Supporting Information

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SI Materials and Methods

Animals. $TSC2^{+/-}$ mice that were generated as described previously (1) were back-crossed for 10 generations to C57BL/6J background. Matings were set up with one WT and one heterozygous mouse to achieve approximately equal numbers of offspring by genotype (half $^{+/+}$, half $^{+/-}$) with either the mother or the father bearing the TSC2 mutation so as to compare $TSC2^{+/-}$ and WT dams. Breeding cages were left undisturbed except for gentle daily checks, removal of sires 1 wk after breeding pairs were set up, and tattooing on P3 by an AIMS tattoo machine, whereby P1 is counted as the day of birth. Mouse tails were collected for genotyping after all experiments had been completed, and weaned mice were group-housed 3–5 per cage before future experiments. All mice were housed in a room set to a 12:12-h light:dark cycle, with lights on at 6 AM, environmental temperature maintained at 22 °C (±2 °C), and humidity ranging from 32 to 68%. All experiments were conducted in accordance to protocols approved by the Institutional Animal Care and Use Committee of the University of California–San Francisco.

Isolation-Induced Vocalizations and Maternal Potentiation. Pups were isolated one-by-one from their home cage and placed in a cardboard recording box situated in an anechoic chamber. During a 5-min isolation period, vocalizations were recorded from an Avisoft Bioacoustics UltraSoundGate CM16/CMPA microphone (Berlin, Germany) capable of accurately recording calls up to 180 kHz, digitized using a PCI data acquisition board (PCI NIDAQ 6251; National Instruments Corp.) at a sampling rate of 366 kHz, and collected and written to disk using custom-built MATLAB software (MathWorks). Each pup was next reunited with its dam and littermates, placed at the end farthest from the nest to allow the dam to retrieve the pup. After a 5-min reunion, that pup was reisolated and recorded for an additional 5 min. Following recordings, pups were weighed and measured for body temperature. All maternal potentiation experiments were performed on P10 pups.

Signal Processing and Analysis of Pup Calls. To detect pup vocalizations in the recorded sound files, we followed a denoising–peak tracking–thresholding approach outlined in detail by Liu et al. (2). The denoising algorithm entailed estimating the background noise and then “subtracting out” this noise from the original signal in the frequency domain. We obtained our noise estimates by visually examining the spectrogram (short-time Fourier transform) and identifying, for each 5-min sound recording, 2-s intervals that contained only background acoustic noise (i.e., no calls, scratches, or other short-time artifacts). Before implementing the spectral subtraction algorithm, all sounds were high-pass filtered (45 kHz cutoff using a 512 pole Finite Impulse Response filter). After denoising, we extracted calls by thresholding the sound file’s amplitude envelope, calculated using a peak-tracing (with delay) algorithm (2). We observed that there were several call types that exhibited very short (<40-ms) “gaps” between two or more frequency components. Following Liu et al., we ensured that these calls were not erroneously broken apart by grouping together any above threshold components that were separated by less than 40 ms. All files were automatically segmented using this algorithm, and identified calls were verified manually by a trained observer viewing the spectrogram to remove any misidentified calls (1.5%, for a total of 46,497 verified calls). Call duration was determined as the time between the start and end of each call, and ICIs were defined as the time between the end of one call and the start of the next. ICIs were determined by log transforming the distribution of ICIs and identifying the largest peak as within-burst intervals and the next smallest peak as interburst intervals. Bursts detected in this way were also verified by a trained observer. Since an absolute voltage to sound pressure calibration was unavailable for our recording setup, we used the log of the voltage level output by the microphone as a proxy for relative call volume. To represent each call by a single frequency, we determined the median frequency of each segmented call by calculating, and then histogramming, the instantaneous frequency. In detail, the procedure was as follows. We calculated the spectrogram for a specific call and compared, for each time-slice, the relative loudness in each frequency bin with that of the mean noise level in those bins. For that time-slice, we defined the instantaneous frequencies as all frequencies in which the relative loudness rose 3 SDs above the relative loudness of the mean noise for that recording. Then we pooled all of these frequencies over all time slices for that call. Finally, we defined the median frequency as the median of the distribution of these pooled frequencies.

