Supporting Information

Mandel-Brehm et al. 10.1073/pnas.1504809112

SI Materials and Methods

Maternal Isolation Assay. Pregnancies were recorded by visual inspection and the day of birth was considered postnatal day 0 (PND 0). For the duration of the experiment (embryonic day 18 to PND 19) the mating pair remained together in the cage and the bedding was changed minimally (PND 8, PND 16) to keep odors unperturbed. USV testing occurred every other day from PND 3–19 during the hours of 9:00 AM to 1:00 PM. Pups were moved from the home cage into the recording environment by holding their tail, and the presence or absence of hindlimb-clasp activity was noted. Pups were kept in a warming cage until all littermates had been tested.

For acoustical analysis, sound files were analyzed using Avisoft SASLab Pro (v4.50). The total 4-min recording time for each trial was used for analysis. Fast Fourier transformation was conducted using Hamming window, 512 FFT Length, 100% frame, and 50% overlap providing 488-Hz frequency resolution and 1.024-ms temporal resolution. USV detection was performed using a fixed amplitude threshold of −60 dB and minimum call duration of 1 ms. For structural analysis, we measured the frequency (Hz) at the point of maximum amplitude and the mean amplitude of each vocalization. For the temporal analysis, we analyzed intercall interval (the time in-between sequential calls that occur less than 100 ms from one another) and interburst interval (time in-between bursts of calls that occur more than 101 ms and less than 500 ms from one another) for each recording (Fig. S7). Background/movement noise was eliminated using an entropy algorithm with a maximum entropy threshold of 0.4. USV data were visually inspected for false-positives and analysis settings were validated with trials of adult mice moving freely about the testing chamber. Zero vocalizations identified in our automated analysis were removed from the dataset or modified.

EEG/EMG Surgeries. Mouse EMG/EEG three-channel headmounts and accessories for subdural cortical EEG recordings were purchased from Pinnacle (Model Nos. 8201, 8202, 8206). On PND 28, animals were anesthesized with continuous flow of 1% isoflourane. Headmounts were glued on to the cranium and pilot holes for screws were made using a 25-gauge needle. Steel screws were set into four fixed locations on the headmount, with two recording electrodes over the posterior left and right cortex and two reference electrodes in the left and right frontal cortex. Screws were electrically coupled to the headmount through quick-setting epoxy applied underneath the screw head. Dental glue was applied to keep headmount in place and screws isolated from external noise. Two single-wire electrodes for EMG recordings were implanted in a small pocket of the nuchal muscles. Animals were injected with 1% flouxetine at the end of surgery and monitored daily while receiving two doses of flouxetine every 24 h for 3 d following surgery. EEG and EMG activity was measured on day 5 following surgery.

EEG Spiking Analysis. Raw EEG files were converted to the generic .edf format [EDF browser v1.22, Teunis van Beelen (Informer Technologies, Inc.)] and then imported into Matlab (Mathworks) for analysis. Fast Fourier transformation of .edf files were processed and visualized using a custom program in Matlab developed by Gary Yellen, Harvard Medical School, Cambridge, MA. EEG signals were transformed to frequency domain and power in frequency bands of 2–4 Hz, 4–8 Hz, 8–12 Hz, 12–20 Hz, and 20–80 Hz. For the power spectrum, the amount of EEG in a band is quantified in units of microvolts squared. Using spectrogram analysis we found that the spikes in EEG possessed harmonics throughout the 4- to 80-Hz range, which reliably caused an increase in power in the 20- to 80-Hz range. Because the majority of power during normal brain activity occurs in the lower frequency bands (<20 Hz), these small "bumps" in power in the higher frequency bands (20–80 Hz) caused by spiking activity, were easy to differentiate. A spiking event was recorded when a change in power >10 dB occurred in the 20- to 80-Hz frequency band. We chose to use a power threshold (dB) instead of amplitude (υV) threshold because of variance in voltage baselines. Spiking events were recorded manually while visually scanning EEG files offline in 10-s window intervals. Our EEG spike analysis was validated against an automated seizure detection algorithm (1). Our results were very consistent with this independent mode of analysis and exhibited a high correlation ($r = 0.97$) between both approaches (Fig. S5).

