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Developmental Delays and Reduced Pup Ultrasonic Vocalizations but Normal Sociability in Mice Lacking the Postsynaptic Cell Adhesion Protein Neuroligin2

Markus Wöhr^{*,#}, Jill L. Silverman^{*}, Maria L. Scattoni, Sarah M. Turner, Mark J. Harris, Roheeni Saxena, and Jacqueline N. Crawley

Laboratory of Behavioral Neuroscience, Intramural Research Program, National Institute of Mental Health, NIH, Bethesda, Maryland 20892, USA

Abstract

Mutations in neurexin and neuroligin genes have been associated with neurodevelopmental disabilities including autism. Autism spectrum disorder is diagnosed by aberrant reciprocal social interactions, deficits in social communication, and repetitive, stereotyped patterns of behaviors, along with narrow restricted interests. Mouse models have been successfully used to study physiological and behavioral outcomes of mutations in the trans-synaptic neurexin-neuroligin complex. To further understand the behavioral consequences of *NEUROLIGIN2* (*NLGN2*) mutations, we assessed several behavioral phenotypes relevant to autism in neuroligin2 null (*Nlgn2*^{-/-}), heterozygote (*Nlgn2*^{+/-}), and wildtype (*Nlgn2*^{+/+}) littermate control mice. Reduced breeding efficiency and high reactivity to handling was observed in *Nlgn2*^{-/-} mice, resulting in low numbers of adult mice available for behavioral assessment. Consistent with previous findings, *Nlgn2*^{-/-} mice displayed normal social behaviors, concomitant with reduced exploratory activity, impaired rotarod performance, and delays on several developmental milestones. No spontaneous stereotypies or repetitive behaviors were detected. Acoustic, tactile, and olfactory sensory information processing as well as sensorimotor gating were not affected. *Nlgn2*^{-/-} pups isolated from mother and littermates emitted fewer ultrasonic vocalizations and spent less time calling than *Nlgn2*^{+/+} littermate controls. The present findings add to the growing literature on the role of neurexins and neuroligins in physiology and behavior relevant to neurodevelopmental disorders.

Keywords

Neuroligin; GABA; inhibition; autism; schizophrenia; anxiety; social behavior; communication; ultrasonic vocalization

[#]Correspondence should be addressed to: Markus Wöhr, (current address), Behavioral Neuroscience, Experimental and Physiological Psychology, Faculty of Psychology, Philipps-University of Marburg, Gutenbergstr. 18, 35032 Marburg, Germany, Fax: +6421 28 23610, Tel: +6421 28 23612, markus.woehr@staff.uni-marburg.de; Or to: Jacqueline N. Crawley, Laboratory of Behavioral Neuroscience, National Institute of Mental Health, Porter Neuroscience Research Center, Building 35 Room 1C903, Bethesda, MD 20892-3730, USA, Fax: +1 301 480 4630, Tel: +1 301 496 7855, ecrawleyj@mail.nih.gov.

^{*}These authors contributed equally

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Introduction

Autism is a neurodevelopmental disorder characterized by aberrant reciprocal social interactions, deficits in social communication, and repetitive, stereotyped patterns of behaviors, along with narrow restricted interests [1]. A strong genetic component in the etiology of autism is indicated by high heritability, with up to 90% concordance in monozygotic twins versus 4-10% in dizygotic twins and siblings [2-5]. Genome-wide and pathway-based association studies have identified a large number of candidate genes for autism [6-9]. Frequencies of highly penetrant mutations in genes associated with synaptic maturation and function [10,11] suggest that autism represents a disorder caused by synaptopathies.

Evidence includes the cases of rare mutations in the genes encoding for neuroligins (*NLGN1*, *NLGN3*, and *NLGN4*) and neurexins (*NRXN1*, *NRXN2*, and *NRXN3*) amongst others. Mutations in the X-linked *NLGN3* and *NLGN4* were among the first mutations shown to cause autism. Jamain et al. [12] detected a missense mutation causing a R451C substitution in a highly conserved *NLGN3* region in two male siblings with autism and their unaffected carrier mother. In a second family, a *NLGN4* 1186insT frameshift mutation appeared in two affected brothers and their asymptomatic mother [12]. This mutation leads to a premature termination of the protein. More recently, several additional genetic alterations in *NLGN1*, *NLGN3*, and *NLGN4* were found in families with autism and intellectual impairment [13-22]. A *de novo* microduplication on chromosome 17p13.1, which includes *NLGN2*, was reported in a patient with intellectual impairment and afebrile seizures [23]. Mutations, translocation events, and deletions associated with autism have been also described in the genes coding for the neurexin binding partners of neuroligins, including *NRXN1*, *NRXN2*, and *NRXN3* [19,24-31]. Interestingly, mutations in *NRXN1* and *NLGN2* have also been reported in patients with schizophrenia [25,26,32-39]. There are indications of some overlapping symptoms between autism and schizophrenia, particularly the negative symptoms such as impoverished speech and social withdrawal [40,41].

Neuroligins form a family of postsynaptic cell adhesion molecules. They bridge the synaptic cleft by forming heterophilic complexes with their presynaptic binding partners, neurexins [10,42]. Neuroligins were first identified as ligands of neurexins [43,44] that were known as receptors for α -latrotoxin, a neurotoxin found in the venom of spiders causing massive synaptic vesicle exocytosis [45]. Neuroligins are crucial for synaptic maturation and function [46] and stabilize synapses, with neuroligin1 (*Nlgn1*) present in excitatory synapses and neuroligin2 (*Nlgn2*) present in inhibitory synapses [10,42,47,48]. Neuroligins have therefore been proposed to regulate the balance between synaptic excitation and inhibition [49-51]. Elevation in excitation was reported to cause a deficit in neuronal information processing and social dysfunction [52] and may contribute to susceptibility for autism [5,53] and associated symptoms such as anxiety and seizures [54,55]. Seizures and anxiety are present in approximately 25% of individuals with autism [7,56,57].

Triple knockout of *Nlgn1*, *Nlgn2*, and *Nlgn3* in mice revealed the vital function of neuroligins in organizing synapses. Triple knockout mice displayed irregular and flat breathing and died shortly after birth due to respiratory failure, likely related to reduced excitatory and inhibitory synaptic transmission in brainstem centers regulating respiration [46]. Mice carrying a deletion in one or two *Nlgns* are viable. Behavioral phenotypes reminiscent of some autism core symptoms were observed in mice lacking *Nlgn1* [58], *Nlgn3* [59], and *Nlgn4* [60].

Deletion of *Nlgn2* leads to impaired inhibitory synaptic transmission as revealed by *in vitro* electrophysiological recordings [61,62], and increased hippocampal excitability, probably

because of impaired GABA_A receptor clustering [63]. At the behavioral level, a previous study reported that *Nlgn2*^{-/-} mice spent less time in the center of an open field, and avoided the light side in the light ↔ dark exploration task for anxiety-like behavior [64]. Pain sensitivity was reduced and rotarod motor coordination was somewhat impaired [64]. Social behavior and memory, however, were not affected by deletion of *Nlgn2* [64]. In contrast, mice overexpressing *Nlgn2* manifested limb claspings and stereotyped jumping behavior [65]. They also showed reduced exploratory locomotion in the open field, anxiety-like behavior in the elevated plus-maze, and impaired social interactions [65].

To further understand the behavioral abnormalities consequent to *Nlgn2* deletion, we conducted a large set of behavioral phenotyping assays in both males and females of all three genotypes, using previously published assays with relevance to the diagnostic and associated symptoms of autism [66]. We observed high reactivity to handling and reduced breeding efficiency and developmental delays that greatly reduced survival in our colony of *Nlgn2*^{-/-} mice. Developmental milestones were evaluated to detect abnormalities that might contribute to poor early survival. Further, pups were assayed for isolation-induced ultrasonic vocalizations, which are important communicative signals, eliciting maternal care behaviors [67-74]. In addition, rodent pup ultrasonic vocalizations have been implicated in stress responses [73,75-78]. Pup ultrasonic vocalizations are enhanced by treatments with anxiogenic substances, while anxiolytic drugs reduce calling levels [79-86]. Anxiety-related behavior was directly tested in adult *Nlgn2*^{-/-} mice, along with measures of general health, rotarod motor learning, exploratory locomotion, social approach, sensory abilities, and sensorimotor gating. Our results extend and replicate previous reports of behavioral abnormalities in *Nlgn2* mutant mice [64,65].

