is pulled along a wire mesh that is held at 45°; its ability to grasp it and begin to climb is recorded (0—no response; 1—animal grasps the mesh) | 10 (3-18)                    | 5.7,9,11,13,15 |
| Negative geotaxis: The pup is placed on an inclined wire mesh (45°) with its head facing down; ability to change its orientation and start walking upward is recorded with a 60-second cut-off (0—no response; 1—animal almost succeeds in changing orientation; 2—animal changes orientation; and starts climbing upward) | 7 (3-15)                     | 5.7,9,11,13,15 |
| Cliff avoidance: The pup is placed on the edge of a cliff, with its forepaws and the head over the edge; the response is positive if the pup turns and crawls away from the cliff (0—absent; 1—present) | 8 (2-12)                     | 13,15       |
| Quadrupled walking: The pup can walk over a distance exceeding its body length (0—absent; 1—present) | 13 (8-18)                    | 9.11,13,15  |

the genotype. Behavioral testing was performed using two batches of mice only (separate to those used in the early USV testing and developmental milestones). This was to ensure enough mice in each genotype group per sex were tested while also keeping the possible confounding batch effects to a minimum. Combining the numbers from both batches, 100 animals were tested (Males: 13 WT, 20 HET and 18 KO and Females: 16 WT, 19 HET and 14 KO). Behavioral testing began at PND 30, when mice are still considered to be juvenile. This is also consistent with the age of testing in previous studies carried out at the Institute of Psychiatry, Psychology and Neuroscience (King’s College London) on Nrnx1α mice by Grayton et al. The first three tests were juvenile play, juvenile three-chamber social approach and rotarod. These tests were carried out before 8 weeks of age (juvenile play: mice aged PND 30; juvenile three-chamber social approach: mice aged PND 37 to 42; rotarod: mice aged 50 to 52). From 8 weeks of age, mice are adult. At this point, the experiments were run in the following order up to the age of 12 weeks: adult social investigation, modified adult three-chamber social approach, marble burying and olfactory habituation. Olfactory habituation/dis-habituation test was conducted on one batch of adult mice only. At least 1 day intertrial interval was included between different tests. All tests were recorded using a camera positioned above the test arenas and movement of each mouse tracked using the EthoVision software (version 3.1). After each trial, boli and urine were removed from the test arena, which was then cleaned with 1% Anistel solution (Tristel Solutions Ltd, UK). At the end of testing, mice were returned to their home cage, which was returned to the housing room. Light levels for each task varied according to the specific task.

2.5 | Juvenile play

Mice were weaned and singly housed at PND 29, 24 hours prior to testing. Each mouse was tested at PND 30 as described previously.25
Testing was conducted in a dimly lit room, illuminated with red light from four cluster lights only (LED cluster red light No. 310-6757; RS Components Northants, UK) with a wavelength of ~705 nm. Each test mouse was placed in a clean test cage (Techniplast cage, 32 × 16 × 14 cm), with a 2 cm thick layer of clean sawdust and habituated to the environment for 5 minutes before the addition of a novel, age- and sex-matched conspecific mouse. A Perspex lid was placed on top of the cage, to contain the mice and to allow the interaction to be recorded. The tail of the conspecific mouse was marked using a permanent marker pen (Pentel, UK) before each test to aid identification. Interactions initiated by the test mouse were recorded for 10 minutes and social play behavior was later scored from the recordings by researchers who were blind to the mouse genotypes. Details of the behavioral measurers scored in this task are listed in Table 2. Following testing, the test and conspecific mice were returned to their respective home cages and housing rooms.

### 2.6 Three-chamber social approach (juvenile mice)

The three-chamber social approach is an assay of mouse sociability and preference for social novelty. Sociability is defined as the subject mouse spending more time in the chamber containing the mouse than in the chamber containing the object. This task was carried out as in Grayton et al. During testing, the subject mouse is presented with the choice of spending time with either a novel mouse or a novel object. This test can be performed as either two or three trials. Because of time constraints, the batches of mice used were subjected to two trials only.

Mice were tested over 4 days, aged PND 37-42. This test was performed using a three-chambered apparatus (rectangular clear perplex three-chambered box, with each chamber 20 cm length × 40.5 cm width × 22 cm height). The box was dimly lit from below (10 lx), and small openings in the dividing walls allowed for easy movement between the chambers. Each chamber was filled with sawdust up to approximately 1 cm. The first trial is a 10 min habituation phase during which the mouse can freely explore the three-chamber apparatus. At the same time, an age- and sex-matched novel conspecific is habituated to sitting under a wire cup in a separate room. After this habituation, the test mouse was briefly confined to the center chamber while a novel object (a black tally counter resembling a mouse in size and shape) and the conspecific mouse were added to the outer chambers. The chamber location of the novel mouse was counterbalanced across all trials to minimize any potential confound because of a preference for chamber location. During the second 10-minute trial, when presented with the choice of spending time in a chamber containing either a novel mouse or a novel object, sociability is defined as a preference for spending time with a novel mouse. During both trials, locomotor activity (distance travelled (cm); velocity (cm/s) and time (s) spent in each chamber) were tracked using the EthoVision software.

### 2.7 Rotarod

The Rotarod is a method used to assess motor learning and coordination in mice. Mice were tested at PND 50-52 using the Rota-Rod 47600 device (Ugo Basile, Milan, Italy). Testing was run over two days and the rotarod was set to forward acceleration from 0 to 40 rpm throughout. On the first day, mice underwent three consecutive trials, 5 minutes in length followed by an hour break, then a further three trials of 5 minutes. This was then repeated the following day to assess motor learning. Latency to fall (s) was recorded, with higher latencies indicating better motor coordination and the increase in latency occurring throughout the 2 days being indicative of motor learning.

