Supporting Information

Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism

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Materials and Methods

Analysis of NL-4 cDNA and Generation of NL-4-KOs. The mouse NL-4 cDNA (GenBank Acc. No. EF694290) was identified by BLAST search using the human NL-4 cDNA sequence. One mouse EST (GenBank Acc. No. AF242658) was detected and the full cDNA sequence was obtained by sequencing the entire cDNA clone using a combination of 5' and 3' RACE-PCR. Pairwise alignment of NL sequences was performed with the GraphAlign software. To generate NL-4-KOs, a 129P2/OlaHsd ES-cell clone (XST093) from BayGenomics (San Francisco, CA) was used. This clone carries a gene trap insertion 340 bp downstream of the first exon of *Nlgn4* (SI Fig. 2*A*), which we confirmed by direct sequencing of PCR products obtained after amplification of mRNA and DNA from ES-cells with primers located in exon 1 of *Nlgn4* (5'-GTACCTCAACCTCTACGTGC-3') and in the lacZ gene of the gene trap insert (5'-ATGGGCGCATCGTAACCGTGC-3') (SI Fig. 2*A*). The insertion leads to the expression of a chimeric mRNA which contains the first coding exon of NL-4, encoding the first 138 residues of NL-4, in frame with the β-galactosidase sequence. The chimeric mRNA was detected by RT-PCR analysis using the forward primer in *Nlgn4* exon 1 (see above) and the reverse primer in the lacZ gene (see above). One chimeric mouse was obtained after blastocyst injection of XST093 ES cells at BayGenomics (San Francisco, CA) and

bred with C57BL/6 females. Mutant animals were obtained after germ line transmission of the ES cells and genotyped using Southern blot analysis after Bam HI and Stu I digestion of genomic tail biopsy DNA (SI Fig. 2*B*) or by PCR on genomic tail biopsy DNA using the following primers: Forward primer in *Nlgn4* exon 1 (see above); reverse primer in intron 1 (5'-CACAGGGACGCGACCTCGC-3') for the WT allele (1038 bp PCR product); reverse primer in the En-2 intron (5'-ACACTCCAACCTCCGCAAACTCCT-3') for the NL-4-KO allele (423 bp PCR product). The mutation was backcrossed into the C57BL/6J background for six generations before behavioral experiments were started.

Protein Analysis and Antibodies. SDS-PAGE and Western blotting were performed according to standard procedures. Subcellular fractionation of mouse brain homogenates was performed as published (1). A specific polyclonal antibody to NL-4 was raised against a peptide (TRAAPSGDPDRDPG) representing residues 686-699 of mouse NL-4. All other antibodies were from Synaptic Systems (Göttingen, Germany), except for antibodies to NL-1, NL-2, NL-3 (2), β-Tubulin (Sigma-Aldrich, Munich, Germany), and PSD95 (Antibodies Incorporated, Davis, CA).

Behavioral Testing. Male WT and NL-4-KO mice (littermates) at 3 months of age were tested in a battery of behavioral tests in the following order: (i) elevated plus maze, (ii) open field, (iii) hole board, (iv) rota-rod, (v) pre-pulse inhibition, (vi) social interaction and memory, (vii) social interaction in pairs, (viii) buried food finding, (ix) sucrose preference, (x) Morris water maze with reversal task, (xi) ultrasound vocalization recording, (xii) resident-intruder test, (xiii) contextual and cued fear conditioning, and (xiv) chemical seizures threshold measurement. All tests were performed in sound-attenuated rooms, between 9.00 h and 17.00 h. Mice were group housed with *ad libitum* access to food and water, except for buried food finding and sucrose preference tests (see detailed descriptions below) with a 12 h - 12 h light-dark cycle (light phase onset at 7.00 h). All experiments were performed with permission of the local authorities (Bezirksregierung Braunschweig) in accordance with the German Animal Protection Law. All experiments and initial quantitative analyses were performed in a 'blind' fashion, i.e. the experimenter had no knowledge of the genotype of the mice tested and analyzed.

Elevated Plus Maze Test. Individual animals were placed on the central platform facing an open arm of the plus-maze (made of grey Perspex® with a central 5 cm x 5 cm central platform, two open arms of 30 cm x 5 cm, and two enclosed arms of 30 cm x 5 cm x 15 cm, with overall illumination at 300 lux). Behavior was recorded by an overhead video camera and a computer equipped with Viewer 2 software (BIOBSERVE, Bonn, Germany) to calculate the time each animal spent in open or closed arms. The proportion of time spent in open arms was used for the estimation of open arm aversion, which is an indicator of fear.

Open Field Test. Spontaneous activity in the open field was tested in a grey circular Perspex® arena (120 cm in diameter, 25 cm high). Individual animals were placed in the center of the open field and were allowed to explore it for 7 min. The behavior was recorded by a computer-linked overhead video camera. Viewer 2 software (BIOBSERVE, Bonn, Germany) was used to calculate the distance traveled and the time spent in the central, intermediate, and peripheral zones of the open field.

Hole Board Test. Individual mice were placed in the centre of the hole board (21 cm x 21 cm x 36 cm transparent Perspex® chamber, non-transparent floor raised 5 cm above the bottom of the chamber with 12 equally spaced holes of 2 cm diameter) and allowed to explore the chamber for 3 min. The distance traveled and the number of holes explored (head dips) were monitored by two layers of infrared photo beams connected to a computer with the AKS software (TSE, Bad Homburg, Germany).

Rota-Rod Test. The rota-rod (Ugo Basile, Comerio, Italy) comprised a rotating drum which was accelerated from 4 rpm to 40 rpm over the course of 5 min. Individual mice were placed on the drum, and once they were balanced, the drum was accelerated. The time in seconds at which the respective animal fell from the drum was recorded using a stop-watch. Each animal went through three consecutive trials, and the longest time on the drum was used for analysis.