To classify calls, each call was output to an image file for viewing in event-scoring software and classification by a trained observer. All calls in the first and last five bursts from each pup were classified to provide a sampling of call types both within and across bursts (14% of all calls, for a total of 6,673 classified calls). The classification scheme used is very similar to that proposed by Scattoni et al. (3), with a few key differences. The “shorts” call type was not used because zooming into each call allowed classification of the call as one of the other simple call types. The “composite” call type by Scattoni et al. was renamed “harmonic” to emphasize the harmonic relationship of the two components, whereas “composite” was used for calls with stacked components that were not in a harmonic relationship. “Two-syllable” calls are any calls with two syllabic components but no stacking, whereas “frequency steps,” as used in this study, are similar to two-syllable calls but with three or more syllables. To prevent confusion, the term harmonics was renamed to “harmonic steps” and defined by the inclusion of components in both syllabic and stacked relationship to one another.

To check for litter effects, we averaged pups within a litter by genotype. Seeing no differences between these values and those from individual pups, we continued our analysis by individual pup (Fig. S5). All processing was conducted through custom-designed MATLAB 7.7 (MathWorks) programs, and call data were stored in a SQLite 3.4.2 (Hipp, Wyrick & Company, Inc.) database and extracted through custom Python 2.5 (Python Software Foundation) scripts. Calls were classified using custom built scoring software (On The Mark 1.0, custom-built by the authors and provided for free as open-source software.).

Pup Retrievals. On P6, pups were removed from their nest, and three pups were returned, one to each corner of the cage away from the nest. The dam was then returned to the nest area facing away from the pups and allowed to retrieve pups under video recording. The first time that the dam picked up each pup was marked as the latency to retrieve that pup. These experiments were performed on a separate cohort from that used for vocalization experiments to avoid potential complications due to prior experience of isolation.

Resident Intruder. On P10, a male C57BL/6J adult (3–4 mo old) intruder mouse was introduced into the home cage while recorded by video camera. The intruder was left in the cage for 15 min before removal, after which video files were analyzed for maternal defense of her nest and home cage. The durations and number of events over which the dam spent above her nest and sniffing or attacking the intruder were measured and compared across dam genotype. The same litters and dams used in pup retrieval experiments were recorded here. All videos were scored using our On The Mark event-scoring software.

Statistics. All effects are reported as significant at $P < 0.05$, and error bars are used to indicate SEM. Statistics were conducted using PASW 18.0 (SPSS) with general linear model repeated-measures analyses. Isolation periods were treated as within-subject effects, whereas dam genotype, pup genotype, and pup gender were between-subject effects. For USV experiments, $n = 26$ (WT dam, WT pup; 11 female, 15 male), $n = 28$ (WT dam, heterozygous pup; 13 female, 15 male), $n = 17$ (heterozygous dam, WT pup; 9 female, 8 male), $n = 25$ (heterozygous dam, heterozygous pup; 17 female, 8 male); $n = 6$ WT dam litters, $n = 5$ heterozygous dam litters. 46,497 calls were segmented and analyzed from USV experiments. For maternal care assays and resident intruder assays, $n = 9$ dams per genotype. To compare median sound frequency distributions, a bootstrapping algorithm was applied in which pups were randomly resampled (with replacement) within each genotype group for 100 repetitions. The fraction of the total number of calls above 75 kHz was computed for each bootstrap, yielding a dataset of these measures for each group. These fractions were then compared by general linear model repeated-measures analyses.

1. Onda H, Lueck A, Marks PW, Warren HB, Kwiatkowski DJ (1999) Tsc2(+/-) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. *J Clin Invest* 104:687–695.
2. Liu RC, Miller KD, Merzenich MM, Schreiner CE (2003) Acoustic variability and distinguishability among mouse ultrasound vocalizations. *J Acoust Soc Am* 114:3412–3422.
3. Scattoni ML, Gandhy SU, Ricceri L, Crawley JN (2008) Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PLoS ONE* 3:e3067.