Materials and methods

Animals and housing

Experiments were performed on neuroligin2 null (*Nlgn2*^{-/-}), heterozygote (*Nlgn2*^{+/-}), and wildtype littermate controls (*Nlgn2*^{+/+}). *Nlgn2*^{+/-} breeders on a mixed background of C57BL/6NCrl, 129S6/SvEvTac, and 129S2/SvPasCrlf were kindly contributed by Frédérique Varoqueaux and Nils Brose [46]. The *Nlgn2* mutant line was generated as previously described [46]. In brief, exon sequences covering the translational start site and at least 380 bp of 5' coding sequence were deleted by homologous recombination in embryonic material. We used a heterozygous breeding strategy to generate offspring of all three genotypes. *Nlgn2*^{+/-} males and females were bred and offspring were housed in a conventional vivarium at the National Institute of Mental Health in Bethesda, MD, USA. Approximately 2 weeks after pairing for breeding, females were individually housed and inspected daily for pregnancy and delivery. The day of birth was considered as postnatal day (pnd) 0. After weaning on pnd 21, mice were socially housed in groups of 2-4 with same-sex partners. All mice were housed in polycarbonate Makrolon IVC cages (369 × 156 × 132 mm, 435 cm²; 1145T; Tecniplast, Milan, Italy). Bedding, paper strips, a nestlet square, and a cardboard tube were provided in each cage. Standard rodent chow and water were available ad libitum. The colony room was maintained on a 12:12 light/dark cycle with lights on at 06:00 h, at approximately 20 °C and 55% humidity. All procedures were in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the National Institute of Mental Health Animal Care and Use Committee.

Genotyping

Mouse tail snips were collected by dissecting 0.5 cm of tail between pnd 7-21 on ice cold ethanol. Tails were digested, genomic DNA isolated and purified using the Qiagen DNAeasy kit according to the manufacturer's instructions (Valencia, CA, USA). After the

extraction, 2.0 µl of DNA in buffer containing ~ 250-400 µg of DNA was amplified by PCR. Genotyping of mouse tail DNA was conducted using standard PCR methods and utilized the following primers: antisense from Neo or wildtype CGC CTA GAC TAC CCT CCC CTC ATA; antisense primer in the deleted region CCC ATC AGT GTA CCA TTC CCT AAA; Neo reverse primer sequence detecting knockout GAG CGC GCG CGG CGG AGT TGT TGA C. Each sample was run twice with the wildtype combination of primers and the knockout combination of primers. Denaturing, annealing, and extension steps were performed using a thermocycler (Mycycler, BioRad, Hercules, CA, USA) at 94 °C for 10 min, 45 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and for 1 cycle 72 °C for 20 min. The PCR reaction was run on a 1.5% agarose gel and stained with ethidium bromide.

General overview

To identify mice, pups were labeled by paw tattoo, using non-toxic animal tattoo ink (Ketchum permanent tattoo inks green paste, Ketchum Manufacturing Inc., Brockville, ON, Canada). The ink was inserted subcutaneously through a 30 gauge hypodermic needle tip into the center of the paw. The tattoo marking for each mouse was coded to allow investigators to score behaviors without knowledge of genotype.

All behavioral tests were performed between 9.00-17.00 h during the light phase of the 12:12 h light/dark cycle. All three genotypes were tested on the same day in randomized order. Investigators were blind to genotypes during all behavioral tests and analysis. Mice were brought to a holding room in the hallway of the testing area at least one hour prior to the start of behavioral testing. Three cohorts of mice were tested. For developmental tasks, separate cohorts of mice were utilized for developmental milestones (cohort 1) and ultrasonic vocalizations (cohort 2) to avoid potential confounds from using previously handled animals. A third cohort of mice was generated to test juvenile and adult behaviors. In cohort 3, order of testing was as follows: (1) elevated plus-maze at age 5–6 weeks, (2) light ↔ dark exploration task at age 6–7 weeks, (3) open field locomotion and rotarod motor learning at age 7–8 weeks, (4) adult social approach at age 8–10 weeks, (5) general health, neurological reflexes, and pain sensitivity at age 9–11 weeks, and (6) acoustic startle and prepulse inhibition of acoustic startle at age 10-12 weeks (7) olfactory habituation/dishabituation at age 12–14 weeks. For each experiment, male and female mice were used in approximately equal proportions. Data from males and females were subsequently compared for sex differences in each behavioral task. No sex differences were observed (not shown in detail).

Behavioral Assays

Developmental milestones and neurological reflexes—For the assessment of developmental milestones and neurological reflexes, pups (N=13 *Nlgn2*^{+/+}, N=10 *Nlgn2*^{+/-}, N=10 *Nlgn2*^{-/-}) were tested according to a modified Fox battery [87,88]. Mouse pups were evaluated on pnd 2, 4, 6, 8, 10, 12, and 14. Each subject was tested at approximately the same time of day. Pups were weighed, and their body length and tail length were measured. Body weight was measured using a palmscale (PS6-250; My Weigh Europe, Hückelhoven, Germany). For body temperature determination a DiGiSense Thermistor Thermometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. Body temperature was measured by gentle application of the thermal probe onto the stomach of the mouse pup. Physical landmarks were scored in pups on a scale of 0-3: extent of pinnae detachment (ear flat against the skin to ear detached; ear canal closed to fully open), eye opening (eyes closed to fully open), incisor eruption (teeth absent to teeth present), and fur development (nude skin to full coat of fur).

To measure the righting reflex, pups were placed on their back on a flat, hard surface, and a stopwatch was used to measure the time that it took them to right themselves on all four paws (maximum: 60 s). To measure negative geotaxis, pups were placed on a level wire mesh screen (8 × 11 cm) that was slanted downwards at an angle of 45°. A stopwatch was used to measure the time that it took the pup to turn around and face up the ramp (maximum: 60 s). Cliff avoidance was measured by placing the front two paws over the edge of a table and gauging the magnitude of their avoidance of the edge (scored on a scale of 0-3, for no response to active avoidance of the precipice). Auditory ability was measured using the Preyer reflex, by snapping two fingers near the pup and gauging the magnitude (0-3) of the startle response. Paw reflex and overall forelimb strength was measured by gauging how strongly the pups grasped the wooden bar of a Q-tip (0-3), how long they could hang from the bar using their front paws (measured by counting; maximum 10 s), how well they maintained contact with a level wire mesh screen when dragged across it (0-3), and how well they climbed up the wire mesh screen (8 × 11 cm) when it was slanted vertically at an angle of 45°. Experimenters were trained until the inter-observer reliability was greater than 95%. An independent group of mice was used to avoid effects of repeated handling on subsequent behavioral testing.

Ultrasonic vocalizations in isolated pups—To elicit ultrasonic vocalizations for the assessment of communication deficits, pups (N=15 *Nlgn2*^{+/+}, N=16 *Nlgn2*^{+/-}, N=13 *Nlgn2*^{-/-}) were isolated from their mother and littermates on pnd 7 for 5 min under room temperature (22-24 °C) as described previously [89-91]. Pups were removed individually from the nest at random and gently placed into an isolation container (10 × 8 × 7 cm; open surface) made of glass, containing clean bedding material. The isolation container was surrounded by a sound attenuating box (18 × 18 × 18 cm) made of 4 cm thick Styrofoam walls. Emission of ultrasonic vocalizations was monitored by an UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics, Berlin, Germany) placed in the roof of the sound attenuating box, 10 cm above the floor. The microphone was connected via an UltraSoundGate 116 USB audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250,000 Hz in 16 bit format by Avisoft RECORDER (version 2.97; Avisoft Bioacoustics). The microphone that was used for recording was sensitive to frequencies of 15-180 kHz with a flat frequency response (±6 dB) between 25-140 kHz. At the end of the 5 min recording session, body weight and body temperature were determined as described above. Prior to each test, behavioral equipment was cleaned using a 70% ethanol solution, followed by water, and dried with paper towels.