Pre-Pulse Inhibition Test. Individual mice were placed in small metal cages (90 mm x 40 mm x 40 mm) to restrict major movements and exploratory behavior. The cages were equipped with a movable platform floor attached to a sensor that recorded vertical movements of the floor. The cages were placed in four sound-attenuating isolation cabinets (TSE, Bad Homburg, Germany). Startle reflexes were evoked by acoustic stimuli delivered from a loudspeaker that was suspended above the cage and connected to an acoustic generator. The startle reaction to an acoustic stimulus, which evokes a movement of the platform and a transient force resulting from this movement of the platform, was recorded with a computer during a recording window of 260 ms and stored for further evaluation. The recording window was defined from the onset of the acoustic stimulus. An experimental session consisted of a 2 min habituation to 65 dB background white noise (continuous throughout the session), followed by a baseline recording for 1 min at background noise. After baseline recording, six pulse-alone trials using startle stimuli of 120 dB intensity and 40 ms duration were applied in order to decrease influence of within-session habituation. These data were not included in the analysis of the pre-pulse inhibition. For tests of pre-pulse inhibition, the 120 dB/40 ms startle pulse was applied either alone or preceded by a pre-pulse stimulus of 70 db, 75 db, or 80 dB intensity and 20 ms duration. An interval of 100 ms with background white noise was employed between each pre-pulse and pulse stimulus. The trials were presented in a pseudorandom order with an interval ranging from 8 to 22 s. Amplitude of the startle response (expressed in arbitrary units) was defined as a difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset. Amplitudes were averaged for each individual animal, separately for both types of trials (i.e. stimulus alone or stimulus preceded by a pre-pulse). Pre-pulse inhibition was calculated as a percentage of the startle response using the following formula:

% pre-pulse inhibition = 100 – [(startle amplitude after prepulse – pulse pair) / (startle amplitude after pulse only) x 100]

Sociability and Social Memory Tests. Sociability and social memory were tested as described (3). The social testing arena was a rectangular, three-chambered box. Each chamber was 20 cm × 40 cm × 22 cm in size. Dividing walls were made from clear Plexiglas, with rectangular openings (35 cm x 35 mm) allowing access into each chamber. The chambers of the arena were cleaned and fresh paper chip bedding was added between trials. The test mouse was first placed in the middle chamber and allowed to explore for five minutes. The openings into the two side chambers were obstructed by plastic boxes during this habituation phase. After the habituation period, an unfamiliar C57BL/6J male mouse (stranger 1), that had no prior contact with the subject mice, was placed in one of the side chambers. The location of stranger 1 in the left vs. right side chamber was systematically alternated between trials. The stranger mouse was enclosed in a small (60 mm x 60 mm x 100 mm), rectangular wire cage, which allowed nose contact through the bars but prevented fighting. The animals serving as strangers had previously been habituated to placement in the small cage. An identical empty wire cage was placed in the opposite chamber. A weighted cup was placed on the top of the small wire cages to prevent climbing by the test mice. Both openings to the side chambers were then unblocked and the subject mouse was allowed to explore the entire social test arena for a 10 min session. The amount of time spent in each chamber and the number of entries into each chamber were recorded by the video-tracking system Viewer 2 (BIOBSERVE, Bonn, Germany). An entry was defined as all four paws in one chamber. At the end of the first 10 min, each mouse was tested in a second 10 min session to quantify social preference for a new stranger. A second, unfamiliar mouse (stranger 2) was placed into the previously empty wire cage. The test mouse had a choice between the first, already-investigated mouse (familiar stranger 1), and the novel unfamiliar mouse (new stranger 2). As described above, measures were taken of the amount of time spent in each chamber and the number of transitions between chambers of the apparatus during the second 10 min session.

Test of Social Interaction in Pairs. The social interaction test was performed in a neutral cage (gray Plexiglas box, 30 cm × 30 cm × 30 cm). During two consecutive days, each individual mouse went through one 10 min session in the neutral cage in order to

habituate to the testing conditions. On day 3 (test day), pairs of unfamiliar mice with the same genotype were placed into the neutral cage for 10 min. Behavior of mice was recorded by a computer and the video-tracking system Viewer 2 (BIOBSERVE, Bonn, Germany). The time spent in social interaction (defined as a staying in close contact) was registered.

Buried Food Finding Test. Starting four days prior to testing, mice received each day several pieces of chocolate cookies of 1.6 g with water *ad libitum*. All mice consumed all cookies within 24 hours. Twelve hours before testing, mice were deprived of food with water *ad libitum*. For testing, individual mice were placed into clear cages (29.5 cm x 18.5 cm x 13 cm), in which a piece of a chocolate cookie was hidden under a 1.5 cm standard bedding at the end of the cage. The mouse was positioned in the right corner at the opposite end of the cage, and the food-finding time, i.e. the time from the moment the mouse was placed into the cage to the time it located the cookie and initiated burrowing, was recorded. As soon as the cookie was uncovered, the mouse was removed from the cage. A fresh cage and bedding was used for each trial, and all mice underwent identical testing procedures.

Sucrose Preference Test. The sucrose preference test was performed using a two bottles procedure, during which mice have free access to both water and a sucrose solution. Animals were first habituated for 48 h to consume water from the two small (100 ml) bottles. After habituation, mice were deprived of water and the sucrose preference was measured during the next 3 days. The first two days served as a habituation to sucrose solution. The results of day 3 were used for the evaluation of sucrose preference. Each day, group-housed mice were placed individually into small plastic cages and were presented for 60 min with two bottles - one with tap water and one with a 2 % sucrose solution. Consumption of water or sucrose solution was measured by weighting the bottles before and after the session. Bottles were counterbalanced across the left and the right sides of the cage, and their position was alternated from test to test. Sucrose preference (percent) was calculated as follows:

Preference = [sucrose solution intake (ml) / total fluid intake (ml)] × 100

Morris Water Maze. Spatial learning and memory was assessed in a water maze (4). A large circular tank (diameter 1.2 m, depth 0.4 m) was filled with opaque water (25±1˚C, depth 0.3 m) and the escape platform (10 cm x 10 cm) was submerged 1 cm below the surface. The swim patterns were monitored by a computer and the based video-tracking system Viewer 2 (BIOBSERVE, Bonn, Germany). The escape latency, swim speed, path length, and trajectory of swimming were recorded for each mouse. During the first two days, mice were trained to swim to a clearly visible platform (visible platform task) that was marked with a 15 cm high black flag and placed pseudo-randomly in different locations across trials (non-spatial training). The extra-maze cues were hidden during these trials. After two days of visible platform training, hidden platform training (spatial training) was performed. For eight days, mice were trained to find a hidden platform (i.e. the flag was removed) that was located at the center of one of the four quadrants of the pool. The location of the platform was fixed throughout testing. Mice had to navigate using extra-maze cues that were placed on the walls of the testing room. Every day, mice went through four trials with an inter-trial interval of 5 min. The mice were placed into the pool facing the side wall randomly at one of four start locations and allowed to swim until they found the platform, or for a maximum of 90 s. Any mouse that failed to find the platform within 90 s was guided to the platform. The animal then remained on the platform for 20 s before being removed from the pool. The next day after completion of the hidden platform training, a probe trial was conducted in order to determine whether mice used a spatial strategy to find the platform or not. The platform was removed from the pool and the mice were allowed to swim freely for 90 s. The percentage of time spent in each quadrant of the pool as well as the number of times the mice crossed the former position of the hidden platform were recorded. In order to investigate the flexibility of cognitive processes in mice, the reversal water maze test was performed. The experimental procedure was identical to the one used for the hidden platform training with the exception that the escape platform was moved from the original position to the neighboring quadrant.

Cued and Contextual Fear Conditioning. Experiments were performed using a computerized fear conditioning system (Video Freeze, Med Associates, St. Albans, VT). The computer was connected to a control unit containing a shock and a tone generator. Training took place in an apparatus consisting of a box (58 cm x 30 cm x 27 cm) with a simple grey interior and a 12 V light attached to the ceiling. The pre-exposure and conditioning context consisted of a Plexiglas chamber (36 cm x 20 cm x 20 cm) placed on a removable shock grid made of stainless steel rods (4 mm in diameter, spaced 6 mm apart). The shock grid was connected to a shocker-scrambler unit delivering shocks of defined duration and intensity. For both contextual and cued fear conditioning, mice were trained within the same session, with the following protocol. The pre-exposure time of 2 min in the conditioning box was followed by a 30 s tone (conditioned stimulus, 10 kHz, 75 dB). Immediately after the end of the tone, the shock (0.4 mA, 2 s) was delivered, and after 15 s the procedure was repeated once more. During the pre-trial period, freezing behavior was monitored. The contextual memory test was performed 24 h after training. Mice were monitored for freezing for 2 min in the same context (chamber and interior) as used for training. The cued (tone-dependent) memory test was performed 26 h after training. Visual context (the interior of the experimental box) was altered by white paper printed with different color and black-and-white patterns (horizontal and diagonal stripes, squares and triangles). A different chamber (25 cm x 20 cm x 15 cm) was used for this test. The floor texture was changed by replacement of the grid floors with smooth Plexiglas floors. The chamber was cleaned with 70 % ethanol between animals. Freezing was monitored in the same mice for 2 min in the absence of tone (preconditioned stimulus freezing) and for 2 min in the presence of a continuous tone (conditioned stimulus freezing). Freezing behavior, defined as the absolute lack of movement (excluding respiratory movements), was recorded by a video camera and analyzed by a computer. The data were converted to the percentage of time animals stayed in freezing.

Resident-Intruder Test. Inter-male aggression was studied in the resident-intruder test (5). Previously group-housed male mice were separated and housed individually for 14 days prior to testing. As standard opponent males we used group-caged males of the

same age from the C57BL/6 strain. A standard opponent was introduced into the cage of the tested resident male and observation started when a tested resident male sniffed the opponent for the first time. The observation was stopped immediately after the first attack (an attack being defined as a bite) in order to prevent wounding, but lasted 5 min if no attack occurred. The latency of attack was recorded by a stop-watch. In a modified version of the resident-intruder setting using an independent cohort of mice, we examined reciprocal social interactions. Male test mice were housed individually for four weeks and then tested in a neutral arena of 50 cm x 25 cm. Individual resident test mice were placed into the arena and left to explore it for 30 min. Subsequently, an unfamiliar C57BL/6 intruder animal of the same age and sex that had previously been group-housed was introduced. Approach and escape behavior was monitored by video recording for four minutes, and analyzed off-line in a blinded fashion (6). Correlation between intruder approach and resident escape behavior was analyzed by Spearman correlation analysis.

Ultrasound Vocalization Analysis. We recorded ultrasonic vocalizations of male mice using the recording software Avisoft SASLab Pro v4.33 at a sampling frequency of 300 kHz. The microphone (UltraSoundGate CM16) was connected to a preamplifier (UltraSoundGate 116), which was connected to a computer (all sound recording hardware and software was from Avisoft Bioacoustics, Berlin, Germany). For the test, male mice were first habituated to the test arena. Subsequently, an unfamiliar female mouse in estrous was put into the same box and the vocalization behavior of the male was recorded for 3 min using AVISOFT Recorder 2.97 (Avisoft Bioacoustics, Berlin, Germany). The same software was used to determine the latency until the first call of the male test mouse and to count the total number of calls. To test for significant differences in call latency and number of calls we used the exact Mann-Whitney test (1-tailed, SPSS 15.0; SPSS, Chicago, IL).

