Supplementary Methods Yang et al.

Subjects

All procedures were approved by the Institutional Animal Care and Use Committees of the National Institute of Mental Health Intramural Research Program, University of California Davis, and Washington State University Vancouver. Generation of the 16p11.2 deletion mice was previously described (Portmann *et al.* 2014). Breeding pairs were imported from Stanford to NIMH to generate Cohort 1, and subsequently rederived at UC Davis to generate Cohorts 2 and 3. +/- males were mated to +/+ females, i.e. inbred on the existing mixed background, which was approximately 94% C57BL/6N and 6% 129P2/Ola and CD-1, and then mated to C57BL/6J females in some cases to ensure survival and colony expansion. To reduce fatalities of +/- pups, pregnant females were supplemented with high-fat rodent chow and fresh fruits. A concentrated liquid dietary supplement (Stat®, Pegasus Laboratories, Pensacola, FL) and vitamin B12 injection were used to treat underweight +/- pups in several cases at NIMH. In cases when +/+ pups outnumbered +/- pups, excess +/+ pups were culled to reduce feeding competition. Juveniles were weaned at 21 days of age and group housed by sex in cages of 2-4 littermates per cage. Weanlings were provided with fresh fruit supplements until 4 weeks of age. Standard rodent chow and tap water were available *ad libitum*. Underweight adults were provided with dietary supplements. In addition to standard bedding, a Nestlet square and a cardboard tube were provided in each cage. The colony room was maintained on a 12:12 light/dark cycle with lights on at 7:00 AM, and at approximately 20°C and 55% humidity. Behavioral testing was conducted between 9:00 AM and 5:00 PM. At UC Davis, all animals were fed with 2018 Teklad global 18% protein rodent diet. No additional supplements were given to breeding cages or weanlings. The colony room was maintained on a 12:12 light/dark cycle with lights on at 7:00 AM, and at

approximately 20°C and 55% humidity. Behavioral testing was conducted between 9:00 AM and 5:00 PM. The survival rate of +/- pups was approximately 60% at both NIMH and UC Davis. Post-mortem pathology reports on deceased +/- included cases of malnutrition, lung congestion and hydrocephalus. No fatalities of +/+ pups were found in NIMH or UC Davis. Genotypes of the mice were identified by visualizing mCherry expression with the DFP-1 dual fluorescent protein flashlight (http://www.nightsea.com/products/dfp-1/ Nightsea, Bredford, MA). The visualization procedure takes place in a dark room. The experimenter holds the tail of the mouse and points the flashlight to its tail/back region, avoiding the eyes. In +/- mice, hairless parts of the body (tail, feet, ear tips) glow fluorescent green under the flashlight. +/+ do not glow green. Accuracy of the mCherry fluorescence visualization method was confirmed by PCR genotyping, as described previously (Portmann *et al.* 2014). The behavioral tests were conducted in the following sequence: elevated plus-maze, light ↔ exploration, open field, male-female interaction, female urine test, novel empty cage, olfactory habituation/dishabituation, nociception, acoustic startle and PPI. An interval of 3-7 days was given between tests.

A) Vocalizations in social settings

Three-phase male-female social interaction test

Two cohorts of young adult males were tested in the three-phase male-female social interaction test, as previously described (Yang *et al.* 2013). Cohort 1, bred and tested at NIMH, employed sexually naïve male subjects between 8 and 12 weeks of age. Female C57BL/6J partner mice of the same age were visually inspected and selected for estrus phase, as previously described (Champlin, Dorr & Gates 1973; Caligioni 2009; Scattoni, Ricceri & Crawley 2011; Brielmaier *et al.* 2012). The male-female social interaction and vocalization test was conducted in an