For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 4.50; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT length, 100% frame, Hamming window, and 75% time window overlap). Correspondingly, the spectrograms were produced at 488 Hz frequency resolution and 0.512 ms of time resolution. Call detection was provided by an automatic threshold-based algorithm (amplitude threshold: -40 dB) and a hold-time mechanism (hold time: 10 ms). Since no ultrasonic vocalizations were detected below 30 kHz, a high-pass filter of 30 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. The accuracy of call detection by the software was verified manually by an experienced user. When necessary, missed calls were marked by hand to be included in the automatic parameter analysis. Total number of ultrasonic vocalizations was calculated for the entire session and in 60 s time bins, to visualize the time course of ultrasonic vocalizations. Additional parameters, based on previous studies of isolation-induced calling [73,74,91,92], including peak frequency and peak amplitude, which were derived from the average spectrum of the entire call, were determined automatically. Peak amplitude was defined as the point with the highest energy within the spectrum. The sound pressure level was calibrated with a synthetic 3 kHz reference sound and measured with a sound level meter (Cat. No. 33-2055,

Radioshack Corporation, Fort Worth, TX, USA). Peak frequency was defined as the frequency at the location of the peak amplitude within the spectrum. In addition, the extent of frequency modulation, i.e. the difference between the lowest and the highest peak frequency within each call, was measured automatically. Temporal parameters included latency to start calling, total calling time, and call duration.

Adult general health and neurological reflexes—General health and neurological reflexes were evaluated in 9-11 week old mice (N=13 *Nlgn2*^{+/+}, N=13 *Nlgn2*^{+/-}, N=10 *Nlgn2*^{-/-}) as previously described [93-99]. General health was assessed on a ranking scale of 0-3 by fur condition, whisker condition, skin color, and body and limb tone. Body weight, using a hand held portable scale (Ohaus, Parsippany, NJ), and basal temperature, using a mouse thermistor probe (Thermalert, Braintree, MA, USA) dipped in olive oil lubricant (STAR fine foods, Fresno, CA, USA) and gently inserted 2 cm into the rectum were also measured. Righting reflex and any occurrences of physical abnormalities were noted.

Empty cage behaviors were scored by placing the mouse into a clean, empty cage and noting incidents of wild running, transfer freezing, stereotypies, grooming, and excessive exploration levels for 3 min. Neurological reflex tests included trunk curl, wire hanging, forepaw reaching, righting reflex, corneal reflex, whisker twitch, pinnae response, eyeblink response, and auditory startle. The reactivity level of the mice was assessed with tests measuring responsiveness to petting, intensity of a dowel biting response, and level of vocalization during handling.

Three 15 min observations of home cage behaviors were conducted at different phases of the circadian cycle (9:00 am, 3:00 pm, and 8:00 pm). Investigators scored incidence of excessive fighting, grooming, stereotypies, lack of huddling with cagemates, and quality of nest building.

Rotarod motor learning—For the assessment of motor coordination and learning in mice (N=8 *Nlgn2*^{+/+}, N=8 *Nlgn2*^{+/-}, N=8 *Nlgn2*^{-/-}), a mouse accelerating rotarod (Ugo Basile, Collegetown, PA) was used as previously described [95,98]. Mice were placed on the rotating drum that accelerated from 4 to 40 revolutions per min over 5 min. Mice were tested for two trials a day, for three consecutive days. The intertrial interval was 1 h. Rotarod performance was scored for latency to fall.

Exploratory behaviors—To assess general exploratory locomotion, mice (N=12 *Nlgn2*^{+/+}, N=11 *Nlgn2*^{+/-}, N=8 *Nlgn2*^{-/-}) were tested in a novel open field environment where their behavior was automatically recorded by a VersaMax Animal Activity Monitoring System (AccuScan Instruments, Inc., Columbus, OH, USA) as previously described [95,98,100,101]. Mice were placed in the center of the open field and allowed to explore the arena for a 30 min session. Total distance traversed in the arena, horizontal activity detected by adjacent beam breaks in the lower photocell panels, vertical activity detected by beam breaks in the z upper photocell panels, and time spent in the center were measured by software linked to the photocell detectors in 5 min time bins. The testing room was illuminated with overhead fluorescent lighting (~ 200 lux).

Social behaviors—For the detection of social deficits relevant to autism, social approach was assayed in mice (N=12 *Nlgn2*^{+/+}, N=9 *Nlgn2*^{+/-}, N=11 *Nlgn2*^{-/-}) using our automated three-chambered apparatus (NIMH Research Services Branch, Bethesda, MD, USA) as previously described [93-95,98-100,102-105]. Novel target mice were 129/SvImJ mice between 12 and 16 weeks of age, of the same sex as the subjects. 129Sv/ImJ was used as the target novel mouse because this strain is generally inactive, passive, and does not exhibit aggressive behaviors towards subject mice. The apparatus was a rectangular, three-

chambered box made of clear polycarbonate. Retractable doorways built into the two dividing walls controlled access to the side chambers. Number of entries and time spent in each chamber were automatically detected by photocells embedded in the doorways and tallied by the software. The test session began with a 10 min habituation session in the center chamber only, followed by a 10 min habituation to all three empty chambers. Lack of innate side preference was confirmed during the second 10 min habituation. The subject was then briefly confined to the center chamber while the clean novel object (an inverted stainless steel wire pencil cup, Galaxy, Kitchen Plus, <http://www.kitchen-plus.com>) was placed in one of the side chambers. A novel mouse previously habituated to the enclosure, was placed in an identical wire cup located in the other side chamber. A disposable plastic drinking cup containing a lead weight was placed on the top of each inverted wire pencil cup to prevent the subject from climbing on top. The side containing the novel object and the novel mouse alternated between the left and right chambers across subjects. After both stimuli were positioned, the two side doors were simultaneously lifted and the subject was allowed access to all three chambers for 10 min. Time spent in each chamber and entries into each chamber were automatically tallied. Time spent sniffing the novel object and time spent sniffing the novel mouse during the 10 min test session were later scored from video recording, by an observer using two stopwatches. The apparatus was cleaned with 70% ethanol and water between subjects. Up to four subject mice were tested in the same room at the same time, using 4 adjacent test chambers as previously described [100].

Anxiety-related behaviors—To evaluate anxiety-related behaviors, two conflict test paradigms were applied, elevated plus-maze and light ↔ dark exploration task, using methods previously described [93,95,98,99,106,107]. *Elevated plus-maze*: Mice (N=11 *Nlgn2*^{+/+}, N=12 *Nlgn2*^{+/-}, N=9 *Nlgn2*^{-/-}) were tested in an elevated plus-maze that consisted of two open arms (30 cm × 5 cm) and two closed arms (30 × 5 × 15 cm) extending from a central area (5 × 5 cm). Room illumination was approximately 30 lux. The test began by placing the subject mouse in the center, facing a closed arm. The mouse was allowed to freely explore the maze for 5 min. Time spent in the open arms and closed arms, and number of entries into the open arms and closed arms, were scored by an investigator, using Observer software (Noldus Information Technology, Leesburg, Virginia). *Light ↔ dark exploration task*: Mice (N=12 *Nlgn2*^{+/+}, N=10 *Nlgn2*^{+/-}, N=10 *Nlgn2*^{-/-}) were tested in the *light ↔ dark exploration* task that was conducted in an automated chamber (NIMH Research Services Branch, Bethesda, MD, USA). The test began by placing the mouse in the light compartment facing away from the partition. The animal was allowed to freely explore the apparatus for 10 min. Time spent in each compartment and number of transitions between the light (~ 600 lux) and dark (~ 3 lux) compartments were automatically recorded.

Sensory abilities—For assessing sensory abilities, four test paradigms were applied: olfactory habituation/dishabituation, acoustic startle and prepulse inhibition of acoustic startle, hotplate and tail flick pain sensitivity tests.