Protein Extraction/Western Blot. Freshly dissected mouse hippocampi or cultured cells (plated embryonic day 16.5 and stimulated day in vitro 7) were collected and dounce homogenized in RIPA buffer [50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, complete protease inhibitor mixture tablet (Roche), 1 mM sodium orthovanadate, 1 mM b-glycerophosphate]. For Western blots, samples were boiled for 5 min in SDS sample buffer, resolved by SDS PAGE, transferred to nitrocellulose, and immunoblotted. Antibodies were diluted 1:1,000 in 3% (vol/vol) BSA with sodium azide and never used more than three times for immunoblotting. Antibodies are as follows: αARC (gift from Paul Worley, Johns Hopkins University, Baltimore); αE6AP (Sigma), αNPAS4 (in-house), αB2-ACTIN (Sigma), and αGAPDH (Abcam). For quantification, each sample was ran in triplicate and normalized to a loading control.

1. Goodrich GS, et al. (2013) Ceftriaxone treatment after traumatic brain injury restores expression of the glutamate transporter, GLT-1, reduces regional gliosis, and reduces post-traumatic seizures in the rat. *J Neurotrauma* 30(16):1434–1441.

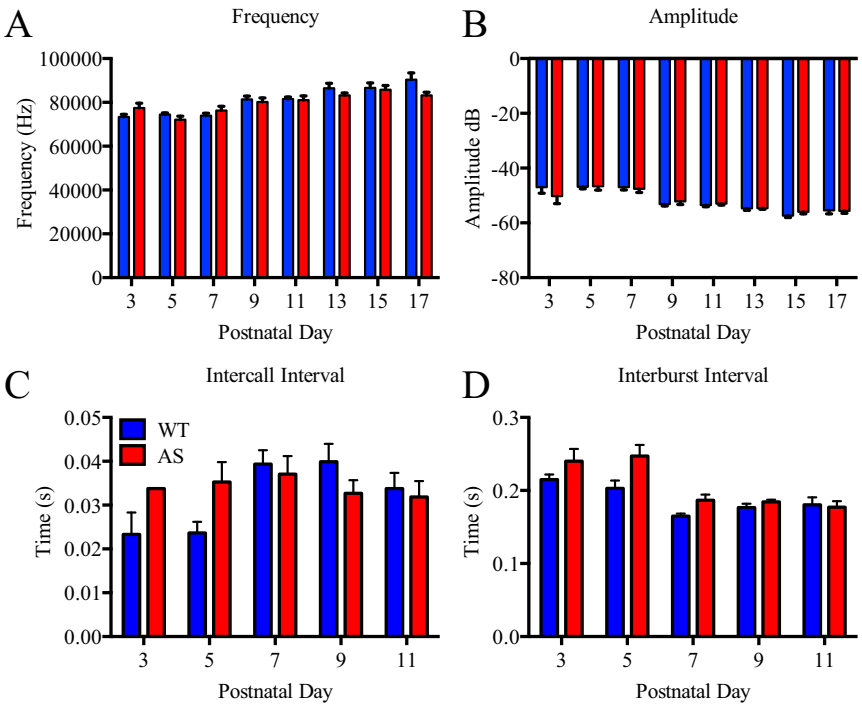

Fig. S1. Structural and temporal features of ultrasonic vocalizations in AS mice are indistinguishable from wild-type littermates. (*A*) Average frequency of vocalizations (WT $n = 8$, AS $n = 9$), (*B*) Average amplitude of vocalizations (WT $n = 8$, AS $n = 9$), (*C*) Average intercall interval (WT $n = 8$, AS $n = 9$), and (*D*) Average interburst interval (WT $n = 8$, AS $n = 9$). Statistics for *A*–*D*: Two-way, nonrepeating-measures ANOVA; Bonferroni multiple comparisons correction.