environmental chamber (ENV-018V; Med Associates, St. Albans, VT, USA). Interior walls were covered with convoluted foam sheets (Uline, Pleasant Prairie, WI**)** to reduce environmental noise**.** Behaviors were recorded by a digital closed-circuit television camera (Panasonic, Secaucus, NJ, USA) positioned 30 cm horizontally from the cage. Dim red light (10 lux) from a desk lamp provided illumination. Ultrasonic vocalizations were recorded with an ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM15; Avisoft Bioacoustics, Berlin, Germany) mounted 2 cm above the testing cage. Sampling frequency for the microphone was 250 kHz; resolution was 16 bits. Phase 1 consisted of a reciprocal interaction session (Cohort 1, 5 min; Cohort 2, 3 min) between the freely moving male and the freely moving unfamiliar estrous female, both sexually inexperienced. The male subject mouse was removed from its home cage and placed in a clean standard mouse cage whose floor was covered with a thin layer of clean bedding. The testing cage was then placed in the sound-attenuating chamber without a cage lid. An estrous B6 female was introduced into the testing cage and the environmental chamber door was closed immediately. At the end of Phase 1, the female was removed from the testing cage and placed in a clean cage outside the experiment room. The male subject was left alone in the testing cage (Phase 2, 3 min). Ultrasonic vocalizations emitted by the male in the absence of the female were recorded. At the end of Phase 2, the same female was returned to the testing cage to rejoin the male subject for the second reciprocal interaction session (Phase 3, 3 min). Each female stimulus mouse interacted with no more than two male subjects a day, with a 2-hour interval between uses. Holding cages were covered with filter-top lids to block odors, and placed outside of the closed door of the testing room. Since male vocalization methods conducted in other laboratories often employ a pre-exposure (Schmeisser *et al.* 2012; Ey *et al.* 2013), modifications were evaluated in Cohort 2.

Male subjects, aged 8-12 weeks, were exposed to an estrous +/+ female for 5 min in a clean mouse cage with fresh bedding 3 days prior to the experiment. On the testing day, the male subject was initially placed in the testing chamber for 3 min alone, during which period its vocalizations in the empty novel environment were recorded. To address the possibility that stimulus females of the B6 inbred strain elicited different responses than stimulus females of the mixed background wildtypes, +/+ females were used as partners for Cohort 2 subjects. Similar results were obtained with B6 and 16p11.2 +/+ stimulus females, in the male Cohorts 1 and 2, respectively, indicating that this methodological difference did not affect findings.

Durations and frequencies of social behaviors were scored from videotapes by a trained investigator using Noldus Observer software (Noldus Information Technology, Leesburg, VA, USA). Social interaction parameters analyzed were nose-to-nose sniffing (sniffing or snout contact in the region around snout/head/neck/mouth), anogenital sniffing (sniffing the anogenital area), body sniffing (sniffing the trunk or limbs), following (walking at the same speed behind the other animal, keeping a distance of 2 cm or shorter), as previously described (Yang *et al.* 2012; Yang *et al.* 2013). The investigator scoring the videos was blinded to genotype by coding the recorded videos, as previously described (Yang *et al.* 2012). Bouts of arena exploration were scored as a measure of general exploratory activity in the novel environment.

To verify that deficits in vocalizations in +/- males are not limited to the male-female interaction scenario, we tested Cohort 3 for female urine-induced USVs, using the same equipment described above. In this test, social odors were presented but no live partner mouse was present. Three days prior to the experiment, each subject male was exposed to a non-littermate estrous +/+ female for 5 min in a clean mouse cage with fresh bedding. On the testing day, each male subject was placed in a testing cage and left alone in the sound attenuating

chamber for 3 min (pre-test). A cotton swab was saturated with 5 µl of fresh urine from an estrous +/+ female, obtained by holding the female by the tail, and gently rubbing its lower abdomen until urine was released into an Eppendorf tube. The end of the wooden stick was quickly taped to one upper corner of the cage, so that the cotton tip was positioned in the middle of the cage, at a level easily reached by the nose of the subject mouse. Vocalizations were recorded for 3 min.

Isolated pup vocalization test

Pups from the NIMH colony were tested for separation-induced USVs as previously described (Scattoni *et al.* 2008; Yang *et al.* 2012). A small number of pups from the NIMH colony were tested on postnatal days 6 and 8. Two full cohorts of pups from the UC Davis colony were tested for USVs on postnatal days 4, 6, 8, 10, and 12 using identical methods. Each pup was gently removed from the nest and placed in a rectangular glass container (10 x 8 x 8.5 cm). The bottom of the container was covered with a thin layer of fresh bedding. The container was immediately placed inside a sound-attenuating environmental chamber (ENV-018V; Med Associates, St. Albans, VT, USA). USVs were recorded for 3 min. At the end of the recording session, the pup was weighed and returned to the nest. Room temperature was maintained at 23 ± 1°C. In addition to call numbers, audio files recorded from pups tested at UC Davis on P8 were analyzed for call categories. A highly experienced investigator manually classified the calls into categories, using definitions of categories as previously described (Scattoni *et al.* 2008).