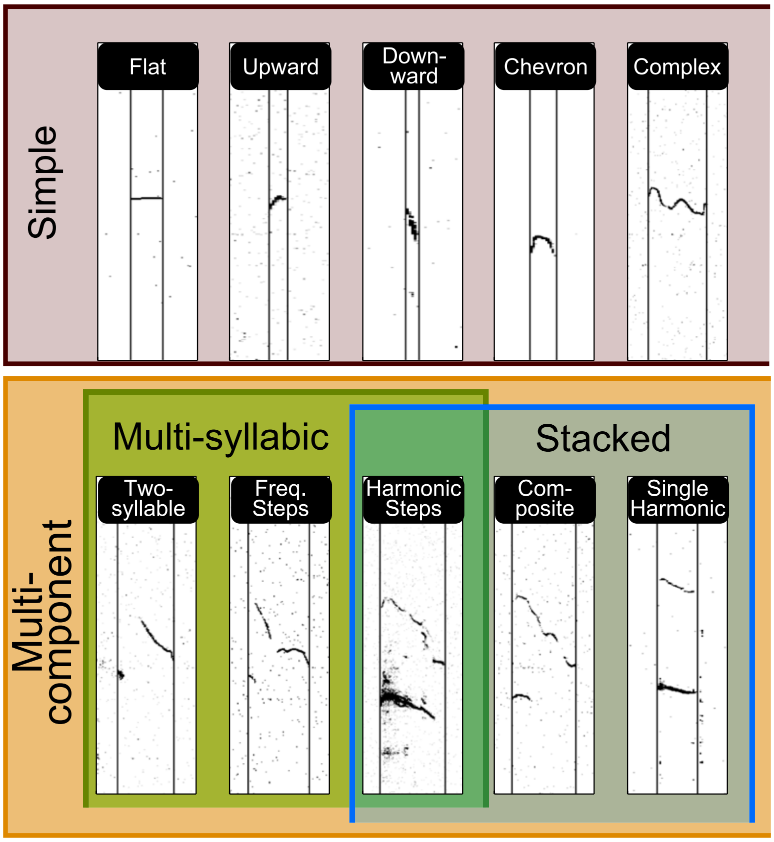

Fig. S1. Calls can be classified based on the number of components within a call as well as the relationship of these components to one another. Simple calls have a single component, whereas multicomponent calls consist of one or more components. Multicomponent calls can be further subdivided into multisyllabic calls, where components are separated by time, or stacked calls, where components are separated in frequency space. A particularly intricate call type, harmonic steps, contains both syllabic and stacked components.