Olfactory habituation/dishabituation: Mice (N=9 *Nlgn2*^{+/+}, N=9 *Nlgn2*^{+/-}, N=9 *Nlgn2*^{-/-}) were evaluated for their responses to non-social and social odors as previously described [95,97,98,108]. The testing room was illuminated with overhead lighting (~ 250-300 lux). Each subject mouse was tested in a clean empty mouse cage containing a thin layer of fresh pinewood bedding. Odor saturated cotton-tipped swabs (15 cm length, Solon Manufacturing Company, Solon, ME, USA) were used to deliver odor stimuli. To reduce novelty-induced exploratory activities, each subject was first habituated for 45 min in the empty testing cage containing one clean dry cotton swab. The test consisted of fifteen sequential 2 min trials: three presentations of plain tap water, three presentations of almond odor (prepared from

almond extract, McCormick, Hunt Valley, MD, USA; 1:100 dilution), three presentations of banana odor (prepared from imitation banana flavor, McCormick, Hunt Valley, MD, USA; 1:100 dilution), three presentations of social odor from social cage 1, three presentations of social odor from social cage 2. Water, almond odor, and banana odor stimuli were prepared by dipping the cotton tip briefly into the solution. Social odor stimuli were prepared by wiping a swab in a zig-zag motion across a soiled cage of unfamiliar mice of the same sex. For each subject, one soiled cage of 129/SvImJ mice and one soiled cage of 129P2-*Pde6b*⁺*Tyr^{c-ch}*/AntJ (FVB/AntJ) provided odors from two novel social strains that differed significantly from the mixed background of the subject mice. Time spent sniffing the swab was quantified with a stopwatch by an observer sitting 2 m away from the testing cage. Sniffing was scored when the nose was within 1 cm of the cotton swab. The intertrial interval was ~1 min.

Acoustic startle and prepulse inhibition of acoustic startle: Mice (N=13 *Nlgn2*^{+/+}, N=11 *Nlgn2*^{+/-}, N=7 *Nlgn2*^{-/-}) were tested for acoustic startle and prepulse inhibition of acoustic startle using the SR Laboratory System (San Diego Instruments, San Diego, CA, USA) as described previously [95,97,98,104,107]. Test sessions began by placing the mouse in the Plexiglas holding cylinder for a 5 min acclimation period. For prepulse inhibition of acoustic startle, mice were presented with each of seven trial types across six discrete blocks of trials for a total of 42 trials, over 10.5 min. The intertrial interval was 10–20 s. One trial type measured the response to no stimulus (baseline movement) and another measured the startle response to a 40 ms 110 dB sound burst. The other five trial types were acoustic prepulse stimulus plus acoustic startle stimulus trials. The seven trial types were presented in pseudorandom order such that each trial type was presented once within a block of seven trials. Prepulse stimuli were 20 ms tones of 74, 78, 82, 86, and 92 dB intensity, presented 100 ms prior to the 110 dB startle stimulus. Startle amplitude was measured every 1 ms over a 65 ms period, beginning at the onset of the startle stimulus. The maximum startle amplitude over this sampling period was taken as the dependent variable. A background noise level of 70 dB was maintained over the duration of the test session.

Hotplate pain sensitivity test: Response to thermal stimulation of the feet was measured in mice (N=12 *Nlgn2*^{+/+}, N=10 *Nlgn2*^{+/-}, N=8 *Nlgn2*^{-/-}) using the hotplate test as previously described [93,95,97,98,104]. Mice were placed on the arena surface which was kept at a constant temperature of 55 °C (IITC Life Science Inc., Woodland Hills, CA, USA). Latency to lift a paw up, lick a paw, jump, rear onto hind paws or shake was recorded. To prevent tissue damage, a cut-off latency of 30 s was applied.

Tail flick pain sensitivity tests: Response to thermal stimulation of the tail was measured in mice (N=11 *Nlgn2*^{+/+}, N=12 *Nlgn2*^{+/-}, N=8 *Nlgn2*^{-/-}) using the tail flick test as previously described [93,95,97,98,104]. Mice were gently restrained with the tail placed in the groove of the tail flick test apparatus (Columbus Instruments, Columbus, OH, USA). An intense infrared light beam was directed at the tail. Latency to move the tail out of the path of the beam was timed automatically by the apparatus. To prevent tissue damage, a cut-off latency of 10 s was applied.

Statistical analysis

Genotype differences were analyzed with One-Way Analysis of Variance (ANOVA) with genotype as the between-subjects factor for pup ultrasonic vocalizations, light ↔ dark exploration, elevated plus-maze behavior, hot plate, tail flick, and acoustic startle reactivity. Time course of ultrasonic vocalizations in isolated pups, prepulse inhibition of acoustic startle, rotarod motor learning, open field locomotion, and olfactory habituation/dishabituation were compared with Repeated Measures ANOVAs with genotype as the

between subject-factor and trials or time as the within-subject factor. Measures of general health, neurological reflexes, and developmental milestones that utilized continuous variables, such as temperature and weight, were analyzed with one-way ANOVAs. Measures of general health and neurological reflexes that utilized a rating of present or absent were analyzed for genotype differences using Chi-squared statistics. Reflexes or physical parameters in general health or developmental milestones that were rated on a 3-point ranking scale were analyzed using non-parametric Kruskal–Wallis for ranks ANOVAs. Social approach was analyzed using a within groups Repeated Measures ANOVA, to compare time spent in the side chambers in the sociability test. Since the times spent in each of the three chambers added to 10 min, and therefore were not independent, the test condition factor compared time spent only in the right versus left chambers. Center chamber times are shown in the graphs for illustrative purposes. Time spent sniffing the novel object versus the novel mouse and entries into the side chambers were similarly analyzed using within groups Repeated Measures ANOVAs. For number of entries during social approach as an internal locomotion control, genotypes were compared by a separate between groups genotype by entries Repeated Measures ANOVA. Significant ANOVAs were followed by posthoc analysis using Bonferroni-Dunn and Newman Keuls. A p-value of < 0.05 was considered statistically significant.

Results

Reduced Breeding Efficiency

From 53 breeding trios, only 28 successful pregnancies resulted, yielding a 53% success rate. Average litter size was 5.7 pups (range of 2 to 8). Low pup yield, high reactivity to handling, and low percentages of successful impregnations proved challenging for colony build-up. Insufficient Ns were obtained to proceed with behavioral analyses beyond those reported herein.

Developmental milestones and neurological reflexes

Figure 1 shows delayed early physical development and neurological reflexes in various parameters in *Nlgn2*^{-/-}. While body weight ($F_{2,30} = 2.01$, $p = 1.17$; Fig. 1A) and fur development ($F_{2,30} = 0.49$, $p = 0.61$, not shown) did not differ between genotypes, delays in physical development and early growth milestones were observed in body length ($F_{2,30} = 6.1$, $p = 0.006$, Newman Keuls posthoc $p < 0.05$ compared to both *Nlgn2*^{+/+} ($q = 4.36$) and *Nlgn2*^{+/-} ($q = 4.27$); Fig. 1B), tail length ($F_{2,30} = 5.26$, $p = 0.04$, $p < 0.05$ compared to *Nlgn2*^{+/+} ($q = 4.58$); Fig. 1C), eye opening ($F_{2,30} = 4.33$, $p = 0.02$, $p < 0.05$ compared to *Nlgn2*^{+/+} ($q = 4.08$); Fig. 1D), and incisor eruption ($F_{2,30} = 4.73$, $p = 0.016$, $p < 0.05$ compared to both *Nlgn2*^{+/+} ($q = 3.21$) and *Nlgn2*^{+/-} ($q = 4.19$); Fig. 1E). Impairments in the grasping reflex were observed in *Nlgn2*^{-/-} ($F_{2,30} = 5.11$, $p = 0.01$, $p < 0.05$ compared to *Nlgn2*^{+/+} ($q = 4.08$) and *Nlgn2*^{+/-} ($q = 3.83$); Fig. 1F). No significant genotype differences were detected on early neurological reflexes, including latencies on the righting reflex ($F_{1,30} = 0.78$, $p = 0.93$; Fig. 1G), acoustic startle reflex ($F_{1,30} = 1.50$, $p = 0.24$; Fig. 1H), and negative geotaxis ($F_{2,30} = 1.33$, $p = 0.29$; not shown).