Analysis of ultrasonic vocalizations

Ultrasonic vocalizations were recorded with an Ultrasound Microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies of 10-180 kHz. Ultrasonic calls were recorded using the Avisoft

Recorder (Version 3.4). Sampling rate was 250 kHz, format 16 bit. Ultrasonic vocalizations were analyzed using Avisoft SASLab Pro software (Avisoft Bioacoustics). Spectrograms were generated for each 1-min audio file, with a FFT-length of 512 points and a time window overlap of 75% (100% Frame, Hamming window). The spectrogram was generated at a frequency resolution of 488 Hz and a time resolution of 1 ms. A lower cut-off frequency of 15 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Calls were inspected visually and classified into eight categories, generally based on criteria described previously (Scattoni, Ricceri & Crawley 2011). Categories analyzed included complex, two-component, upward, downward, chevron, short, frequency steps, and flat. "Unstructured" and "composite" described in a previous study (Scattoni, Ricceri & Crawley 2011) were rarely detected in the present cohorts, and were not analyzed. Summary statistics were generated by Avisoft SASLab Pro and analyzed using STATISTICA (StatSoft, Inc.). Cohort 1 was analyzed for call categories during minutes 1, 2, 3 as previous described (Scattoni, Ricceri & Crawley 2011; Yang *et al.* 2013). Only animals that emitted calls were included in the category quantification. In contrast, for call number analysis, all subject mice were included. Genotype differences in call numbers were analyzed by comparing total calls in minutes 1, 2, 3 of each phase between +/+ and +/-.

B) Tests of functional hearing

Acoustic startle and prepulse inhibition

We previously detected lack of acoustic startle in our line of 16p11.2 deletion mice (Portmann *et al.* 2014). However, the Mills 16p11.2 deletion line (Horev *et al.* 2011) appear to display normal hearing (personal communications, Anne Churchland, Cold Spring Harbor Laboratory; Ted Abel, University of Pennsylvania). To confirm and extend our previous findings, acoustic startle

and prepulse inhibition were conducted in another independent cohort of +/- and +/+, using the SR-Laboratory System (San Diego Instruments, San Diego, CA) as described previously (Silverman *et al.* 2011; Yang *et al.* 2012). The subject was placed in the Plexiglas holding cylinder for a 5 min acclimation period. For the next 8 minutes, the mouse was presented with each of six trial types across six discrete blocks, for a total of 36 trials. The intertrial interval was 10–20 s. One trial type measured the response to no stimulus (baseline movement). The other five trial types measured *acoustic startle responses* to 40 ms sound bursts of 80, 90, 100, 110 or 120 dB at 10kHz. The six trial types were presented in pseudorandom order such that each trial type was presented once within a block of six trials. 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. Background noise level of 70 dB SPL was maintained over the duration of the test session.

Sensorimotor gating was tested in +/+ and +/- mice using *prepulse inhibition of acoustic startle*. Each subject was presented with 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. Baseline movement was measured in the absence of stimulus. Maximum startle response to a 40 ms 110 dB tone was measured without a prepulse stimulus. The other five trial types each consisted of an acoustic prepulse stimulus (20 ms tones of 74, 78, 82, 86, and 92 dB intensity) and an acoustic startle stimulus (110 dB stimulus). The seven trial types were presented in pseudorandom order, such that each trial type was presented once within a block of seven trials. Startle amplitude was measured every 1 ms over a 65 ms period which begins at the onset of the startle stimulus. The maximum startle amplitude over this sampling period was recorded for analysis. A background noise level of 70 dB was similarly maintained over the duration of the prepulse test session.