Seizure Testing. Testing was conducted when mice were 8-10 weeks of age using a single subcutaneous injection of pentylenetetrazole (PTZ; Sigma-Aldrich, Munich, Germany). Mice were taken from their home cages and placed individually into clean

Plexiglas cages (15 cm x 20 cm x 20 cm) for 10 min before PTZ injection. Based on the results of pilot experiments, PTZ was injected at a dose of 50 mg/kg. After injection, mice were returned immediately to the cubicle and observed for 30 min. We defined the following phases in the continuum of behavioral responses to subcutaneous PTZ injection: (i) Hypoactivity. This phase was characterized by a progressive decrease in motor activity until the animal came to rest in a crouched or prone position with the abdomen in full contact with the cage bottom. (ii) Partial clonic seizure activity affecting the head. (iii) Partial clonic seizure activity affecting head and forelimbs. Partial or focal seizures were brief, typically lasting 1 or 2 s, and often accompanied by vocalizations. Partial seizures occurred either individually or in multiple discrete episodes before generalization. (iv) Generalized clonus with sudden loss of upright posture and/or whole body clonus involving all four limbs and tail. The duration of generalized seizures was variable but typically involved behavioral changes lasting for 30-60 s, followed by a quiescent period. Most mice exhibited multiple generalized seizures irrespective of their subsequent status for tonic hind limb extension. (v) Tonic-clonic (maximal) seizure, i.e. generalized seizure characterized by tonic hind limb extension. (vi) Death due to asphyxia. Tonic-clonic maximal seizures were associated with death, although some mice recovered spontaneously. Latencies to all behavioral manifestations of seizures were recorded manually by the experimenter. The seizure score was calculated according to Ferraro and coauthors (7) with slight modifications. Latencies (inverse) to immobility (I), partial clonus of the head (HC), partial clonus of the forelimb (FC), generalized clonus (GC), tonic-clonic (TC), and death (D) were summed to assign each mouse a seizure score according to the following equation:

Seizure score = (0.1) (1/I latency) + (0.2) (1/HC latency) + (0.3) (1/FC latency) + (0.4) (1/GC latency) + (0.5) (1/TC latency) + (0.6) (1/D latency)

At the PTZ dose used, all mice exhibited partial and generalized clonic seizures. Only a small proportion of mice developed tonic-clonic seizures and died. The weighting factors (from 0.1 to 0.6) were included as a means of incorporating a measure of the progressive nature of the PTZ-induced seizure phenotype into the severity rating

because generalized clonus is regarded as a more significant event than partial clonus, and tonic hind limb extension is regarded as the most severe component of the phenotype followed by death. With this type of analysis, the seizure score reflects the degree of progression of the seizure syndrome in each mouse.

MRI Volumetry. Upon completion of the behavioral analysis, tested mice were anesthetized in a chamber with 5 % isofluorane, and then intubated and kept under anesthesia with 1-1.5 % isofluorane in O_2 and room air (1:1.5). *In vivo* brain volumetry was performed by MRI at a field strength of 2.35 T (Bruker Biospin, Rheinstetten, Germany) using a T1-weighted three-dimensional FLASH sequence as described (8), reaching an isotropic resolution of 117 μm. We determined the total brain volume (excluding bulbus olfactorius, cerebellum and brainstem), and separately the size of the lateral and third ventricles, the cerebellum, and the brainstem by manually drawing respective regions-of-interest on up to 50 contiguous horizontal MRI sections. The experimenter performing the analyses was not aware of the genotype of the mice.

Statistical analysis. Unless stated otherwise in Experimental Procedures, the data, given in figures and text are expressed as mean ± SEM, and data were compared by Kruskal-Wallis analysis of variance (ANOVA) with post hoc planned comparisons, or by ANOVA for repeated measurements and χ^2 test where appropriate, using SPSS v.14 software (SPSS, Chicago, IL). A p value below 0.05 was considered to be significant.

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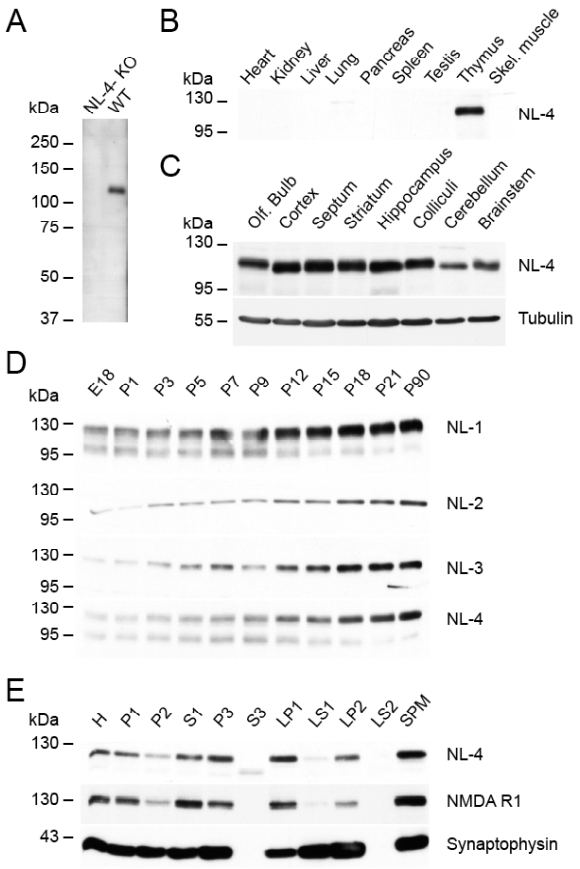
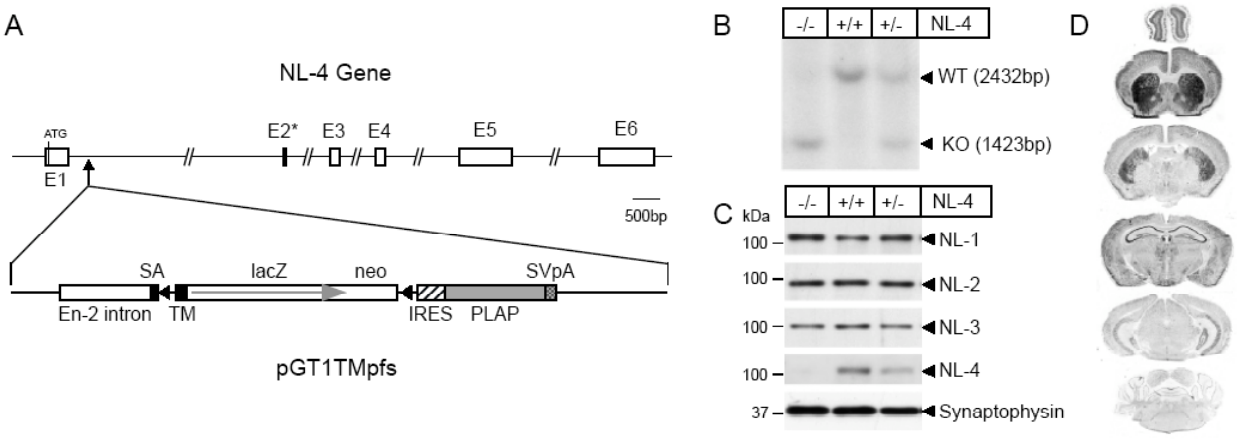
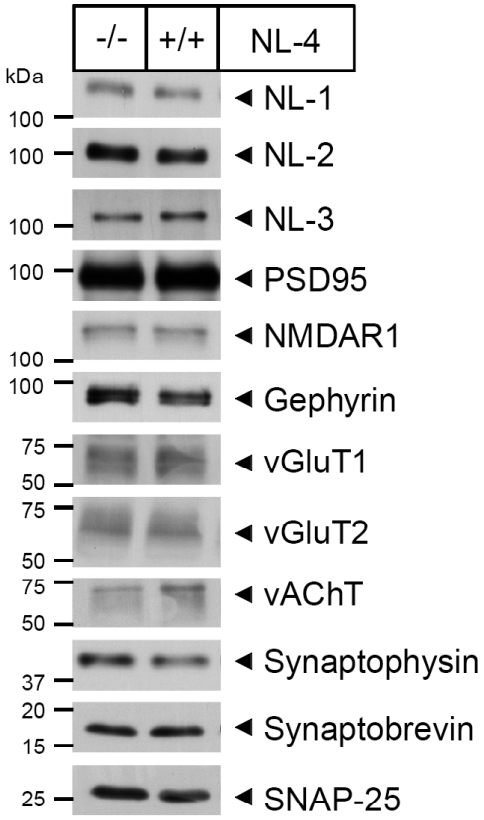