### Table 2 Juvenile Play behaviors

| Juvenile play behaviors                      | Sniffing of the body above the shoulders | Behavior where the mouse moves near the other mouse without making direct contact with the mouse | Partners are mutually sniffing each other’s anogenital region, while moving in tight circles together with their reciprocal following movements | Mice groom each other | Test animal is being groomed by the conspecific | Test animal pushes its own snout or the whole anterior part of its body under the conspecific’s body and rests for at least 3 seconds | Test animal is lying flat or standing still (eyes closed or open) while maintaining close physical contact with the conspecific | Test animal passes between the wall of the cage and the body of the conspecific by pushing its own body through the narrow space available | Test animal crawls underneath the conspecific’s body, crossing it transversely from one side to the other | Test animal crawls over the conspecific’s body, crossing it transversely from one side to the other |
|---------------------------------------------|-----------------------------------------|-------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|----------------------------|-----------------------------------------------|--------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Investigative behaviors                    | Social snifing                          | Following                                                                                        | Mutual circle                                                   | Social grooming           | Social rest                                   | Push under                                                                                       | Social inactive                                                                                       | Play-soliciting behaviors | Push past                                                                 | Crawl under                                                                                                           | Crawl over                                                                                                           |

Note. The number, latency and duration of each behavior were recorded.
2.8 | Adult social investigation

Social investigations of adult mice were assessed as described previously. Mice were tested over 3 days, aged PND 65-67. Mice were transferred to a clean cage, identical to their normal home cage (Techniplast cage, 32 × 16 × 14 cm) containing only sawdust at a height of 2 cm, 1 hour before testing to habituate. The testing area was dimly lit from below (10 lx). The tails of the conspecific mice were marked with a pen (Pentel, UK) so they can be identified in the recording. During testing, mice were transferred into the testing room in their “new” home cage, and an age and sex-matched novel conspecific mouse was put into the cage with them. The mice could interact and recorded for 5 minutes. When aggression was observed for prolonged periods (longer than 30 seconds in one continuous bout or >2 minutes total cumulative duration), the trial was stopped and the conspecific mouse was removed. In this study, no trials exceeded the threshold for terminating the trials early. Following testing, the conspecific was removed and taken back to its home cage, and the test mouse was taken out of the test room, enrichment added to the cage and returned to the housing room. Social behaviors initiated by the test mice were scored from the recordings by researchers who were blind to the genotypes of the mice. The details of the measures scored can be seen in Table 3 and (see References 38, 39).

2.9 | Three-chamber social approach (adult conspecific mice)

Individuals with ASD may display abnormal social cues. Based on this, we performed the three-chamber social approach task again in the mice as young adults (approximately PND 72) following the protocol described above for juvenile mice but with the position of the test mouse and the conspecific mouse reversed. The aim was to see whether the conspecific mouse would spend less time with a knock-out test mouse under a wire cage than it would with a wild-type or heterozygote.

2.10 | Marble burying

Marble burying is a test used to evaluate repetitive behavior in mouse models. Testing was performed in a dimly lit test room (10 lx) when mice were at approximately PND 80. For each mouse, a Techniplast cage (32 × 16 × 14 cm) was filled with 5 cm sawdust and 12 marbles were placed equidistant from each other in a 3 × 4 array covering ⅔ of the arena, while the remaining ⅓ of the cage was left clear for the addition of the mouse. Upon addition of the mouse to the empty ⅓ of the cage, a clear Perspex lid was used to cover the cage and contain the mouse while allowing recording to take place by an overhead camera. The test ran for 30 minutes, during which time the mouse could freely explore the cage and bury marbles. The number of buried marbles was checked at 10, 20 and 30 minutes. (Marbles that were buried up to at least ⅔ of their height were recorded as buried). After 30 minutes, the mouse was removed and returned to its home cage.

### Table 3

| Social investigation behaviors | Social sniffing | Sniffing of the body above the shoulders |
|-------------------------------|----------------|-----------------------------------------|
| Following                     | Behavior where the test mouse moves near the conspecific without making direct contact with the mouse |
| Mutual circle                 | Partners are mutually sniffing each other’s anogenital region, while moving in tight circles together with their reciprocal following movements |

| Affiliative behaviors | Social grooming | Mice groom each other |
|-----------------------|-----------------|-----------------------|
| Social rest           | Test animal is being groomed by the conspecific |
| Push under            | Test animal pushes its own snout or the whole anterior part of its body under the conspecific’s body and rests for at least 3 seconds |

| Social interaction-soliciting items | Social inactive | Test animal is lying flat or standing still (eyes closed or open) while maintaining close physical contact with the conspecific |
|------------------------------------|----------------|-----------------------------------------------------------------|
| Crawl under/over                    | Test animal crawls underneath/over the conspecific’s body, crossing it transversely from one side to the other (only number recorded, as duration cannot be reliably estimated) |
| Push past                           | Test animal passes between the wall of the cage and the body of the conspecific by pushing its own body through the narrow space available (only number recorded, as duration cannot be reliably estimated) |

**Note.** The number, latency and duration of each behavior were recorded.

2.11 | Olfactory habituation/dishabituation

Olfactory habituation is used to assess deficits in olfaction. As social behavior relies heavily on olfaction, this test is a necessary control for the interpretation of the social behavior. Previously a buried cookie test showed that there were no olfactory deficits in Nrxn1α knock-out mice, however, a recent study showed that outcomes of
the buried cookie test and olfactory habituation testing can differ given the former test can be influenced by the mouse’s appetitive behavior. Therefore, this test was performed on the second batch of test mice to ensure there are no olfactory deficits. This test was performed in batch 2 only, in which there were 45 mice (Males; 6 WT, 8 HET and 8 KO and Females; 8 WT, 7 HET and 8 KO). Animals were tested at approximately PND 85 in their home cage, which had been cleaned out 3 days prior to testing, with all enrichment removed and a fresh cage lid to minimize interfering odors. Following a 10 minute habituation the mouse was exposed to three odors in turn: water (control/no odor; 50 μL), banana essence (nonsocial; 50 μL; 1:100 dilution; Uncle Roy’s, Moffat, UK) and urine collected from a novel, sex-matched conspecific (social, 25 μL). Each odor was presented on a cotton-tipped wooden applicator three times and for a period of 2 minutes each time with an interval of roughly one minute while the next cotton bud was prepared. The total time (s) spent by the mouse sniffing each cotton bud during every trial was recorded. Habituation to an odor was defined as a decrease in sniffing over consecutive presentation of the same odor, and dishabitation as an increase of sniffing when a new odor is presented.