Auditory brainstem response

To directly assess hearing ability, we measured auditory brain stem responses (ABR) to pure tones across a wide frequency range, using methods previously described (Mahrt *et al.* 2013). ABR experiments were conducted at Washington State University Vancouver (WSUV), using mice transferred from UC Davis. Animals were switched to a reverse 12h:12h light/dark cycle after arriving at WSUV, and were allowed to acclimate for a week before the ABR experiment. All experiments were conducted in the dark phase. To explore age-related hearing loss associated with the background strain, one cohort was tested at 27-32 weeks of age (middle-aged) and the second cohort was tested at 6-7 weeks of age (young adult). Each mouse was anesthetized with ketamine/xylazine (100 mg/kg and 5 mg/kg respectively, i.p.) and placed on a heating pad to maintain body temperature at 37°C. ABRs were recorded in a sound attenuating chamber using standard subcutaneous needle electrodes (Grass Technologies, Warwick, RI, USA). The active electrode was parallel to the right mastoid and the reference electrode was at the vertex of the skull. Pure tone stimuli were generated and ABR were recorded with custom software. Brainstem responses were amplified and filtered (2 kHz; 100 Hz-3 kHz; Dagan Corporation, Minneapolis, MN, USA), band-pass filtered (500-6,000 Hz; Krohn-Hite, Brockton, MA, USA), passed through a spike enhancer (Fredrick Haer, Bowdoin, ME, USA) and then digitized (10,000 samples/s; Microstar Laboratories, Bellevue, WA, USA). Rare myogenic artifacts greater than 30 µV were rejected by the software. Pure tone stimuli (5 ms duration, 1 ms rise/fall time, repetition rate 10/s) were delivered via a high-speed data acquisition board (400,000 samples/s; Microstar Laboratories, Bellevue, WA, USA) and presented free-field via an Infinity Emit leaf-tweeter speaker placed 10 cm from the right ear and 45° from midline. The speaker was regularly calibrated with a 1/4 inch calibrated microphone (Bruel and Kjaer model 4135) placed in the

position normally occupied by the mouse's ear. Speaker output was flat between 6 and 50 kHz. The speaker used to test young mice had a gradual decrease in sound pressure of about 1.9 dB per 10 kHz between 50 to 100 kHz, with a maximum output of 80 dB SPL at 100 kHz. The speaker used to test middle-aged mice had a gradual decrease in sound pressure of about 1.7 dB per 10 kHz between 50 to 100 kHz, with a maximum output of 83 dB SPL at 100 kHz. Distortion components were buried in the noise floor, at least 50 dB SPL below the signal level, as measured by custom-designed software performing a fast Fourier transform (FFT) of the digitized microphone signal. Hearing thresholds to tones between 8 and 32 kHz were determined in steps of 4 kHz because this is their most sensitive hearing range. Hearing threshold to tones between 50 and 100 kHz were determined in steps of 10 kHz. Hearing threshold was defined as the lowest sound pressure level (SPL) in which a recognizable and repeatable waveform was present. Responses were sampled over a 15 ms window with a 1 ms stimulus onset delay. The recording window was averaged over 512 repetitions for intensities at least 10 dB SPL above threshold and averaged over 1024 repetitions for intensities within 5 dB SPL of threshold. For +/- mice, responses were averaged over 1024 repetitions for each frequency at the maximum intensity available. Because no ABR responses were detected in +/- mice in our preliminary experiments, we used the repetition rate of 1024 at all frequencies to be certain that there was no response. Each mouse was tested once and average thresholds and standard deviations were calculated.

C) Sensory responses

Nociception

To evaluate potential confounds that could arise from hyposensitivity or hypersensitivity to aversive stimuli, we conducted two standard nociception tests, using methods previously

described (Silverman *et al.* 2011; Yang *et al.* 2012). For the *hot plate test*, the subject was placed on a 55^{o}C surface (IITC Life Science Inc., Woodland Hills, CA). Latency to first response, including licking or shaking paws, was recorded. For the *tail flick test*, the subject was gently restrained with the tail placed in the groove of the tail flick monitor (Columbus Instruments, Columbus, OH). An infrared photobeam was directed at the tail. Latency to flick the tail out of the path of the beam was recorded. To prevent tissue damage, a cut-off latency of 30s was employed in both tests.