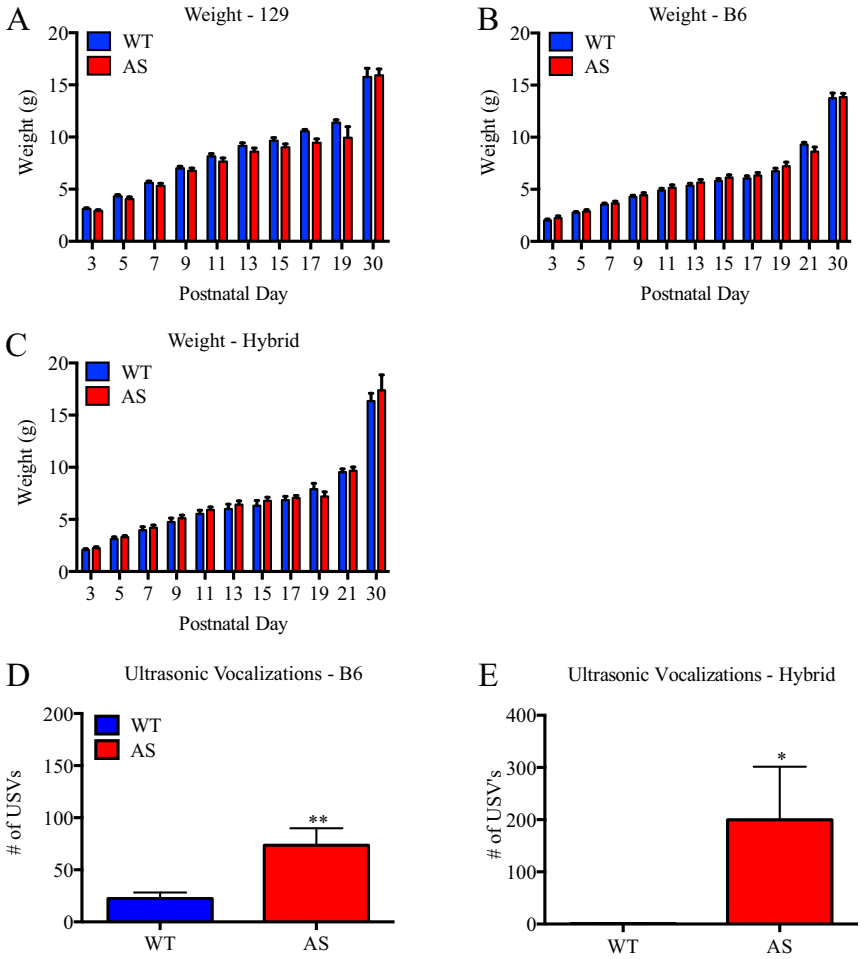

Fig. S2. USV phenotype in AS mice is not due to differences in postnatal weight or repetitive handling. (*A–C*) Weights of WT and AS littermates during early postnatal development on a 129 background (WT $n = 11$; AS $n = 7$), B6 background (WT $n = 19$, AS $n = 15$), and hybrid background (WT $n = 11$, AS $n = 10$), respectively. (*D* and *E*) USVs measured from naïve wild-type and AS mice at PND 16 as done previously (see Fig. 1). AS mice exhibited significantly more USVs than wild-type littermates on a B6 background (WT $n = 13$; AS $n = 15$) and hybrid background (WT $n = 7$; AS $n = 5$). Statistics for *A–C*: Two-way, nonrepeating measures ANOVA; Bonferroni multiple comparisons correction. Statistics for *D* and *E*: Unpaired two-tailed *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars represent SEM.