2.12 Statistical analysis

Data were analyzed using GraphPad Prism version 7.00 for Windows (GraphPad Software, California). Unless otherwise specified, data were analyzed using either two-way ANOVA or a two-way repeated measures ANOVA, as appropriate, followed by Tukey’s multiple comparisons test. Sex and genotype were main factors but when there was a statistically significant sex x genotype interaction, the effect of genotype was analyzed within each sex using one-way ANOVA or repeated measures ANOVA, as appropriate, followed by Tukey’s multiple comparisons test. USV and motor behavior analysis was performed using a mixed-model Analysis of Variance with repeated measures but with the genotype as factor and PND or call categories as the repeated measures. Since no main effect of sex (or the interaction between genotype and sex) were detected, USV and motor behavior were combined. For developmental milestone testing, all data were analyzed using nonparametric Kruskall-Wallis followed by multiple comparisons of mean ranks (z scores), since these data are ordinal. One sample t-tests were used to assess chamber preference in the three-chamber social approach task.

3 RESULTS

3.1 Ultrasonic vocalizations

3.1.1 Nrxn1α KO mice showed abnormal ultrasonic vocalizations

Although the ontogenetic profile of pups’ ultrasonic vocalizations emitted from age PND 2 to 12 did not follow an inverted U-shape, there was a main effect of day ($F_{(4,436)} = 3.148, P = .0144$). Starting from PND 4, both WT and KO pups rapidly increased the ultrasonic vocalization emission with a peak at PND 12, while the profile of emission appeared quite flat in HET pups. Moreover, the ultrasonic calling rate varied across genotype ($F_{(2,109)} = 6.485, P = .0022$). Figure 1A showed that KO mice emitted less vocalizations than their WT and HET littermates upon separation from their mother and siblings, though these results did not reach statistical significance (genotype x postnatal day $F_{(8,436)} = 0.840, P = .5681$) to apply a post hoc comparison.

Analysis of the USVs emitted across the first 12 postnatal days of age detected significant differences between WT, HET and KO pups in the mean call duration (day; $F_{(4,436)} = 61.406, P < .0001$; genotype: $F_{(2,109)} = 29.018, P < .0001$; see Figure 1B). Post hoc comparisons performed on the two-way interaction genotype x postnatal day ($F_{(8,436)} = 3.806, P = .0002$) indicated that KO pups emitted shorter calls than WT and HET pups at every PND assessed ($P < .01$), except on PND 8 when KO showed different call duration only compared with HET pups. On PND 2, mean duration of vocalizations also differed across WT and HET pups ($P < .01$). Peak of the frequency and amplitude did not differ between genotypes (respectively, $F_{(2,109)} = 0.191, P = .8262; F_{(2,109)} = 0.584, P = .5596$, data not shown).

Analysis of neonatal vocal emission did not vary when accounting for body weight and body temperature through a linear mixed model.

3.1.2 Classification of ultrasonic vocalizations into distinct call categories within Nrxn1α KO mice

Figure 1C-G (and Figure S1) illustrate the genotype-dependent variation in frequency of calls at PND 2 to PND 12. Analysis of the frequency of call types at PND 2 showed that both HET and KO pups (respectively, $P < .05, P < .01$) emitted fewer complex calls than WT, while KO pups produced less two components calls compared with HET ($P < .01$), see Figure 1C. A significantly higher number of short calls was emitted by KO compared with WT and HET pups ($P < .01$) after posthoc comparison performed on genotype x calls subtype interaction ($F_{(16,872)} = 4.560, P < .0001$). As illustrated in Figure 1B, a genotype-dependent effect was found at PND 4 ($F_{(2,109)} = 12.294, P < .0001$), with KO emitting significantly less complex and two-components calls and more short calls ($P < .01$) than the other two genotypes (genotype x calls subtype interaction: $F_{(16,872)} = 8.352, P < .0001$).

At PND 6 (Figure 1E), KO pups produced significantly less two-components and more short calls ($P < .01$) than WT and HET (genotype: $F_{(2,109)} = 6.085, P < .01$; genotype x calls subtype interaction: $F_{(16,872)} = 11.764, P < .0001$). At this specific time point, the ultrasonic emission differed within the downward call subtype with KO pups emitting less calls of this type in comparison to WT ($P < .05$).

At PND 8 (Figure 1F), similar to the vocal profile analyzed at PND4, KO pups emitted less complex calls than HETs and less two-components and more short calls ($P < .01$) than WT and HETs (genotype: $F_{(2,109)} = 6.406, P < .001$; genotype x calls subtype interaction: $F_{(16,872)} = 4.442, P < .0001$). Over the last postnatal day of recording...
FIGURE 1  Ultrasonic vocalizations (USVs) in Nrxn1α WT, HET and KO pups in response to social separation during a 3-minute-session. (A) Mean (± SEM) number and (B) Mean (± SEM) duration of ultrasonic vocalizations on PND 2, 4, 6, 8 and 12. (C-G) Production of ultrasonic vocalizations by call category at PND 2 (C); at PND 4 (D); at PND 6 (E); at PND 8 (F); at PND 12 (G). Data derived from 33 WT (15 males and 18 females), 58 HET (26 males and 32 female), 21 KO (11 males and 10 females). After post hoc (Tukey test), levels of significance indicated by **P < .01 KO vs HET and WT; $$$P < .01 KO vs HET; ¥¥P < .05 or ¥¥¥P < .01 KO vs WT; by £P < .05 or ££P < .01 HET vs WT.
(PND 12), KO emitted less complex and more short calls (\( P < .01 \)) than WT and HETs (genotype: \( F_{(2,109)} = 4.658, P < .05 \); genotype x calls subtype interaction: \( F_{(16,872)} = 5.672, P < .0001 \)). Moreover, KO emitted less two-components call subtypes (\( P < .05 \)) than WT pups (Figure 1G).

### 3.1.3 Differences in body temperature, body weight and righting reflex in Nrxn1α mice

Pups’ body temperature, body weight and righting reflex (measured as latency to turn back onto all four paws when placed on the back) were measured each day immediately after ultrasonic vocalization recording. Analysis of the body temperatures measured across the first 12 postnatal days of age detected a main effect of genotype (\( F_{(2,109)} = 3.419, P < .0363 \)) and day (\( F_{(4,436)} = 275.255, P < .0001 \)). Figure 2A illustrates KO mutants showed a decreased temperature when compared with other genotypes, though these results did not reach statistical significance (genotype x postnatal day (\( F_{(8,436)} = 1.640, P = .1114 \)) to apply a post hoc comparison.