Ultrasonic vocalizations in isolated pups

A genotype difference was detected in the number of ultrasonic vocalizations emitted by pups ($F_{2,41} = 4.23$, $p = 0.02$; Fig. 2A). *Nlgn2*^{-/-} emitted fewer ultrasonic vocalizations than *Nlgn2*^{+/+} (Bonferroni-Dunn posthoc tests: $p = 0.02$; all other p-values > 0.05). Call rate in *Nlgn2*^{-/-} was reduced to ~ 30-40% of *Nlgn2*^{+/+}. *Nlgn2*^{+/-} displayed an intermediate phenotype. Total calling time was also affected by genotype ($F_{2,41} = 3.66$, $p = 0.04$; Fig. 3B). *Nlgn2*^{-/-} spent less time calling than *Nlgn2*^{+/+} littermates ($p = 0.04$; all other p-values > 0.05). As there was no genotype differences in the latency to start calling ($F_{2,41} = 0.31$, $p =$

0.74; Fig. 3A) and duration of calls ($F_{2,41} = 2.31$, $p = 0.11$; Fig. 3C), this reflects a reduced call repetition rate, i.e. call rate per min, in *Nlgn2*^{-/-}. Genotype did not affect peak frequency of calls ($F_{2,41} = 1.62$, $p = 0.21$; Fig. 3D), peak amplitude of calls, i.e. loudness ($F_{2,41} = 0.14$, $p = 0.87$; Fig. 3E), and call frequency modulation ($F_{2,41} = 0.75$, $p = 0.48$; Fig. 3F), showing that *Nlgn2*^{-/-} were physically able to produce calls similar to *Nlgn2*^{+/-} and *Nlgn2*^{+/+}.

When analyzing the time course of the emission of ultrasonic vocalizations during the 5 minute isolation period, no genotype differences were observed in the first minute ($F_{2,41} = 0.52$, $p = 0.60$; Fig. 2B) However, in *Nlgn2*^{+/+} increasing numbers of calls were detected across the one minute time bins in the five minute isolation test ($F_{4,56} = 5.00$, $p = 0.002$; Fig. 2B). No such increase in call numbers was detected in *Nlgn2*^{+/-} ($F_{4,60} = 1.90$, $p = 0.12$; Fig. 2B) and *Nlgn2*^{-/-} ($F_{4,48} = 0.71$, $p = 0.42$; Fig. 2B). The time course difference was seen in the last minute of the 5 minute isolation session, when *Nlgn2*^{+/+} emitted more calls ($t_{14} = 3.15$, $p = 0.007$), but *Nlgn2*^{+/-} ($t_{15} = 1.84$, $p = 0.09$) and *Nlgn2*^{-/-} ($t_{12} = 0.71$, $p = 0.49$) did not, resulting in a genotype difference in the last minute ($p = 0.006$; p-values for all other comparisons > 0.05). Body weight and temperature at the end of the isolation session did not differ between genotypes ($F_{2,41} = 1.68$, $p = 0.20$ and $F_{2,41} = 0.88$, $p = 0.42$; respectively), and were not correlated with the number of ultrasonic vocalizations emitted ($r = 0.26$, $p = 0.09$ and $r = 0.09$, $p = 0.96$, respectively).

Adult general health, empty cage and home cage observations and neurological reflexes

Adult *Nlgn2* mice were evaluated for general health and neurological reflexes at 9-11 weeks of age (Table 1). The three genotypes scored similarly on physical measures of body weight ($F_{2,33} = 1.28$, $p = 0.29$) and body temperature ($F_{2,33} = 0.20$, $p = 0.82$). Genotypes did not differ, and scored in the normal range for other physical characteristics including fur condition, balding patches, whisker appearance, body and limb tone, skin pigmentation, and piloerection. Observations of empty cage behaviors revealed no abnormalities in general activity, no instances of wild running, stereotypies or transfer freezing. No high levels of grooming were observed in the empty cage. *Nlgn2*^{-/-} were similar to littermates and in the normal range on home cage activity, nest building, huddling, and grooming. Fighting or sitting outside of the huddle were rarely observed. Reflexes including the righting reflex, forepaw reaching, corneal reflex, whisker and ear twitching, auditory startle, and wire hang were all present and within the normal range. Reductions in the trunk curl reflex exhibited a strong trend toward significance (Chi squared = 5.81, $p = 0.054$). Strong reactivity to handling, shown in Table 1 as struggling and/or audible vocalizations, was observed in *Nlgn2*^{-/-} (Chi squared = 9.22, $p = 0.009$).

Rotarod motor learning

Figure 4 illustrates performance on the accelerating rotarod task, which quantifies motor coordination, balance, and gait. Latency to fall increased across the 3 trials, as expected, representing a significant effect of training ($F_{2,42} = 6.40$, $p = 0.004$). *Nlgn2*^{-/-} displayed shorter latencies to fall as compared to *Nlgn2*^{+/-} ($F_{2,21} = 6.05$, $p = 0.008$, Newman Keuls posthoc $p < 0.05$; $q = 4.67$). *Nlgn2*^{+/+} showed unusually short latencies to fall, perhaps related to the complex genetic background on which the mutation had been generated. Since no significant interaction was detected between genotype and training ($F_{4,42} = 0.99$, $p = 0.42$), individual trials by genotype were not analyzed.

Exploratory behaviors

Figure 5 illustrates parameters of exploratory locomotion in the Accuscan open field arena. Across the 30 minute session, the time course for total distance traversed by all three genotypes declined as expected, representing habituation to the novel open field ($F_{5,125} = 7.26$, $p < 0.001$, Fig. 5A). Total distance scores were reduced in *Nlgn2*^{-/-} ($F_{2,27} = 4.18$, $p =$

0.02, Fig. 5A, Newman Keuls posthoc $p < 0.05$ as compared to both *Nlgn2*^{+/+} ($q = 3.74$) and *Nlgn2*^{+/-} ($q = 3.44$)). The time course showing declining horizontal activity by all genotypes also represented normal habituation ($F_{5,120} = 3.56$, $p = 0.005$; Fig. 5B). Horizontal activity was significantly reduced in *Nlgn2*^{-/-} ($F_{2,27} = 3.31$, $p = 0.048$; Fig. 5B, $p < 0.05$ as compared to *Nlgn2*^{+/-} only ($q = 3.63$)). Vertical activity did not change over the 30 minute test period ($F_{5,135} = 0.71$, $p = 0.76$; Fig. 5C). Vertical activity was lower in *Nlgn2*^{-/-} ($F_{2,27} = 8.11$, $p = 0.002$, Fig. 5C, $p < 0.05$ as compared to both *Nlgn2*^{+/+} ($q = 4.76$) and *Nlgn2*^{+/-} ($q = 5.31$)). Time spent in the center of the test arena increased, representing habituation to the arena, over the time course ($F_{5,125} = 6.72$, $p < 0.001$). Time in the center of the arena was reduced in *Nlgn2*^{-/-} ($F_{2,27} = 6.80$, $p = 0.004$, Fig. 5D, $p < 0.05$ as compared to both *Nlgn2*^{+/+} ($q = 2.92$) and *Nlgn2*^{+/-} ($q = 5.22$)).

Social behaviors

Sociability, defined as spending more time with the novel mouse than with the novel object, was seen in all genotypes (*Nlgn2*^{-/-}: $F_{1,10} = 16.60$, $p < 0.001$; *Nlgn2*^{+/-}: $F_{1,8} = 4.69$, $p = 0.06$; *Nlgn2*^{+/+}: $F_{1,11} = 18.80$, $p < 0.001$; Fig. 6A). All genotypes spent more time sniffing the novel mouse than the novel object (*Nlgn2*^{-/-}: $F_{1,10} = 18.00$, $p = 0.002$; *Nlgn2*^{+/-}: $F_{1,8} = 10.90$, $p = 0.01$; *Nlgn2*^{+/+}: $F_{1,11} = 5.90$, $p = 0.03$; Fig. 6B). Number of entries, an internal control for locomotion, was affected by genotype ($F_{2,28} = 4.90$, $p = 0.015$; Fig. 6C). *Nlgn2*^{-/-} displayed lower total entries into the two side chambers than both *Nlgn2*^{+/+} and *Nlgn2*^{+/-} (Newman Keuls posthoc $p < 0.05$ as compared to both *Nlgn2*^{+/+} ($q = 3.83$) and *Nlgn2*^{+/-} ($q = 3.76$)). All genotypes showed the normal absence of innate chamber side bias during the 10 minute habituation phase before the start of the sociability test ($F_{2,20} = 0.16$, $p = 0.78$; Fig. 6D).