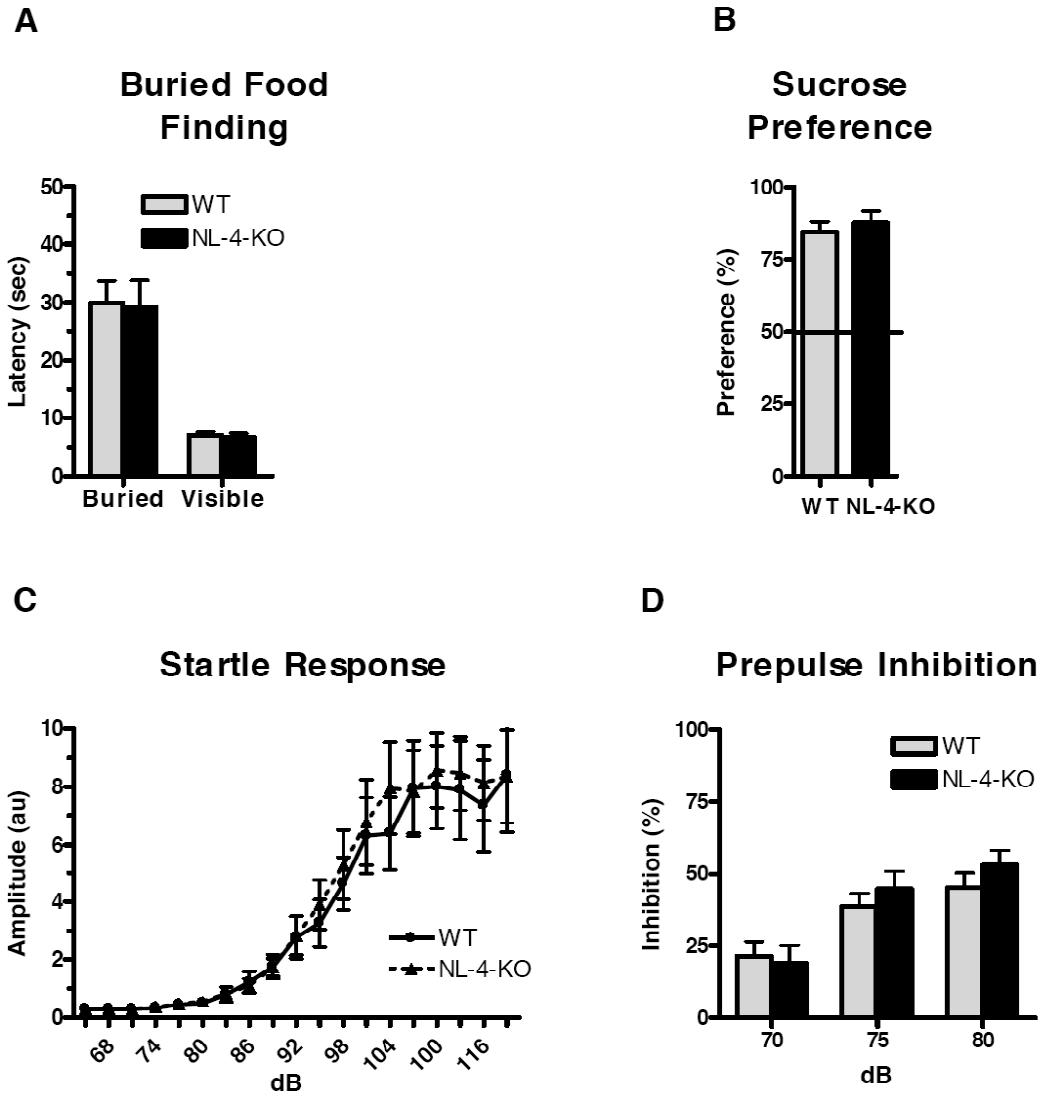
SI Fig. 1. Expression profile of NL-4 protein. (*A*) A specific antibody to NL-4 was tested in Western blot analyses of whole brain homogenates from a WT and an NL-4 KO mouse (20 μg per lane separated by SDS-PAGE). The antibody detects a single band of the predicted size in WT brain homogenate but not in NL-4 KO brain homogenate, demonstrating that the antibody is monospecific. (*B*) Homogenates of the indicated tissues (20 μg per lane) were separated by SDS-PAGE and analyzed by Western blotting with the NL-4 specific polyclonal antibody. (*C*) Homogenates of the indicated brain regions (20 μg per lane) were separated by SDS-PAGE and analyzed by Western blotting with the NL-4 specific polyclonal antibody and with an anti-β-Tubulin antibody. (*D*) Brain homogenates of mice at the indicated developmental stages (E, embryonic day; P, postnatal day; 20 μg per lane) were separated by SDS-PAGE and analyzed by Western blotting with the NL-4 specific polyclonal antibody and with antibodies to NL-1, NL-2, and NL-3. (*E*) Brain subcellular fractions (20 μg per lane) were separated by SDS-PAGE and analyzed by Western blotting with the NL-4 specific polyclonal antibody and antibodies to the NMDA receptor subunit NMDA R1, a postsynaptic marker, and Synaptophysin, a synaptic vesicle marker. Fractions are designated as follows: H, homogenate; P1, nuclear pellet; P2, crude synaptosomal pellet; S1, supernatant after synaptosome sedimentation; P3, light membrane pellet; S3, cytosolic fraction; LP1, lysed synaptosomal membranes; LS1, supernatant after LP1 sedimentation; LP2, crude synaptic vesicle fraction; LS2, cytosolic synaptosomal fraction; SPM, synaptic plasma membranes.