Olfactory habituation/dishabituation

The ability to smell non-social and social odors was evaluated using methods described previously (Yang & Crawley 2009). Each subject mouse was tested in a clean empty mouse cage containing a thin layer of fresh bedding. Odor-saturated cotton-tipped swabs (6 in. length, Solon Manufacturing Company, Solon, Maine) were used to deliver odors. To reduce novelty-induced exploration, each mouse was habituated in the empty testing cage containing one clean dry cotton swab for 45 minutes before testing. The test consisted of fifteen 2-min trials: three trials with plain tap water, followed by three trials with banana odor (prepared from imitation banana flavoring, McCormick, Hunt Valley, MD; 1:100 dilution), three trials with vanilla odor (prepared from vanilla extract, McCormick, Hunt Valley, MD; 1:100 dilution), three trials with social odor from social cage 1, three trials with social odor from social cage 2. Water, banana odor, and vanilla 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 C57BL/6J mice were the sources of the two social odors. Time spent sniffing the swab was recorded with a stopwatch by an observer sitting 2 meters away from the testing cage. Sniffing

was scored when the nose was within 1 cm of the cotton swab. The inter-trial interval was approximately 1 minute.

D) Stress and anxiety-related behaviors

Considering the possibility that stress responses and generalized anxiety could influence vocalizations in adult mice in a social setting, and in separated pups (Insel, Hill & Mayor 1986; Rowlett *et al.* 2001), we observed behaviors in the home cage, and conducted standard tests for anxiety-related behaviors in mice.

Home cage observations

Home cage behaviors were observed in the vivarium for three 15-min sessions at 7 AM, 1 PM, and 8 PM. The investigator stood 2 feet from the ventilation rack and observed home cages for 15 min, noting scattered nesting, altered sleeping, excessive fighting, and high levels of repetitive behaviors.

Elevated plus-maze

The elevated-plus maze test for anxiety-related behaviors were conducted as previously described (Chadman *et al.* 2008; Silverman *et al.* 2010; Brielmaier *et al.* 2012; Yang *et al.* 2012). The *elevated plus-maze* consisted of two open arms (30 x 5cm) and two closed arms (30 x 5 x 15 cm) extending from a central area (5 x 5 cm), and elevated 50 cm from the floor. Room illumination was approximately 30 lux. The subject mouse was placed in the center of the maze, 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 transitions

The *ligh t↔ dark exploration test* for anxiety-like behaviors was conducted as previously described (Silverman *et al.* 2011; Yang *et al.* 2012) in an automated chamber consisting of a small, dark enclosed side and a larger illuminated side with no lid (NIMH Research Services Branch, Bethesda, MD)**.** The subject mouse was placed 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 (350 lux) and dark (3 lux) compartments were automatically recorded.

Open field activity

Open field exploratory activity was evaluated as previously described (Silverman *et al.* 2011; Yang *et al.* 2012). Each animal was tested in a VersaMax Animal Activity Monitoring System (Accuscan, Columbus, OH) for a 60 min session. Total distance traversed, horizontal activity, vertical activity, and time spent in the center were automatically measured.

Motor stereotypies

To understand the prevalence of spontaneous motor stereotypies in 16p11.2 deletion mice, we scored spontaneous jumping and backflipping in a novel empty cage test in a second independent cohort, using methods previously described (Portmann *et al.* 2014). To evaluate the consistency of stereotypic circling and backflipping behaviors across ages, mice were tested at 6, 8, and 10-12 weeks of age. Each subject was placed in an empty clean mouse cage, with a 0.25 cm layer of clean bedding lining the bottom. The cage was covered with a metal wire lid and a plastic filter top. Behaviors were recorded for 60 minutes and analyzed for the last 10 minutes. An observation was scored every 30 seconds for a total of 20 samples. Occurrences of circling,

hanging on the wire, backflipping, self-grooming, resting, and exploration were recorded for each observation sample.

Statistical analysis

USV data were not normally distributed and were analyzed with non-parametric tests. Non-parametric Mann-Whitney U test was used to compare genotype differences in USV call numbers and call repertoires in each phase. Hearing threshold data from the ABR experiments were non-normally distributed, and Kruskal-Wallis test was used to compare age differences in ABR responses in young and middle-aged +/+ mice. Repeated Measures ANOVA was used to analyze pup USVs and body weights across days, olfactory habituation and dishabituation response, and adult open field activity across 60 min. One-way ANOVA was used to analyze genotype differences in call frequencies, social sniff behaviors during male-female social interaction, anxiety-like behaviors in the elevated plus-maze and light ↔ dark exploration, and nociception responses in hot plate and tail flick tests.

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