Body weight differed significantly between genotypes (\( F_{(2,109)} = 9.385, P < .0002 \)) across the five days of testing (\( F_{(4,436)} = 4785.520, P < .0001 \)). Post hoc comparisons performed on the two-way interaction genotype x day (\( F_{(8,436)} = 13.589, P < .0001 \)) showed that KO pups were lighter than HET at PND 2 and HET and WT pups from PND 4 through PND 12 (\( P < .01 \)), see Figure 2B.

As expected, significant differences were found in righting reflex latencies over time (day: \( F_{(4,436)} = 216.029, P < .0001 \)), with all three genotypes reaching the full development of the reflex on PND 12 (genotype: \( F_{(2,109)} = 1.305, P = .2755 \)). However, a significant deviation from normative motor development was present in KO pups. Post hoc comparisons performed on the interaction genotype x day (\( F_{(8,436)} = 2.313, P < .0195 \)) indicated that KO pups spent more time to turn their body on PND 4 (\( P < .01 \)) than littermate controls and HETS (Figure 2C).

### 3.1.4 Nrxn1α deletion was associated with altered spontaneous motor behaviors

Several abnormalities in the acquisition of the spontaneous motor responses were also detected during the first two postnatal weeks. From PND 2 to 6, when pups are generally unable to move around properly, they pivot with the forelegs. Pivoting duration rapidly increased across the days of observation as showed by the main effect of the age (\( F_{(2,218)} = 14.019; P < .001 \)). Statistical analysis also showed a main effect of the genotype for pivoting duration (\( F_{(2,109)} = 10.838; P < .001 \)) but not for the interaction (genotype x postnatal day (\( F_{(4,218)} = 1.589, P = .1781 \)), see Figure 3A. A similar motor alteration was observed for duration of wall climbing, measured from PND 6 to 12 when pups start to move around using all four legs.
The time spent in performing wall climbing increased from PND 6 to 12 ($F_{(2,218)} = 27.883; P < .001$) and a genotype difference was also detected ($F_{(2,109)} = 37.03; P < .0275$); but not a significant interaction genotype x postnatal day ($F_{(4,218)} = 1.522, P = .1968$). A hyperactive profile of Nrxn1α KO pups was confirmed by the analysis of both duration and frequency in locomotion (respectively, (Figure 3B).
Figure 3C,D). The locomotor activity rapidly increased across the days of observation (duration: $F_{(2,218)} = 96.050$; $P < .0001$ and frequency $F_{(2,218)} = 139.088$; $P < .0001$). Posthoc comparisons performed on the two-way interaction genotype × days (duration: $F_{(4,218)} = 6.890$; $P < .0001$; frequency $F_{(4,218)} = 3.837$; $P < .0049$) indicated that KO mice were more active than HET and WT on PND 12 ($P < .01$). To confirm this, ANOVA showed a main effect of the genotype in the number of immobility events ($F_{(2,109)} = 10.128$; $P < .0001$) and a significant interaction between genotype × days ($F_{(8,436)} = 22.23$; $P < .0249$). Post hoc comparisons reported that KO pups performed a reduced number of immobility events when compared with HET and WT pups on PND 12 ($P < .01$), see Figure 3E.

However, when a fine motor coordination and equilibrium were required, Nrxn1α KO pups exhibited a less active profile. KO mice exhibited a lower number of face washing episodes than the other two genotypes at PND 12 ($F_{(2,109)} = 3287$; $P < .0411$), suggesting a clear motor deficit in KO pups in the ability to stand up on their hindlimbs and wash their faces with the forelimbs (Figure 3F). Moreover, Figure 3G,H show that KO pups have a lower number of head shaking and head rising and head shaking episodes than HET and WT pups. ANOVA detected a main effect of genotype on frequency of head rising ($F_{(2,109)} = 3.871$; $P < .0238$) and head shaking ($F_{(2,109)} = 2.912$; $P = .0586$) but not a statistical significance for genotype × postnatal day interaction (respectively, $F_{(8,436)} = 0.977$, $P = .4210$; $F_{(8,436)} = 0.856$, $P = .5538$). No differences between genotypes were observed for curling, side or circling behaviors (data not shown).

3.2 | Developmental milestones

3.2.1 | Body weight was significantly lower in male Nrxn1α KO mice, but not female Nrxn1α KO mice

Male and female body weight was measured at PND 3, 5, 7, 9, 11, and 15. There was a significant effect of genotype ($F_{(2,76)} = 6.45$, $P < .01$) but no significant sex difference ($F_{(2,76)} = 0.14$) or interaction with genotype ($F_{(2,76)} = 1.22$) across PND 3-15. KO mice had significantly lower body weight at PND 9: $F_{(2,76)} = 3.61$, $P < .01$, PND 11: $F_{(2,76)} = 7.74$, $P < .001$, PND 13: $F_{(2,76)} = 8.39$, $P < .001$ and PND 15: $F_{(2,76)} = 7.34$, $P < .001$. This reduction in body weight was only observed in male KO mice. Female body weight did not differ significantly between genotypes at any specific age (Figure S2). In adulthood, there is no significant sex difference (sex factor: $F_{(1,64)} = 1.78$) or sex by genotype interaction ($F_{(2,64)} = 0.46$), see Figure S3. Body weight of Nrxn1α KO mice is significantly but only slightly reduced in both males and females (genotype factor: $F_{(2,64)} = 14.25$, $P = .001$, Figure S3).

3.2.2 | Body length was significantly lower in male Nrxn1α KO mice, but not female Nrxn1α KO mice

Male and female body and tail length were measured at PND 3, 7 and 11 (Figure S4, panels A-D). There was a significant effect of genotype ($F_{(2,76)} = 3.145$, $P < .05$) but no significant sex difference ($F_{(2,76)} = 0.01$) or interaction with genotype ($F_{(2,76)} = 1.17$) across PND 3-11. Body length was only significantly shorter in KO mice at PND 11: $F_{(2,76)} = 3.96$, $P < .05$. This effect on body length was only observed in male KO mice. However, there was not a significant difference between male tail length and in females no changes to body length or tail length were observed.

3.2.3 | Mice with Nrxn1α deletion showed delays in vertical screen

The vertical screen grasp test measures the ability of the pup to hold on to a wire mesh screen when pulled gently by the tail across the screen in a vertical position. Vertical screen score was measured in mice at PND 5, 7, 9, 11, 13 and 15 (Figure 4E,F). Both male and female Nrxn1α KO mice showed significant delays in grasping the vertical screen on the final day of testing, PND 15, in comparison to WT and HET littermates (male mice: $H(2, n = 39) = 9.29$, $P = .0096$, female mice: $H(2, 43) = 10.27$, $P = .0059$).