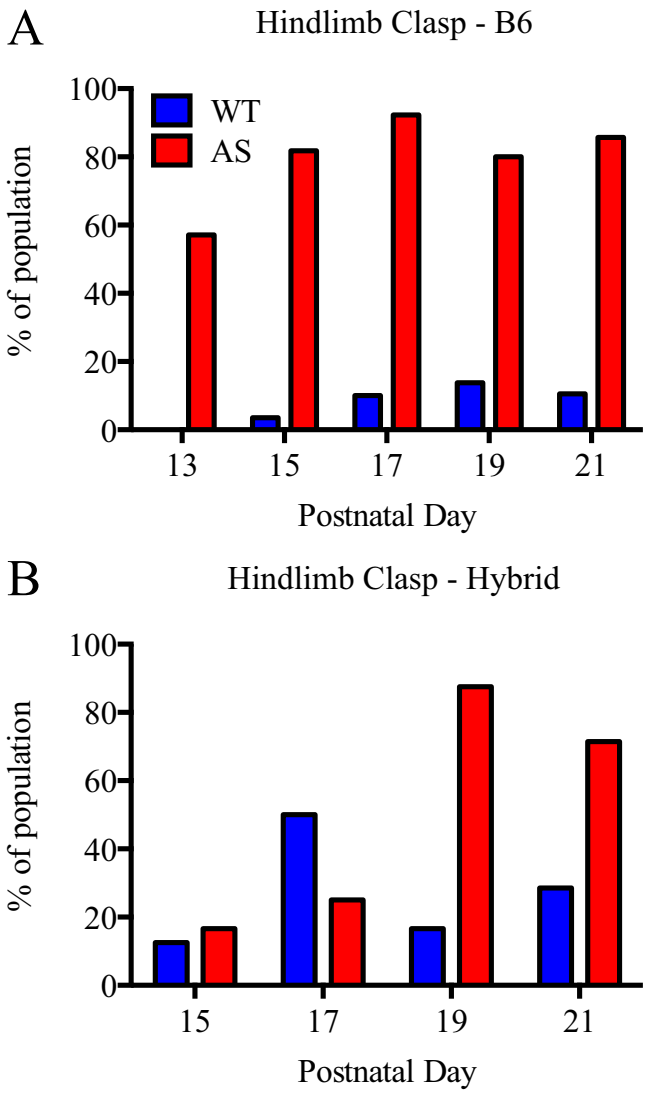

Fig. S3. AS mice have increased frequency of hindlimb clasping in early development compared with wild-type littermates. Hindlimb-clasp activity compared between wild-type and AS mice from PND 3 through PND 21. (*A*) B6 genetic background (WT n= 5–16; AS n= 7–9) and (*B*) Hybrid genetic background (WT $n = 9$; AS $n = 7$–10).

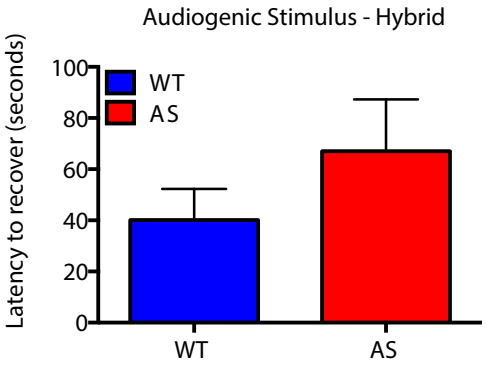

Fig. S4. Audiogenic stimulus phenotype in AS mice becomes less robust in adulthood. Wild-type and AS mice were subjected to the audiogenic stimulus assay throughout the adult-period PND 42–90. Latency to recover was indistinguishable between wild-type and AS mice littermates (WT $n = 8$; AS $n = 10$). Statistics: unpaired two-tailed *t* test.

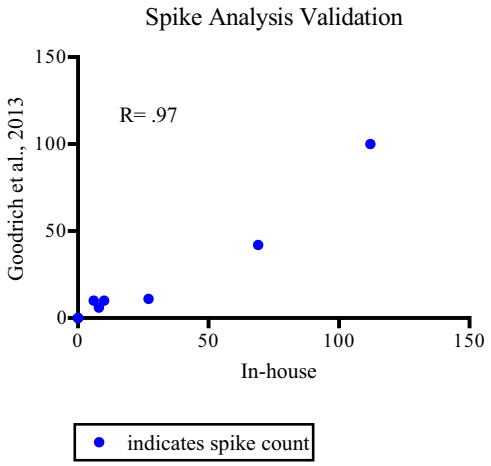

Fig. S5. Validation of spiking analysis for EEG recordings. Total number of spiking events was measured manually (see *SI Materials and Methods*) in-house. To validate of our analysis, a subset of EEG files (WT $n = 3$; AS $n = 4$) were analyzed with an automated spike detection program (1). Pearson's correlation was used for statistical analysis. $R = 0.97$.