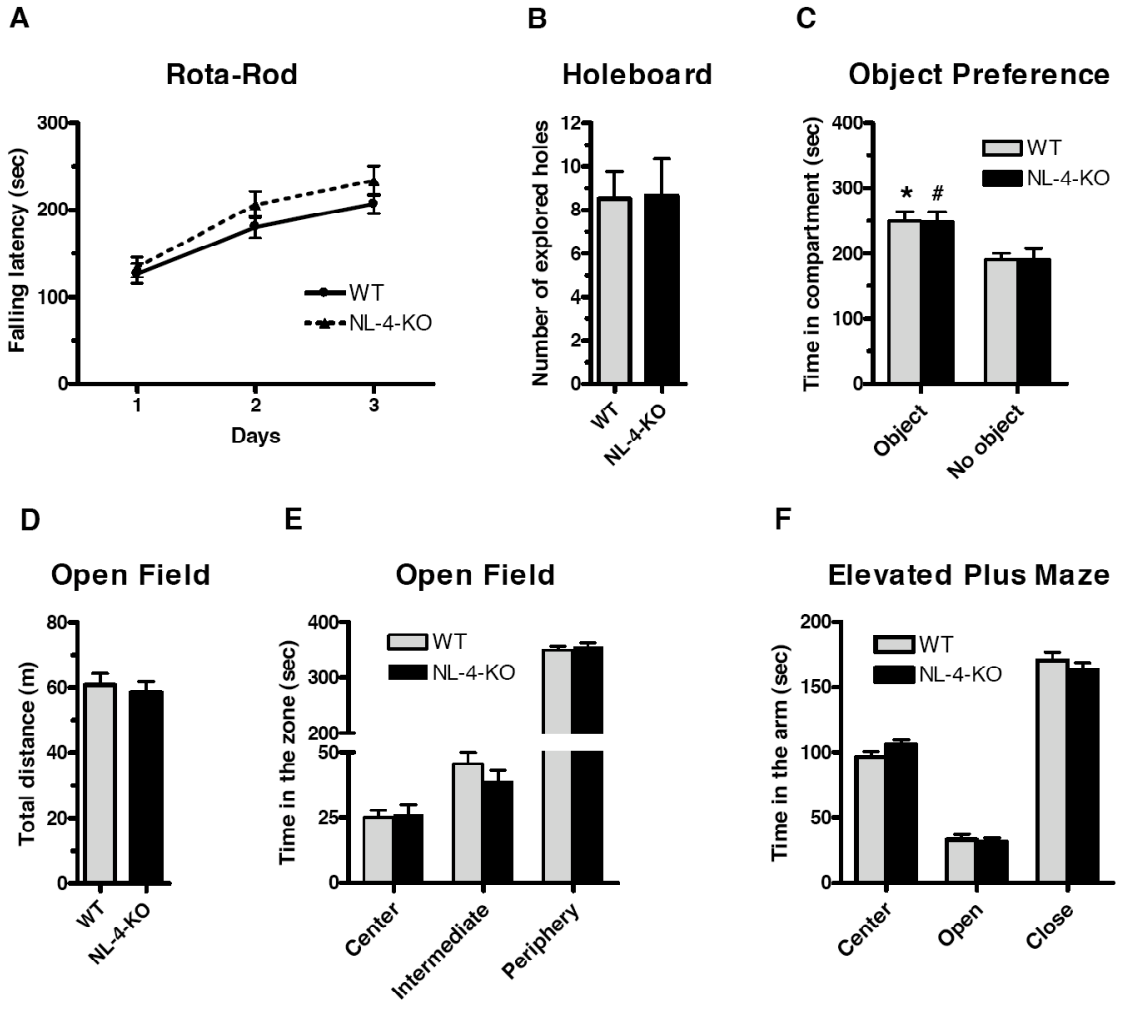
SI Fig. 2. NL-4-KO, detection of the NL-4 mutation, and expression pattern of the NL-4 promoter. (*A*) Representation of the first six exons of the NL-4 gene (E1-E6) and the insertion site of the gene trap vector pGT1TMpfs. The gene trap vector contains an En-2 intron and splice acceptor site (SA) upstream of a transmembrane domain (TM), followed by lacZ (β-galactosidase) and a neomycin resistance fusion gene (neo), an internal ribosomal entry site (IRES), and a placental alkaline phosphatase gene (PLAP) with a polyadenylation signal (SVpA). The probe used for the Southern blot analysis of genomic DNA is located in the first NL-4 exon (E1). The insertion resulted in a new splice acceptor (SA), leading to a chimeric mRNA with 138 amino acids from NL-4 in frame with β-galactosidase. (*B*) Southern blot analysis of genomic DNA from WT (+/+) and heterozygous (+/-) or homozygous (-/-) NL-4-KO mice. DNA was digested with BamH I and Stu I. Bands representing the WT and NL-4-KO (KO) alleles are indicated on the right. (*C*) Western blot analysis of adult brain homogenates (20 μg per lane separated by SDS-PAGE) from WT (+/+) and heterozygous (+/-) or homozygous (-/-) NL-4-KO mice. Blots were probed with polyclonal antibodies to NL-1, NL-2, NL-3, and NL-4, and a monoclonal antibody to Synaptophysin. No modification in the expression levels of NL-1, NL-2, or NL-3 were detectable in NL-4-KOs. (*D*) β-Galactosidase activity (expressed under the control of the NL-4 promoter) in brain sections from a NL-4-KO, detected by a standard dye reaction with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. *In situ* hybridization experiments yielded very similar data (data not shown).