Anxiety-related behaviors

Elevated plus-maze—Genotypes did not differ in anxiety-related measures obtained in the elevated plus-maze, including the percentage of time spent on the open arms ($F_{2,30} = 0.62$, $p = 0.55$; Fig. 7A) and the number of open arm entries ($F_{2,30} = 1.44$, $p = 0.25$; Fig. 7B). Locomotor activity in the elevated plus-maze, however, differed between genotypes. Total entries into both open and closed arms were reduced in *Nlgn2*^{-/-} ($F_{2,30} = 5.57$, $p = 0.009$; Fig. 7C) as compared to *Nlgn2*^{+/+} and *Nlgn2*^{+/-} (Newman Keuls posthoc $p < 0.05$ as compared to both *Nlgn2*^{+/+} ($q = 3.26$) and *Nlgn2*^{+/-} ($q = 4.66$)). The reduced number of arm entries seen in *Nlgn2*^{-/-} is consistent with lower exploratory behaviors in the open field and during the social approach task.

Light ↔ dark exploration task—The behavioral profile in the light ↔ dark exploration task differed between genotypes on the number of transitions between the light and dark chambers ($F_{2,30} = 5.86$, $p = 0.007$; Fig. 7D) and the time spent in the dark chamber ($F_{2,30} = 3.54$, $p = 0.04$; Fig. 7E). *Nlgn2*^{-/-} made fewer transitions between the light and dark compartment as compared to *Nlgn2*^{+/-} (Newman Keuls posthoc $p < 0.05$ as compared to *Nlgn2*^{+/-} only ($q = 4.84$)). *Nlgn2*^{-/-} spent more time in the dark chamber than *Nlgn2*^{+/+} and *Nlgn2*^{+/-} ($p < 0.05$ as compared to both *Nlgn2*^{+/+} ($q = 3.91$) and *Nlgn2*^{+/-} ($q = 3.91$)). Latency to enter the dark chamber was not affected by genotype ($F_{2,30} = 0.64$, $p = 0.53$; Fig. 7F). The behavior displayed by *Nlgn2*^{-/-} in the light ↔ dark exploration task indicates an increased level of anxiety-like behavior, or a confound due to reduced general exploratory locomotion.

Sensory abilities

Olfactory habituation/dishabituation—Olfactory habituation/dishabituation confirmed normal olfactory abilities in all three genotypes (Fig. 8). No genotype differences were

detected on habituation to repeated exposures to water ($F_{2, 24} = 0.56$, $p = 0.58$), banana ($F_{2, 24} = 0.78$, $p = 0.46$), social odor from unfamiliar cage 1 ($F_{2, 24} = 0.01$, $p = 0.98$), and social odor from a different, unfamiliar cage 2 ($F_{2, 24} = 0.48$, $p = 0.62$). *Nlgn2*^{+/-} differed from their littermates on the amount time spent sniffing the almond odor ($F_{2, 24} = 9.46$, $p < 0.001$; Newman Keuls posthoc $p < 0.05$ as compared to *Nlgn2*^{+/+} ($q = 4.9$) and *Nlgn2*^{+/-} ($q = 5.7$)). The first presentation of the water-soaked swab elicited moderate sniffing that declined across the second and the third exposure to water (habituation; $F_{2, 24} = 10.0$, $p < 0.001$). The next presentation, a swab soaked in almond extract, elicited more sniffing (dishabituation; $F_{1, 24} = 10.0$, $p = 0.004$), which declined across the second and third presentation of the almond odor (habituation; $F_{2, 24} = 72.8$, $p < 0.001$). A similar response pattern was observed during the subsequent presentation of banana odor (dishabituation; $F_{1, 24} = 33.48$, $p < 0.001$; followed by habituation; $F_{2, 24} = 30.27$, $p < 0.001$). Robust sniffing resumed at a high level to the first presentation of a social odor, a swab swiped across the floor of a cage of novel 129/SvImJ mice (dishabituation; $F_{1, 24} = 98.59$, $p < 0.001$), and declined across the next two presentations of social odor from the same cage (habituation; $F_{2, 24} = 75.99$, $p < 0.001$). A second social odor from another unfamiliar cage of FVB/AntJ mice elicited renewed sniffing (dishabituation; $F_{1, 24} = 51.34$, $p < 0.001$), which again declined across the next two presentations (habituation; $F_{2, 24} = 64.39$, $p < 0.001$). No genotype differences were detected on dishabituation from water to almond ($F_{2, 24} = 1.15$, $p = 0.33$), almond to banana ($F_{2, 24} = 1.03$, $p = 0.37$), banana to social odor from unfamiliar cage 1 ($F_{2, 24} = 0.02$, $p = 0.97$), and from social odor 1 to social odor 2 from the different, unfamiliar cage ($F_{2, 24} = 0.93$, $p = 0.41$).

Acoustic startle and prepulse inhibition of acoustic startle—Reactivity to a sudden 110 dB intense acoustic tone did not differ between genotypes, reflecting similar baseline startle responses ($F_{2,28} = 0.13$, $p = 0.88$; Fig. 9A). Inhibition of the startle response by prepulses of 74 – 92 dB was not affected by genotype ($F_{2,28} = 0.54$, $p = 0.59$; Fig. 9B). Prepulse intensity affected startle response inhibition as expected ($F_{4,112} = 13.60$, $p < 0.001$). There was no interaction between genotype and prepulse intensity ($F_{8,112} = 0.50$, $p = 0.85$). Normal prepulse inhibition of acoustic startle seen in *Nlgn2*^{-/-} indicates intact sensorimotor gating.

Hotplate and tail flick pain sensitivity test—In the hotplate test, no genotype difference was detected. A trend toward increased latency to react was observed in *Nlgn2*^{-/-} ($F_{2,28} = 2.96$, $p = 0.07$; Fig. 10A), suggesting reduced pain sensitivity in *Nlgn2*^{-/-}. Genotypes did not differ in the tail flick test ($F_{2,28} = 0.84$, $p = 0.44$; Fig. 10B).

Discussion

Mutations in genes coding for components of the trans-synaptic neurexin-neurologin complex have been implicated in the etiology of autism, schizophrenia, and intellectual impairment [12-39]. Mouse models with homologous mutations have revealed behavioral phenotypes relevant to some aspects of those disorders [58-60,64,65,109-111]. We conducted a sequence of behavioral assays in *Nlgn2*^{-/-} mice to further understand the behavioral consequences of *NLGN2* mutations, extending previously reported phenotypes of *Nlgn2* mutant mice with the *Nlgn2*^{-/-} from the same original line [46,64].

Developmental milestones were delayed in *Nlgn2*^{-/-} pups on some parameters, including shorter body length, later eye opening, later incisor eruption, and reduced grasping of a small rod. In contrast, adult general health measures were generally normal in *Nlgn2*^{-/-} mice, with the exception of their robust high responsivity to handling. *Nlgn2*^{-/-} mice performed in normal ranges on measures of social and non-social olfaction, and on acoustic startle reactivity. Nociception showed a trend for longer latencies on the hotplate pain sensitivity

test in *Nlgn2*^{-/-} mice, but genotype differences were not significant, in contrast with significantly longer latencies previously reported by Blundell et al. [64].