3.2.4 | Male mice with Nrxn1α deletion showed delays in negative geotaxis

Negative geotaxis involves placing the pup on an inclined plane with the pup’s head facing downward. The ability of the pup to change orientation to face up the plane was assessed. Testing was carried out on PND 9, 11, 13 and 15 (Figure 4G,H). Male Nrxn1α KO mice showed severe delays in orientating themselves upward at PND 13 ($H(2, n = 39) = 8.04$, $P = .02$) and PND 15 ($H(2, n = 39) = 20.10$, $P < .0001$) with most pups failing to hold themselves on the wire mesh during the trial on each day. Female Nrxn1α KO did not show significant delays in completing this test but there was no significant sex difference in negative geotaxis.

3.2.5 | Nrxn1α KO mice displayed delayed ear canal opening

Ear canal opening was measured at PND 13 and 15; however, the first opening was not observed until PND 15 (Figure 5A). Therefore, there is not likely to have been an effect of the delayed opening of the ear canal on the repertoire of the ultrasonic vocalizations reported in our study. The differences in USV were reported prior to when the ear canal opens which was after PND 13. Pups are deaf until PND 15 but they can emit ultrasonic vocalizations during the first postnatal weeks for eliciting maternal retrieval. Both male and female KO mice showed a significant delay in ear opening, with almost all WT and HET pups displaying full ear canal opening by PND 15 while less than half of KO littermates reached the milestone at this stage (Males: $H(2, n = 39) = 19.85$, $P < .0001$ and females: $H(2, n = 43) = 12.87$, $P = .002$).
3.2.6 | **Nrxn1α KO mice showed a delay in cliff avoidance**

Cliff avoidance was used as a measure of gross visual ability, during which a pup was placed on an apparatus with an opaque side and a clear Perspex side resembling a cliff-like drop. The pup must turn away from danger and crawl onto the opaque part of the apparatus. This developmental milestone was measured at PND 15 only, since the test requires pups’ eyes to be open (Figure S5). Male Nrxn1α KO mice showed significant delays in cliff avoidance, with most KO pups running off the
apparatus immediately (H(2, n = 39) = 20.13, P < .0001). Female Nrxn1α KO mice did not show significant delays or any sex difference in cliff avoidance, however, there was a strong trend toward a delay in cliff avoidance compared with WT littermates (H(2, n = 43, P = .06).

Nrxn1α deletion was not associated with reduced grasping reflex, surface righting, fur appearance, incisor eruption, horizontal screen, quadrupled walking, auditory startle, tactile startle or eye opening (data not shown).

3.3 Deletion of Nrxn1α did not cause olfactory deficits

The Nrxn1α deletion did not result in olfactory deficits in mice (Figure S6). Normal habituation/dishabituation profiles were observed in all groups characterized by a decrease in sniffing latencies between the first and last exposure to particular odor, followed by reinstatement of sniffing when a new odor was presented and marked increase in sniffing durations for the social condition. This confirms that social data was not confounded by differences in olfaction between WT, HET and KO mice of either sex.

3.4 Male juvenile Nrxn1α HET and KO mice showed altered social investigative behavior

For analysis of juvenile social behavior, scored behaviors were grouped into investigative, affiliative, and play-soliciting behaviors as listed in Table 3. A strong sex effect was present between males and females (sex × genotype interaction F(2,93) = 5.18, P < .01), therefore the effects of genotype within each sex were analyzed separately by one-way ANOVA. In males, no significant differences were seen in the frequency of investigative behaviors, however both Nrxn1α HET and KO mice spent significantly less time engaging in investigative behaviors than control littermates (F(2,48) = 7.42, P = .002, Figure 7). When individual behaviors were analyzed, duration of sniffing behavior (F(2,48) = 7.06, P = .002) was the only significantly different investigative behavior as was seen in the juvenile data, while following and mutual circle behaviors were not significant (Figure 8). The frequency and duration of affiliative behaviors was also lower in male KO mice and behaviors reached significance when summed together (F(2,48) = 5.25, P = .009 and F(2,48) = 6.03, P = .005, respectively, Figure 7). In addition, HET and KO mice also showed greatly increased levels of aggression, compared with WTs (frequency of attack, F(2,48) = 4.99, P = .01; duration of attack, F(2,48) = 7.48, P = .002 and latency of attack, F(2,48) = 14.3, P < .0001, see Figure 9). All other behaviors did not differ between genotypes. In females, a deletion in Nrxn1α was not associated with alterations in investigative, affiliative or social investigative behaviors (Figure S8). Aggressive behaviors were only observed in 2 out of 14 KO female mice but not in any WT or HET female mice so aggression in female mice is not expressed at a sufficiently robust level to comment on a possible genotype effect.

3.6 Nrxn1α deletion did not result in social abnormalities in the juvenile three-chamber social approach task

During Trial 1, the mouse could freely explore the three chambers of the apparatus. Analyses were performed to ensure there was no preference for either side of the chamber that could confound the results of Trial 2 (Figure S9). One sample t-tests confirmed that there was not a significant preference for time spent in the left vs the right chamber for any of the groups of mice. In addition, their movement was tracked, and the distance moved and velocity in each part of the apparatus recorded. There was no significant sex × genotype interaction on distance moved (F(2,93) = 0.95) or velocity (F(2,93) = 1.70) so both sexes where analyzed together. Both male and female KO mice moved a significantly shorter total distance during Trial 1 (genotype F(2,93) = 29.97, P < .0001, see Figure S10). For total velocity, both male and female KO mice (genotype F(2,93) = 36.05, P < .0001) showed significantly lower velocities than their HET and WT littermates (Figure S10).

During trial 2, a novel mouse (social cue) and a novel object (nonsocial cue) were placed in the left and right chambers and the time spent in each chamber was tracked and recorded. Analyses were performed using the % time spent in the novel mouse chamber measure. There was no significant effect of genotype (F(2,93) = 1.70) and no significant sex × genotype interaction (F(2,93) = 0.26) on preference for the social cue in trial 2. However,
the typical response of preference for a social cue was not seen in any of the male mice based on one sample t-tests comparing preference for time spent in the social vs the object cue chamber for any of the groups of male mice. (Figure S11). The premise of this task is that control (WT) mice are expected to show a preference for the social cue and this suggests that the test has not worked in juvenile male mice and cannot be interpreted. In females, a preference for the social cue was seen (one sample t-tests: \( t(15) = 8.19 \ P < .001 \) WT; \( t(17) = 6.93 \ P < .0001 \) HET; \( t(14) = 2.61 \ KO \ P < .05 \)), suggesting that the protocol worked in juvenile female mice (Figure S11). The three-chamber social approach task was only validated using adult mice, so it is possible that juvenile mice do not show robust social approach behavior as assessed by this task.