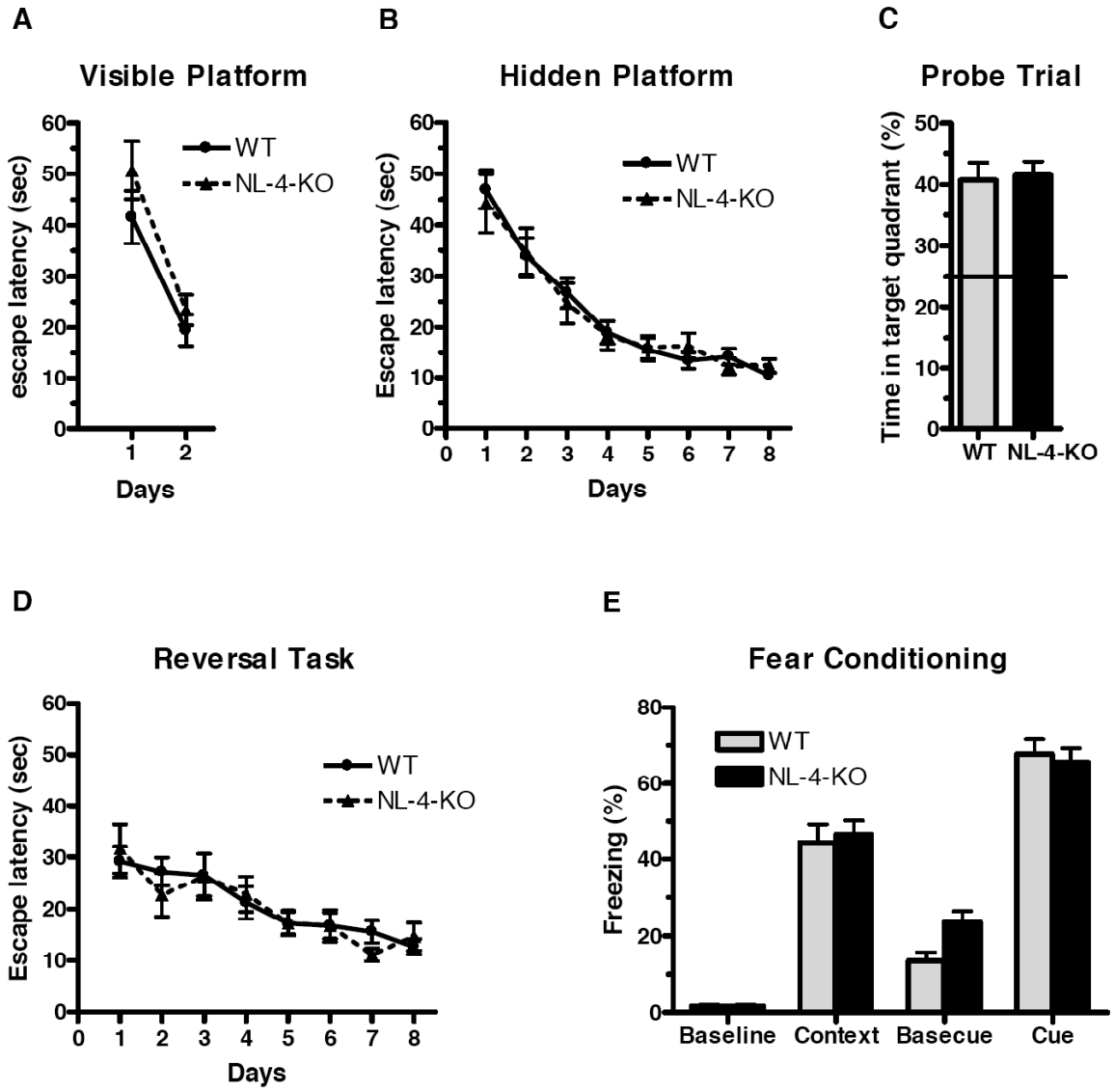
SI Fig. 3. Expression of NLs, NL interaction partners, and selected synaptic proteins in NL-4-KOs. Western blot analysis of brain homogenates from a WT (+/+) and an NL-4-KO mouse (-/-) (20 μg per lane separated by SDS-PAGE) using antibodies to the indicated proteins. All of the tested proteins are expressed at similar levels in WT and NL-4-KO brain. vGluT, vesicular glutamate transporter; vAChT, vesicular acetylcholine transporter; NMDA R1, R1 subunit of the NMDA receptor; PSD95, postsynaptic density protein of 95 kDa; SNAP-25, synaptosomal associated protein of 25 kDa.