As in the study by Blundell et al. [64], *Nlgn2*^{-/-} mice displayed an anxiety-like phenotype in the light ↔ dark exploration task. Blundell et al. [64] further observed increased levels of anxiety-like behaviors in the open field arena as assessed by fewer center entries and a reduction in the time spent in the center in *Nlgn2*^{-/-} mice and similar findings were obtained in the present study, although the locomotor phenotype was more prominent than the anxiety-like phenotype. The picture is completed by greater reactivity to handling observed in *Nlgn2*^{-/-} mice in the present study. The anxiety-like phenotype seems to be in line with decreased inhibitory synaptic transmission seen in *Nlgn2*^{-/-} mice [61,62]. However, in the present study, elevated plus-maze behavior was normal on the measures relevant to anxiety. As the light intensities used for open field testing (~ 200 lux) and the light ↔ dark exploration task (light compartment: ~ 600 lux) were higher than the light intensity used for elevated plus-maze testing (~ 30 lux), it appears possible that the low light intensity used has rendered the elevated-plus-maze testing less anxiogenic and thus masked genotype differences. The behavioral data gathered, however, do not support this possibility as the percentage of time spent in open arms was around 20-30%, which is consistent with the literature [93,95,98,99,107]. The low percentage of time spent in open arms clearly shows that open arms were anxiogenic. Importantly, anxiety-related test findings were probably confounded by low exploratory activity in both studies. In fact, fewer transitions and more time in the dark in the light ↔ dark exploration task could easily be caused by reduced exploration.

Nlgn2^{-/-} mice showed reduced locomotion in a number of behavioral tests, including vertical activity in the open field, total entries in the elevated plus-maze, and total entries in the social approach task. Lower locomotor activity observed in several behavioral tests could be due to lower general exploratory activity or minor motor impairment. In support of motor deficits, *Nlgn2*^{-/-} mice performed slightly worse on rotarod learning, consistent with a previous study [64], and displayed a grasping reflex deficit during early development.

Prepulse inhibition deficits were reported in a small percentage of individuals with autism [112-113]. Our findings did not reveal any evidence for impaired sensorimotor gating in *Nlgn2*^{-/-} mice. Normal sensorimotor gating was reported in *Nlgn3*^{-/-} and *Nlgn4*^{-/-} mice [59,60,93], although deficits in prepulse inhibition of acoustic startle were seen in mice lacking *Neurexin1a* [111]. Genetic alterations in *NRXN1* are associated with the occurrence of schizophrenia in humans [25,26,32-37,39].

Social approach, assayed in our automated three-chambered social approach apparatus [93-95,98-100,102-105], was normal in all genotypes. Intact social behavior in *Nlgn2*^{-/-} mice is consistent with a previous report by Blundell et al. [64]. Using a similar version of the three-chambered social approach apparatus, they observed normal social approach and normal social memory in *Nlgn2*^{-/-} mice. Reciprocal social interactions also showed no genotype differences in the previous knockout study [64]. In contrast, mice overexpressing *Nlgn2* spent less time interacting with each other in a reciprocal social interaction test and did not show significant sociability in the social approach apparatus [65].

Mouse vocalizations in the ultrasonic range have been extensively characterized in isolated pups and in adults during social interactions [114-117]. *Nlgn2*^{-/-} pups emitted fewer ultrasonic vocalizations and spent less time calling than *Nlgn2*^{+/+} littermate controls when socially isolated from mother and littermates. Since there was no genotype difference in the latency to start calling, these data represent a lower call repetition rate, i.e. calls per min, in

Nlgn2^{-/-} than *Nlgn2*^{+/+} pups. Call rate in *Nlgn2*^{-/-} was reduced to ~30-40% of *Nlgn2*^{+/+} pups. *Nlgn2*^{+/-} pups displayed an intermediate phenotype.

Varoqueaux et al. [46] reported that triple knockout of *Nlgn1*, *Nlgn2*, and *Nlgn3* leads to irregular and flat breathing mice, followed by death shortly after birth due to respiratory failure. Reduced call rates observed in *Nlgn2*^{-/-} pups in the present study may similarly have been caused by respiratory deficits. However, genotypes did not differ on acoustic call characteristics such as call duration, peak frequency, peak amplitude and frequency modulation. Ultrasonic vocalizations emitted by *Nlgn2*^{-/-} were virtually identical to ultrasonic vocalizations emitted by *Nlgn2*^{+/+} pups. This indicates that the respiratory system, including the innervations of vocal tract and related substrates necessary for the production of ultrasonic vocalizations, were intact in *Nlgn2*^{-/-} pups. In fact, Varoqueaux et al. [46] reported that all single *Nlgn* knockouts evaluated for respiratory deficits by whole-body plethysmography approached ventilation rates of wildtype controls. As pups tested for isolation-induced ultrasonic vocalizations did not differ in body weight and temperature, the reduced call rate observed in *Nlgn2*^{-/-} pups is therefore more likely be explained by deficits in higher-order processes mediating the initiation of ultrasonic vocalization emission.

Isolation-induced ultrasonic vocalizations are closely linked to the individual's predisposition to show anxiety-related behavior in mice [77] and rats [73,75,76,78] and can be enhanced by anxiogenic substances, while anxiolytic substances reduce calling levels [79-86]. Anxiety-related behaviors, including the emission of ultrasonic vocalizations in isolation, are known to be influenced by early environmental factors, particularly maternal care [73,74]. This is particularly relevant as *Nlgn2*^{-/-} mice were reported to display an increased level of anxiety-like behaviors [64] and reduced breeding efficiency and developmental delays were observed in the present study. It would therefore be interesting to assess maternal behavior in future studies. However, the present findings did not indicate a strong anxiety-like phenotype, and the observed low locomotor activity is likely to have confounded elevated plus-maze and light ↔ dark transition scores. Further, if an anxiety-like phenotype was present in *Nlgn2*^{-/-} pups, one would have expected more, not less, pup ultrasonic calling. Similarly, in a rat study, low levels of maternal care were found to be associated with increased, but not decreased numbers of isolation-induced ultrasonic vocalizations [73].

Therefore, it is tempting to assume that the reduced ultrasonic calling behavior seen in *Nlgn2*^{-/-} pups reflects a communication deficit. In fact, it is well known that isolation-induced ultrasonic vocalizations emitted by mouse pups serve important communicative functions and are crucial for pup survival. As shown in playback experiments, mouse pup ultrasonic vocalizations elicit maternal search and retrieval behavior [68-72,74]. Reduced levels of pup ultrasonic vocalizations or unusual calling patterns were detected in several genetic mouse models of autism and speech impairments [89-91,93, 118-128].

Stereotypies and repetitive behavior were not observed in mice of any genotype when placed in an empty cage, during other tasks, or during home cage observations, although excessive self-grooming behavior has been reported in a number of autism models [58,98,102,104,111,129]. Repetitive grooming was also not observed in mice overexpressing *Nlgn2* [65].

In summary, *Nlgn2*^{-/-} mice displayed normal social behaviors and sensory abilities, but reduced exploratory activity, impaired rotarod performance, and delays on a substantial number of developmental milestones. No motor stereotypies or repetitive behaviors were detected. Our findings are consistent with some of the previous findings in this line of knockout mice [64]. Interestingly, *Nlgn2*^{-/-} pups isolated from their mother and littermates

emitted fewer ultrasonic vocalizations and spent less time calling than *Nlgn2*^{+/+} littermate controls. It would therefore be interesting to test *Nlgn2*^{-/-} mice in behavioral assays developed to assess communication in juvenile and adult mice. Using the well-established BTBR T+tf/J (BTBR) mouse model of autism [66], we found that BTBR mice emit unusual ultrasonic vocalizations as juveniles [130] and display low scent marking and minimal ultrasonic vocalization responses to female olfactory cues as adults [101], along with their strong behavioral phenotypes relevant to the first and third diagnostic symptoms, impairments in social behavior and high levels of repetitive behavior [99-104].

Reduced breeding efficiency and high reactivity to handling resulted in low numbers of adult mice available for further behavioral assessment, e.g. adult communication, reciprocal social interactions, and cognitive abilities. Future studies, using alternative molecular methods, such as forebrain inducible transgenic mice or an siRNA microinjection approach, may be able to increase breeding efficiency and hence uncover additional phenotypes relevant to autism that may be masked due to low general exploratory activity or minor motor impairment in the present study. Taken together present and previous findings [64], our results support a role for *Nlgn2* in synaptic functions relevant to several fundamental behaviors in mice, although social interactions appear to be remarkably unaffected.