### 3.7 Conspecifics spent more time with adult female Nrxn1α KO mice than WT and HET littermates in the modified three-chamber social approach task

This version of the three-chamber social approach task used conspecifics in the place of test mice, and test mice (WT, HET and KO) in the place of “novel mouse.” As expected, during trial 1 no differences in total distance moved or total velocity was seen between conspecific mice and no preference for either side of the chamber was seen. There was no effect of genotype (\( F_{2,93} = 2.06 \)) and no significant sex × genotype interaction (\( F_{2,93} = 2.31 \)) on preference for the social cue in Trial 2, see Figure S12. This suggests that HET and KO male and female mice do not give off abnormal social cues as the conspecific mice did not spend less time in their chamber than with WT or HET.
Mice were assessed for motor learning and co-ordination using the rotarod task. Each mouse underwent three trials per session with two sessions per day, for 2 days. Although there was evidence of motor learning between day 1 and 2 (within factor: $F_{(3, 279)} = 44.78$, $P < .0001$), there were no significant differences in performance between WT, HET and KO mice in either males or females (genotype: $F_{(2, 93)} = 2.13$), see Figure S13.

3.9 | Nrxn1α deletion was not associated with repetitive behaviors in mice

Presence of repetitive behaviors was assessed in mice using the marble burying protocol. The numbers of marbles buried did not differ...
significantly between WT, HET and KO mice of either sex, see Table 4.

**FIGURE 7**  Profile of investigative, affiliative and social interaction-soliciting behaviors in adult male *Nrxn1α* mice. Mean (± SEM) number of investigative behaviors (A) did not differ between groups. Mean (± SEM) duration of investigative behaviors (B), number (C) and duration (D) of affiliative behaviors were reduced in KO mice. Number (E) and duration (F) of social interaction-soliciting behaviors were unchanged between groups. Data derived from 13 WT, 20 HET and 18 KO males. After post hoc (Tukey) test, levels of significance indicated by *P < .05 and **P < .01 WT vs HET and KO; $P < .05$ HET vs KO

4 | DISCUSSION

Ultrasonic vocalization testing showed decreases in the number of pups’ calls and altered sonographic profile in *Nrxn1α* KO mice (Figures 1, S1). These findings may be indicative of a loss in the complexity of USV production with *Nrxn1α* KO mice being less able to produce syllables with several internal frequency changes and preferring to emit calls with a short duration. Many studies have investigated USV communication in mouse pups carrying deletions in genes or regions associated with ASD. SHANK1 is a gene that encodes a synaptic scaffolding protein and has previously been associated with ASD.\textsuperscript{48} Isolation-induced pup USVs were studied in Shank1 (−/−) null mutant, Shank1 (+/−) heterozygous and Shank1 (+/+ ) wildtype littermate controls between PND3 and 12. Shank1 (−/−) pups were found to vocalize less than their littermates with a prominent genotype difference arising on PND 6, bearing similarity to the findings in the current study. The effects of social context also were tested in Shank1 mutants using bedding from an unfamiliar adult conspecific, rather than clean bedding, in a second round of testing. The same genotype-dependent deficit was seen again.\textsuperscript{49} Reductions in USV calls have also
be seen in neuroligin mouse models including Nlgn2 KO mouse pups,37 mouse pups carrying the human R451C mutation in Nlgn350 and a loss of function mutation in Nlgn4.51 Conversely, pups on the BTBR genetic background called more loudly and frequently when separated from their litter in comparison to mice maintained on the C57BL/6J (B6) background.24 Mice on this background have previously shown autism-like behavioral deficits.25 Initially this difference was thought to be because of differences in body size, since the BTBR mice are larger and may therefore have larger thoracic sizes. However, when compared with other strains of mice with similar body weights, the abnormality persisted.

The early vocal repertoire has been also studied in many other ASD animal models from a qualitative point of view, detecting spectral and temporal properties of the calls and classifying them into different categories.24,52 Similar to Nrxn1α mice, Reeler mutant pups produced a narrowed repertoire of calls, specifically on PND 6 and 8, limiting their vocal repertoire to the two-components, chevron and complex calls; while Dab1 mutant pups showed a limited call pattern restricted to short and downward calls.23,53 A poor USV repertoire also has been detected in Cd157 KO pups from PND 3 until 10 compared with a rich repertoire in WT mice.54 These seemingly conflicting findings may simply indicate that the animal models reflect the diversity
observed in patients across the different ASD, including the atypical vocalizations. Notably, remarkable similarities have been observed between human baby and mouse pups in terms of physiological factors involved in the production of distress vocalizations and a variety of acoustical parameters describing them (i.e., amplitude, fundamental frequency, formant frequencies, timing of onset). Since the genetic component as a physiological factor plays a pivotal role in producing the neonatal vocalizations, some human studies focused on siblings of an older child with ASD, considered at high-risk (HR) for ASD. HR infants later diagnosed with ASD showed an atypical pattern of crying, characterized by shorter duration of crying episodes, compared with low-risk infants. In a similar manner, our in-depth analysis detected a decreased average duration of the calls and an increased production of the short call type in Nrxn1α mutant pups. From a translation point of view, studying the call rate and the spectrographic features of USVs in genetic animal models such as mice with Neurexin 1α deletion, may be useful to understand socio-communicative abnormalities in human infants, with the aim of identifying the developmental origin and early behavioral signs of ASD.