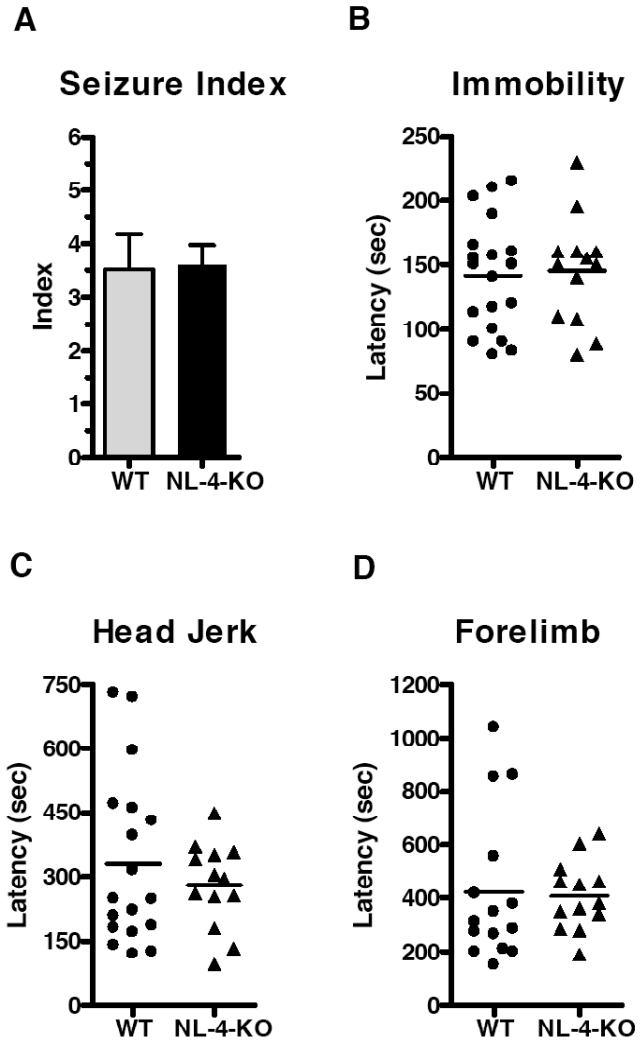
SI Fig. 4. NL-4-KO mice show no deficits in sensory functions and sensorimotor gating. (*A*) Buried food-finding test of olfaction. (*B*) Sucrose preference test for taste sensation and anhedonia. (*C*) Stimulus-response curve for auditory-based startle reaction as a behavioral evaluation of hearing. (*D*) Prepulse inhibition of the acoustic startle response as a measure of sensorimotor gating. No significant differences between genotypes were observed (n=20, WT; n=18, NL-4-KO).



SI Fig. 5. Normal locomotor, exploratory, and anxiety behavior in NL-4-KO mice. (*A*) Rota-rod test for motor performance and coordination. (*B*) Holeboard test for exploratory activity. (**c**) Object preference test for general curiosity towards inanimate objects. The asterisk and cross indicate a significant preference for the object in both, WT and NL-4-KO mice. (*D,E*) Open field test for general locomotor activity (*D*) and anxiety (*E*). (*F*) Elevated plus maze test for anxiety. No significant differences between genotypes were observed (n=20, WT, and n=18, NL-4-KO, for *A,B,D-F*; n=18, WT, and n=16, NL-4-KO, for *C*).



SI Fig. 6. Normal learning and memory processes in NL-4-KO mice. (*A-D*) Morris water maze task for hippocampus-dependent learning and memory. (*A*) Visible platform test for estimating vision, swimming capacity, and motivation to find the rescue platform. (*B*) Hidden platform test for spatial memory and navigation. (*C*) Probe trial for confirmation of a spatial strategy to find the escape platform. (*D*) Reversal training for assessing cognitive flexibility, i.e. the ability of mice to modify previously acquired skills. (*E*) Classical fear conditioning test for hippocampus-dependent (freezing upon context) and hippocampus-independent (freezing upon cue) learning and memory. No significant differences between genotypes were observed (n=20, WT; n=17, NL-4-KO).



SI Fig. 7. Normal seizure propensity in NL-4-KO mice. Seizures were induced by pentylenetetrazol injection. (*A*) Seizure index as a cumulative measure characterising the overall seizure propensity was calculated according to the following formula:
Seizure score = (0.1) (1/I latency) + (0.2) (1/HC latency) + (0.3) (1/FC latency) + (0.4) (1/GC latency) + (0.5) (1/TC latency) + (0.6) (1/D latency)
with (I) immobility, (HC) partial clonus of the head, (FC) partial clonus of the forelimb, (GC) generalized clonus (GC), (TC) tonic-clonic seizures, and (D) death.
(*B-D*) Latencies of different components of seizure progression in mice. (*B*) Immobility; (*C*) partial clonus of the head; (*D*) partial clonus of the forelimbs. No significant differences between genotypes were observed (n=13-19, WT; n=13, NL-4-KO).

SI Table 1. Overview of the behavioral tests performed on male WT and NL-4-KO littermates

Test Type	Changes in NL-4-KOs
Sensory	
Buried food finding	ns
Sucrose preference	ns
Startle response	ns
Prepulse inhibition	ns
Morris water maze (visible platform)	ns
Locomotion and Exploration	
Rota-rod	ns
Open field (total distance travelled)	ns
Hole board	ns
Object preference	ns
Anxiety	
Open field (areas visited)	ns
Elevated plus maze	ns
Seizure Propensity	
PTZ induced seizure	ns
Learning and Memory	
Morris water maze (hidden platform)	ns
Morris water maze (probe trial)	ns
Morris water maze (reversal task)	ns
Fear conditioning (context)	ns
Fear conditioning (cue)	ns
Social Behavior	
Time in contact in arena (pairs)	↓
Tripartite chamber (stranger vs. empty, time)	↓
Tripartite chamber (stranger vs. empty, visits)	↓
Tripartite chamber (stranger vs. familiar, time)	↓
Tripartite chamber (stranger vs. familiar, visits)	ns (↓)
Resident intruder test (attack latency)	↑
Resident intruder test (% of attacking mice)	↓
Vocalization	
Latency to first call	↑
Number of calls	↓

ns, no significant changes; ↑, significant increase; ↓, significant decrease