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Highlights

- Mutations in neurexin and neuroligin genes have been associated with neurodevelopmental disabilities including autism
- Deep behavioral phenotyping of mice lacking the postsynaptic cell adhesion protein neuroligin2
- Developmental delays and reduced pup ultrasonic vocalizations in *Neuroligin2* knockout mice
- Normal sociability in *Neuroligin2* knockout mice

Fig. 1. Developmental milestones and neurological reflexes in *Nlgn2* mouse pups

(A) Body weight, (B) body length, (C) tail length, (D) eye opening, (E) incisor eruption, (F) grasping reflex, (G) righting reflex, and (H) acoustic startle during early development. Solid line: *Nlgn2*^{+/+} wildtype littermate control mice; dashed line: *Nlgn2*^{+/-} heterozygote mice; dotted line: *Nlgn2*^{-/-} null mutant mice. Data are presented as means $\pm$ standard errors of the mean, for all figures. * $p < 0.050$ vs. *Nlgn2*^{+/+} (general difference); # $p < 0.050$ vs. *Nlgn2*^{+/-} (general difference). *Nlgn2*^{-/-} pups displayed delays on several developmental milestones.

Fig. 2. Number of ultrasonic vocalizations in isolated *Nlgn2* mouse pups

(A) Total number of ultrasonic vocalizations emitted during the 5 min isolation from mother and littermates. (B) Time course for the number of ultrasonic vocalizations emitted for each 1 min time bin across the 5 min isolation session. Black bar: *Nlgn2*^{+/+} wildtype littermate control mice; striped bar: *Nlgn2*^{+/-} heterozygote mice; white bar: *Nlgn2*^{-/-} null mutant mice. * $p < 0.050$ vs. *Nlgn2*^{+/+} (difference in min 5). *Nlgn2*^{-/-} pups displayed reduced numbers of ultrasonic vocalizations.

Fig. 3. Detailed spectrographic analysis of ultrasonic vocalizations in isolated *Nlgn2* mouse pups (A) Latency to start calling, (B) total calling time, (C) duration of calls, (D) peak frequency, (E) peak amplitude, and (F) frequency modulation of calls emitted during the 5 min isolation from mother and littermates. Black bar: *Nlgn2*^{+/+} wildtype littermate control mice; striped bar: *Nlgn2*^{+/-} heterozygote mice; white bar: *Nlgn2*^{-/-} null mutant mice. * $p < 0.050$ vs. *Nlgn2*^{+/+}. All genotypes displayed intact production of ultrasonic vocalizations.

Fig. 4. Rotarod motor learning in adult *Nlgn2* mice

Latency to fall, scored across three consecutive days. Solid line: *Nlgn2*^{+/+} wildtype littermate control mice; dashed line: *Nlgn2*^{+/-} heterozygote mice; dotted line: *Nlgn2*^{-/-} null mutant mice. # *p* < 0.050 vs. *Nlgn2*^{+/-} (general difference). *Nlgn2*^{-/-} mice displayed reduced rotarod motor learning (as compared to *Nlgn2*^{+/-}).

Fig. 5. Exploratory behaviors in adult *Nlgn2* mice
 (A) Total distance travelled, (B) horizontal activity, (C) vertical activity, and (D) center time during open field exploration. Solid line: *Nlgn2*^{+/+} wildtype littermate control mice; dashed line: *Nlgn2*^{+/-} heterozygote mice; dotted line: *Nlgn2*^{-/-} null mutant mice. * p < 0.050 vs. *Nlgn2*^{+/+} (general difference); # p < 0.050 vs. *Nlgn2*^{+/-} (general difference). *Nlgn2*^{-/-} mice displayed reduced exploratory locomotion.

Fig. 6. Social behaviors in adult *Nlgn2* mice

(A) Chamber time, (B) sniff time, (C) number of entries during the three-chambered social approach task. (D) Chamber time during the 10 min habituation phase before the start of the sociability test. Black bar: novel mouse (A & B), right chamber (C & D); striped bar: center chamber (A & D); white bar: novel object (A & B), left chamber (C & D). * $p < 0.050$ novel mouse vs. novel object, within genotype. All genotypes displayed intact sociability on the social approach task.

Fig. 7. Anxiety-related behaviors in adult *Nlgn2* mice

(A) Percentage open arm time, (B) open arm entries, and (C) total arm entries on the elevated plus-maze. (D) Light ↔ dark transitions, (E) time in dark chamber, and (F) latency to enter the dark chamber in the light ↔ dark exploration task. Black bar: *Nlgn2*^{+/+} wildtype littermate control mice; striped bar: *Nlgn2*^{+/-} heterozygote mice; white bar: *Nlgn2*^{-/-} null mutant mice. * p < 0.050 vs. *Nlgn2*^{+/+}; # p < 0.050 vs. *Nlgn2*^{+/-}. *Nlgn2*^{-/-} mice displayed a partial anxiety-like phenotype.

Fig. 8. Sensory abilities in adult *Nlgn2* mice - Olfactory habituation/dishabituation
Sniff time across presentation of different non-social (almond and banana) and social odors (129/SvImJ and FVB/AntJ). Solid line: *Nlgn2*^{+/+} wildtype littermate control mice; dashed line: *Nlgn2*^{+/-} heterozygote mice; dotted line: *Nlgn2*^{-/-} null mutant mice. # $p < 0.050$ vs. *Nlgn2*^{+/+}. All genotypes displayed intact olfactory habituation/dishabituation.

Fig. 9. Sensory abilities in adult *Nlgn2* mice - Acoustic startle and prepulse inhibition of acoustic startle

(A) Startle reactivity in response to a 110 dB startle stimulus. (B) Prepulse inhibition of startle reactivity with prepulses of 74, 78, 82, 86, and 92 dB (no prepulse = NO STIM). Black bar: *Nlgn2*^{+/+} wildtype littermate control mice; striped bar: *Nlgn2*^{+/-} heterozygote mice; white bar: *Nlgn2*^{-/-} null mutant mice. All genotypes displayed intact startle and prepulse inhibition.

Fig. 10. Sensory abilities in adult *Nlgn2* mice – Hotplate and tail flick pain sensitivity tests
 (A) Latency to respond in the hotplate pain sensitivity test, (B) latency to respond in the tail flick pain sensitivity test. Black bar: *Nlgn2*^{+/+} wildtype littermate control mice; striped bar: *Nlgn2*^{+/-} heterozygote mice; white bar: *Nlgn2*^{-/-} null mutant mice. All genotypes displayed normal reactivity to nociceptive stimuli.

Table 1
General health, empty cage behaviors, and neurological reflexes in adult *Nlgn2* mice

Genotypes	+/+ N=13	+/- N=13	-/- N=10	p-value
Body weight	25.9±2.8	23.1±2.3	20.5±1.1	NS
Body temperature	37.1±0.24	37.3±0.16	37.3±0.27	NS
Fur quality (3 pt scale)	2	2	2	-
Piloerection	0%	0%	0%	-
Bald patches (%)	10%	4%	0%	NS
Missing whiskers (%)	0%	0%	0%	
Body tone (3 pt scale)	2	2	2	-
Limb tone (3 pt scale)	2	2	2	-
Skin color (3 pt scale)	2	2	2	-
Physical abnormalities	0%	12%	20%	NS
Wild running (%)	0%	0%	0%	-
Transfer freezing (%)	10%	8%	20%	NS
Stereotypies (%)	0%	0%	0%	-
Grooming (3 pt scale)	1.5	1.3	1.7	NS
Exploration (3 pt scale)	1.9	1.9	2	NS
Trunk curl (%)	95%	72%	60%	0.054
Wire hanging (s)	45.5±5.52	56.7±2.24	54.4±4.37	NS
Forepaw reaching (%)	100%	100%	100%	-
Righting reflex (%)	100%	100%	100%	-
Corneal (%)	100%	93%	100%	NS
Whisker twitch (%)	100%	100%	100%	-
Ear twitch (%)	100%	100%	100%	-
Auditory startle (%)	100%	100%	100%	-
Struggle/vocalization (%)	5%	16%	50%	0.009*
Dowel biting (3 pt scale)	1.7	1.6	1.5	NS