Concomitant with the assessment of vocal responsiveness, spontaneous motor behaviors have been assessed to evaluate motor coordination and balance skills acquired by pups during the first two postnatal weeks (Figures 2 and 3). In several ASD animal models, deficits in motor abilities have been detected in early development in addition to socio-communicative deficits. In the current study, Nrxn1α KO mice expressed an excessive pivoting behavior at PND 2, 4 and 6 when they were partially able to move with the posterior forelimbs, as well as an increased ability to climb the wall of the glass container at PND 6, 8 and 12. Over the second postnatal week, pups start acquiring a better motor performance until they are able to fully move around the whole cage. At PND 12, Nrxn1α KO spent many time walking and exploring the cage since they had increased locomotor activity and reduced immobility behavior, suggesting they had a

| TABLE 4 | Mean (± SEM) number of marbles buried by Nrxn1α mice at 10, 20 and 30 minutes |
|----------|---------------------------------------------|
|          | 10 minutes | 20 minutes | 30 minutes |
| WT (M)   | 9 (1.0)    | 9 (0.4)    | 10 (0.5)   |
| HET (M)  | 7 (0.7)    | 9 (0.6)    | 10 (0.3)   |
| KO (M)   | 5 (1.1)    | 7 (1.3)    | 8 (1.5)    |
| WT (F)   | 8 (0.7)    | 9 (0.4)    | 10 (0.5)   |
| HET (F)  | 9 (0.7)    | 10 (0.4)   | 11 (0.3)   |
| KO (F)   | 7 (1.8)    | 9 (1.8)    | 9 (1.6)    |

Note. No differences were observed between groups of mice. Data derived from 13 WT, 20 HET and 18 KO males and 16 WT, 19 HET and 14 KO females.
hyperactive motor profile compared with WT and HET pups. In line with these findings, the early behavioral characterization of Chd8 HET mouse model described signs of abnormal motor development in the first 2 weeks after birth, including an increase in time spent moving compared with littermates, indicative of hyperactive spontaneous behavior. However, when Nrx1α KO were tested to detect subtle motor competences, they failed to show appropriate performances. Overall, the episodes of head shaking and head rising behaviors were reduced in Nrx1α KO during PND 2 to 12. Face washing was reduced in Nrx1α KO compared with WT and HET, particularly at PND 12, suggesting that Nrx1α KO pups did not show a mature motor profile when they need to have an appropriate equilibrium. As Nrx1α KO, Reeler pups showed a deficit in face washing and wall climbing at PND 12 compared with control mice. This and other studies carried out in ASD animal models, such as Reeler, Synapsin, CHD7 and CHD8 mice, showed a delayed or abnormal development of the motor system during the first 12 PNDs. Thus, it could be very useful to evaluate the neonatal motor profile in the context of behavioral phenotyping of ASD animal models, since early motor abnormalities also have been reported in infants and children diagnosed with ASD. These motor alterations could influence the acquisition of socio-communicative functioning and on other aspects of the development over postnatal life, resulting in deficits in ultrasonic vocalizations in mice.

To further assess developmental effects in the Nrx1α mutant, mice were tested through a battery of developmental milestones (Figures 4, 5 and 5). In males, body weight, body and tail length were significantly lower in KO mice than WT and HET littermates from around PND 9 until the end of testing (Figures 4a and 52). These measures in females were unaffected by the Nrx1α deletion. This could be associated with some of the other developmental delays seen in males in this part of the study. Both male and female ear opening was delayed in KO mice at PND 15 (Figure 54); however, eye opening, fur appearance and incisor eruption were normal. Body weight and length are unlikely to be responsible since females did not show any delays in growth. Both male and female KO mice show significant delays grasping the vertical screen at PND 15 in which the pup must both grasp the screen and begin to climb (Figure 4). This may suggest reduced grasp reflexes; however, the horizontal screen and grasp reflexes measurements were unaffected. Body weight is also unlikely to have affected the finding since female body weight was not affected by Nrx1α deletion making the cause of this delay unclear. Negative geotaxis was affected in male KO mice from PND 13 to PND 15, while KO females did not show any delays (Figure 4). In this case it is possible that the reduced male body weight may have been responsible. Male KO mice showed a severe deficit in cliff avoidance at PND 15 (Figure 55). When placed on the apparatus, male KO mice appeared to charge off immediately, while HET and WT mice were able to reverse away from the edge, back to safety. Female KO mice also showed a trend toward this behavior. Cliff avoidance is used as a measure of visual ability; however, given the odd behavior of the KO mice in this task, it is unlikely that this task was providing an assessment of visual ability. Grayton et al. reported higher levels of anxiety in adult male mice. It is possible that a higher level of anxiety is also present in pups. If so, running off the apparatus may be an attempt to run away from the experimenter. Similar findings have been found in other studies of developmental milestones. NLGN2 is a member of the neurexin family of proteins that form trans-synaptic complexes with neurexins and, like Nrx1α, is associated with both ASD and schizophrenia. Homozygous Nlg2 KO mice were tested between PND 2 and PND14 and showed delays in certain developmental milestones including shorter body length, later eye opening, later incisor eruption and reduced grasp reflex. As was seen in our study, these mice showed no delay in righting reflex or acoustic startle. By adulthood, these measures were normal. A study of mouse pups with the human R451C mutation in Nlgn3 showed minor developmental differences in comparison to the WT, including slightly different growth rates and slower righting reflexes at postnatal days 2 to 6. Unlike the findings in our study, developmental milestones and growth rates were accelerated in a study of the BTBR mouse. The mice were tested between PND2 and PND14 and had significantly higher body weight and tail length and earlier eye opening and incisor eruption than B6 mice. They also showed earlier completion of forelimb grasp reflex, vertical screen and cliff aversion tests. However, they showed delays in negative geotaxis and righting reflex (the former of which was also seen in the Nrx1α KO mice). It is possible that their larger size could have accounted for some of these differences and the earlier eye opening may account for the earlier cliff aversion.