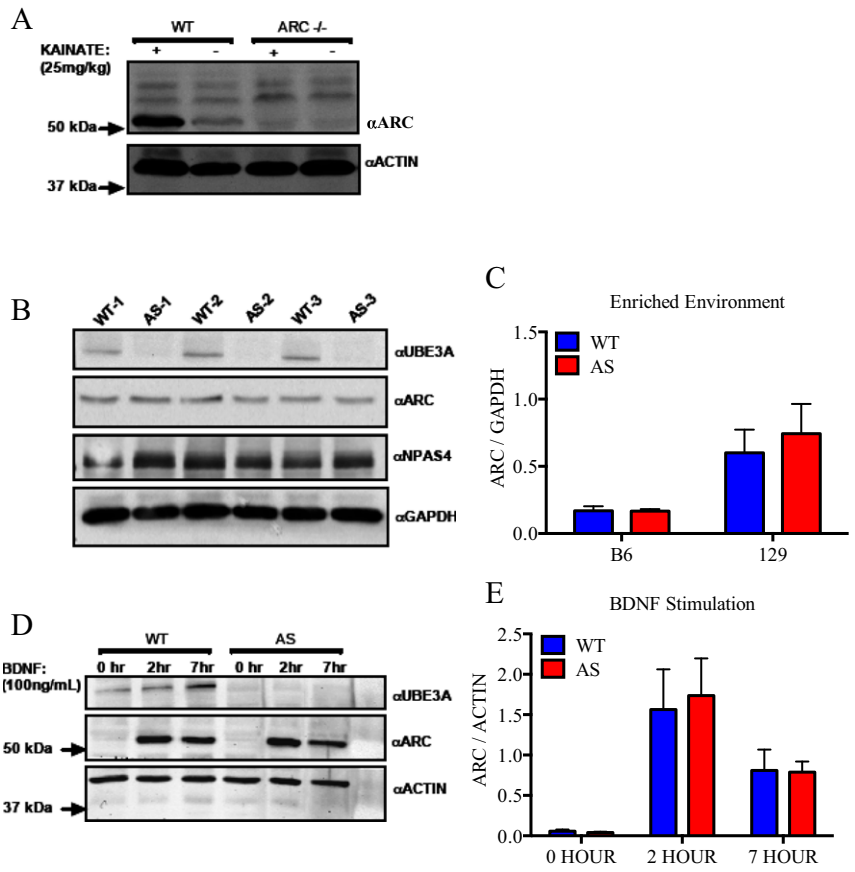

Fig. S6. ARC protein levels are unchanged between wild-type and AS mice. ARC protein levels in AS and wild-type mice were analyzed in different contexts. (*A*) Western blot with validation of ARC antibody. Wild-type ($Arc^{+/+}$) and Arc knockout ($Arc^{-/-}$) mice were exposed to Kainic acid (25 mg/kg) for 2 h. Hippocampal lysates were prepared and analyzed using Western immunoblotting with anti-ARC antibodies (provided by Paul Worley, Johns Hopkins University, Baltimore). Note the prominent band at 50 kDa appears with stimulation and is absent in the $Arc^{-/-}$ lysate. (*B*) Representative Western blot from wild-type and AS littermates exposed to 2 h of enriched environment. Hippocampal lysates were immunoblotted with αUBE3A, αARC, αNPAS4, and αGAPDH. (*C*) Quantification of Fig. S6*B* Western blot shows no statistical difference between ARC in AS and WT mice. ARC levels normalized to GAPDH. (WT $n = 3$; AS $n = 3$). (*D*) Representative blot of WT and AS cortical neuronal cultures stimulated for 0, 2, or 7 h with 100 ng/mL brain-derived neurotrophic factor (BDNF). (WT $n = 3$; AS $n = 3$). (*E*) Quantification of *D* reveals no difference in ARC protein levels between wild-type and AS mice. (*C* and *E*) Whole-cell lysates were quantified using Bradford assay and ran in triplicate for quantification. Fifty-micrograms of protein were loaded for Western blot analysis and protein levels were normalized to GAPDH or ACTIN loading control. One-way ANOVA was used for statistical analysis.

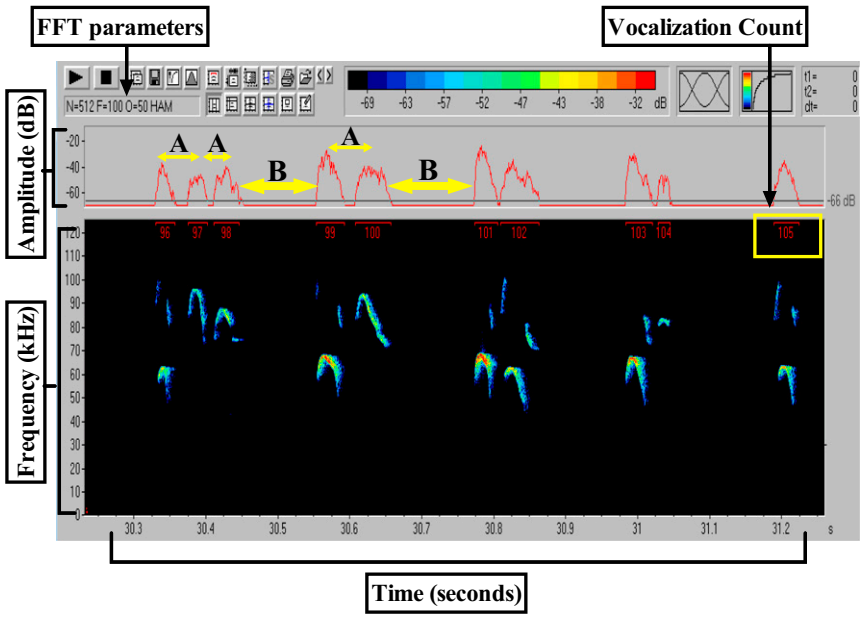

Fig. S7. Screenshot of USV analysis depicting intercall and interburst interval. Maternally isolated USVs are visualized and analyzed using Avisoft Sound Analysis Software. Examples of the intercall interval are depicted as "A" and examples of the interburst interval are depicted as "B." Time window is approximately 1 second.