This study characterized the behavior of juvenile and adult Nrx1α KO mice in detail, using mice at earlier developmental stages than previously described and on mice backcrossed onto a single genetic background (C57BL/6J). Adult male KO mice show significantly increased levels of aggression and abnormalities in social behavior. These findings replicate those in. Social behaviors could be analyzed and interpreted as they were presented, since olfactory ability in mice was not a confounding factor. The olfactory habituation test performed during this study confirmed that there are no olfactory deficits in HET and KO Nrx1α mice (Figure 56) as previously reported. During the juvenile play protocol, male KO and HET mice showed a significant reduction in investigative behaviors only, which persisted into adulthood (Figures 5 and 6). Analysis of the individual investigative behaviors showed that the grouped behaviors were significant mainly because of sniffing duration times. This is particularly compelling since sniffing behavior is an active behavior in which mouse contact occurs, while the following and mutual circle behaviors do not necessarily involve contact. Therefore, a clear decrease in active contact is observed in KO mice. The effects of genotype on social behavior was mainly observed in the duration, rather than number of, bouts. One possible explanation for this effect primarily on duration of social investigation is that the drive to explore, and initiate an interaction with, a social cue was not altered but that it was the extent/complexity of the social interaction which was reduced in mice with the Neurexin deletion. In adulthood, KO mice also showed decreased investigative and affiliative behaviors and high levels of aggression in the social interaction test (Figures 7 and 8). Increases in aggression were present in the HET mice as well as the KO mice.
suggested a strong association with Nrxn1α and aggressive behaviors (Figure 9). The presence of increased aggression and decreased investigative behaviors in HET mice is interesting since it shows evidence of a heterozygote effect, which is particularly important when considering that the majority of Nrxn1 deletions in human patients are found in the heterozygous condition. Biallelic deletions in Nrxn1 are extremely rare, with only three reported cases.62,63 Most recently, a fraternal twin brother and sister were found, both sharing a biallelic deletions in the Nrxn1 gene. This was the first study of biallelic deletions in which the patients clearly displayed autism-like behaviors and aggressive behaviors.64 The decreased investigative behaviors that are present in juvenile HET mice were no longer present in adulthood. It is possible that having one functioning Nrxn1α gene could compensate for the loss of the other copy as mice enter adulthood, meaning that changes to affiliative and investigative behaviors are not seen (although aggression does appear). Overall these findings suggest that in males the deleterious effects of Nrxn1α on behavior are present in juveniles and become more severe in adulthood and in the homozygous condition, providing evidence for the role of Nrxn1α in aggressive behaviors in both mice and humans.

One possibility for the adult behavioral abnormalities such as aggression seen in male mice could be that they are a consequence of early social dysfunction. Similarly, human literature discusses how early frustration in children with ASD who cannot interact with other children can result in certain behaviors such as avoidance or venting and tension releasing behaviors (both verbal and nonverbal).65,66 Female behavior appeared to be unaffected by Nrxn1α deletion during these two tests (Figures S7 and S8). Although no significance or trends were reached, the profiles appear to show a slight decrease in investigative and affiliative behaviors in juvenile, but not in adult female mice. As in the previous study by Grayton et al,8 there were no differences in the level of aggression seen in adult female KO mice. Differences in aggression exist between male and female mice.67-69 With males tending to show higher levels of aggression toward intruder mice than females. Female mice typically only show aggression to intruders during lactation and rearing pups.69 It would be interesting to study the social behavior of female HET and KO mice with offspring in a future study. Social abnormalities were also observed during the three-chamber social approach testing, however, the findings indicated that the protocols had not worked successfully and cannot be fully interpreted (Figures S9-S12). Two protocols were run, a juvenile three-chamber social approach and an adult version that was modified so that the test mice were under the wire cup as a “novel mouse” and the arena was explored by conspecifics. No previous publications have used the three-chamber social approach test in juveniles or with these modified positions, so it is possible these tests are not robust in mice and validation of the use in juveniles and modified three-chamber task are warranted.

In addition to social behaviors, repetitive behaviors and motor learning behaviors were assessed. Adult body weight was found to be lower in male and female Nrxn1α KO mice (Figure S3). This could potentially confound locomotor activity and should be considered when interpreting results of these tests. The rotarod is a method used to assess motor learning and co-ordination in mice.37 There were no differences in rotarod performance between genotypes in either sex (Figure S13). Therefore, on the pure genetic background, the Nrxn1α deletion was not associated with deficits in motor learning. However, it has previously been reported that on the mixed genetic background (C57BL6/SV129) Nrxn1α KO mice exhibit enhanced motor learning on the rotarod.16 Etherton et al16 also observed increases in repetitive behavior measured by observing the number of self-grooming bouts and time spent self-grooming in Nrxn1α KO mice. In both the marble burying protocol in this study and the self-grooming assessment by Grayton et al8 using a pure genetic background (C57BL/6J), genotype effects on repetitive behavior were not seen. Further to this, when looking at the data profile, the male KO mice appear to bury less marbles than their WT and HET littermates (Table 4). Following this finding, the video recording was checked at 10 and 20 minutes to ensure that mice were not burying and then uncovering marbles but still no significant differences were seen. We can conclude from this study and that by Grayton et al8 that Nrxn1α deletion is not robustly associated with repetitive behavior. Although repetitive behavior is seen in many patients with ASD (DSM-V, American Psychiatric Association, 2013) the fact it is not seen in this study does not mean the Nrxn1α mouse cannot be used as a model of ASD. Mouse models are unlikely to reproduce every possible feature of a disorder given ASD are complex disorders and not because of variation in one gene.

As this study presents evidence of a developmental trajectory for mouse behavior, future research using this mouse model should aim to characterize animal behavior at younger juvenile ages than described here. The first step that should be considered is to perform the juvenile play task at P21 rather than P30. In this study P30 was chosen so that tests could be run at the same time points as those in Grayton et al,8 since our study aimed to replicate several the findings from Grayton's paper. Ideally, we would like to run the task at even younger ages, however, running social tests below P21 becomes difficult since rodents younger than P21 do not tend to exhibit fully developed exploratory behaviors.70 In addition, if performing testing before weaning it would be difficult to establish whether observed behavioral abnormalities were related to the mother or to pup-pup interactions.

In conclusion, we present evidence for a role of Nrxn1α deletion in social behaviors, one of the core symptom domains affected in ASD. This suggests that deletions within the Nrxn1 gene in patients could be responsible for the social impairments. The Nrxn1α deletion also causes developmental delays in young mice aged from approximately PND 9 to PND 15, reduced and restricted vocal repertoire, as well as early motor abnormalities. Though there were no repetitive behaviors present, the purpose of mouse models is not to directly mimic every symptom associated with a disorder. Furthermore, this is the first study to describe a developmental trajectory in Nrxn1α KO mice, since many earlier studies have focused on adult mice. The deleterious effects of Nrxn1α deletion have been shown to be present in juvenile mice but become more severe in adulthood, while in females this is not the case. It is well known that more males than are affected by ASD in the human population.71 The reasons for this apparent "protective effect" in females are unknown and could be explored in
this mouse model since it appears to reflect the sex differences seen in humans.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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

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