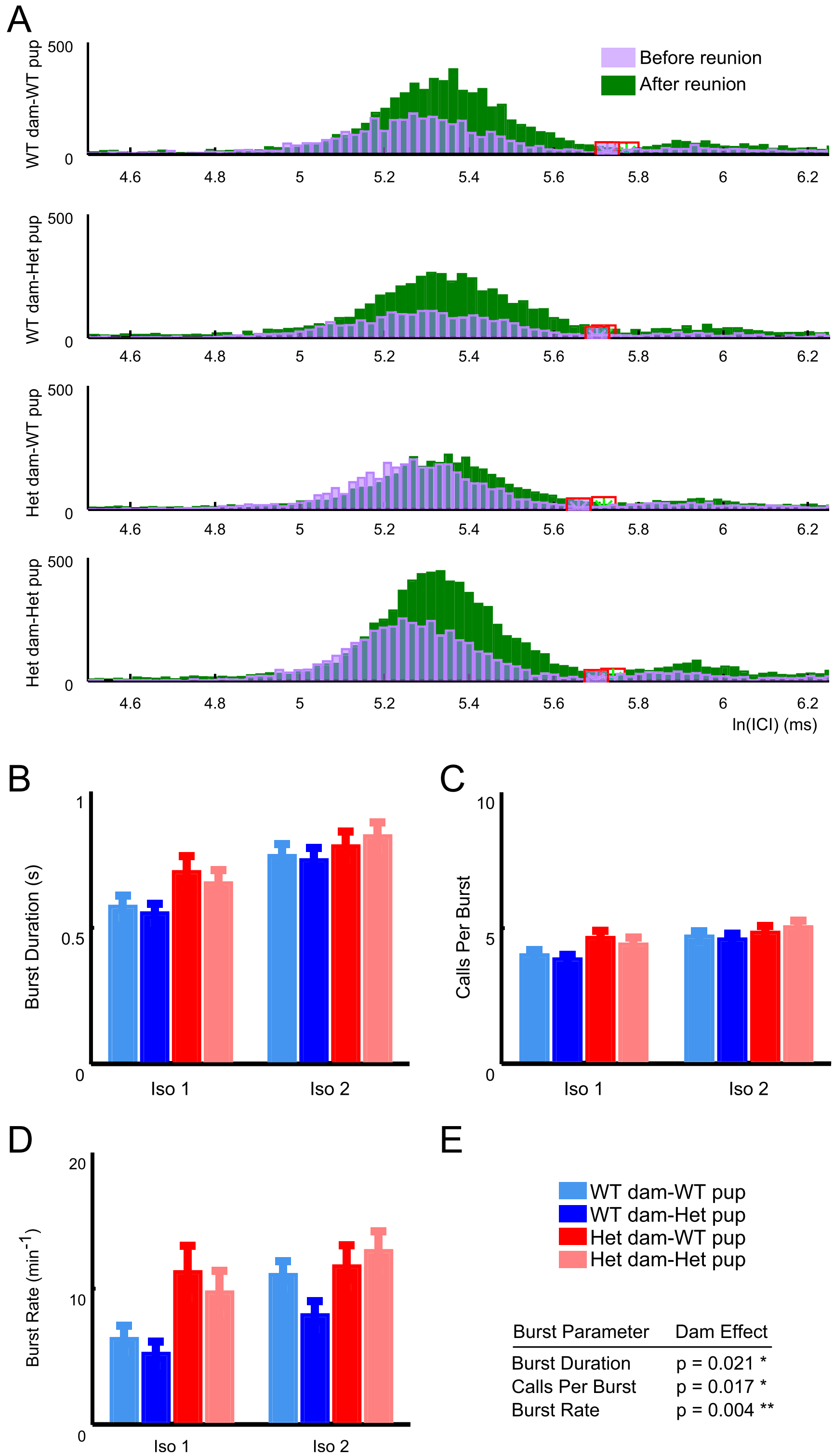

Fig. S2. Burst analysis revealed more intense burst parameters in pups from heterozygous dams. (*A*) Histograms of the natural log of the ICIs reveal a larger peak followed by a smaller peak. The larger peak corresponds to intraburst intervals, whereas the smaller peak corresponds to interburst intervals (the period between the last call of one burst and the first call of the next burst). Calls from isolation 2 (green) are overlaid on calls from isolation 1 (blue) to show potentiation in call numbers as well as shift in interburst interval in pups born to heterozygous dams. (*B–D*) Mean burst duration ($P = 0.021$) (*B*), calls per burst ($P = 0.017$) (*C*), and rate ($P = 0.004$) (*D*) increased following reunion and were significantly higher in pups born to heterozygous dams, mimicking individual call parameters. (*E*) Statistics summary shows significant dam main effects on burst parameters.

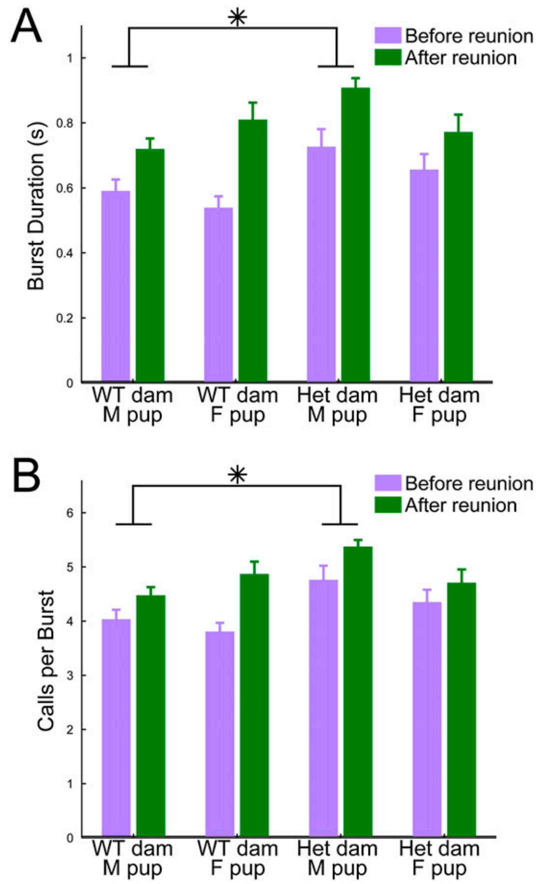

Fig. S3. Males from heterozygous dams were susceptible to changes in additional burst parameters. (*A* and *B*) In addition to higher burst rates, burst analysis revealed that male pups from heterozygous dams vocalized at longer burst durations ($P = 0.035$) (*A*) and higher calls per burst ($P = 0.022$) (*B*) than did males pups from WT dams.

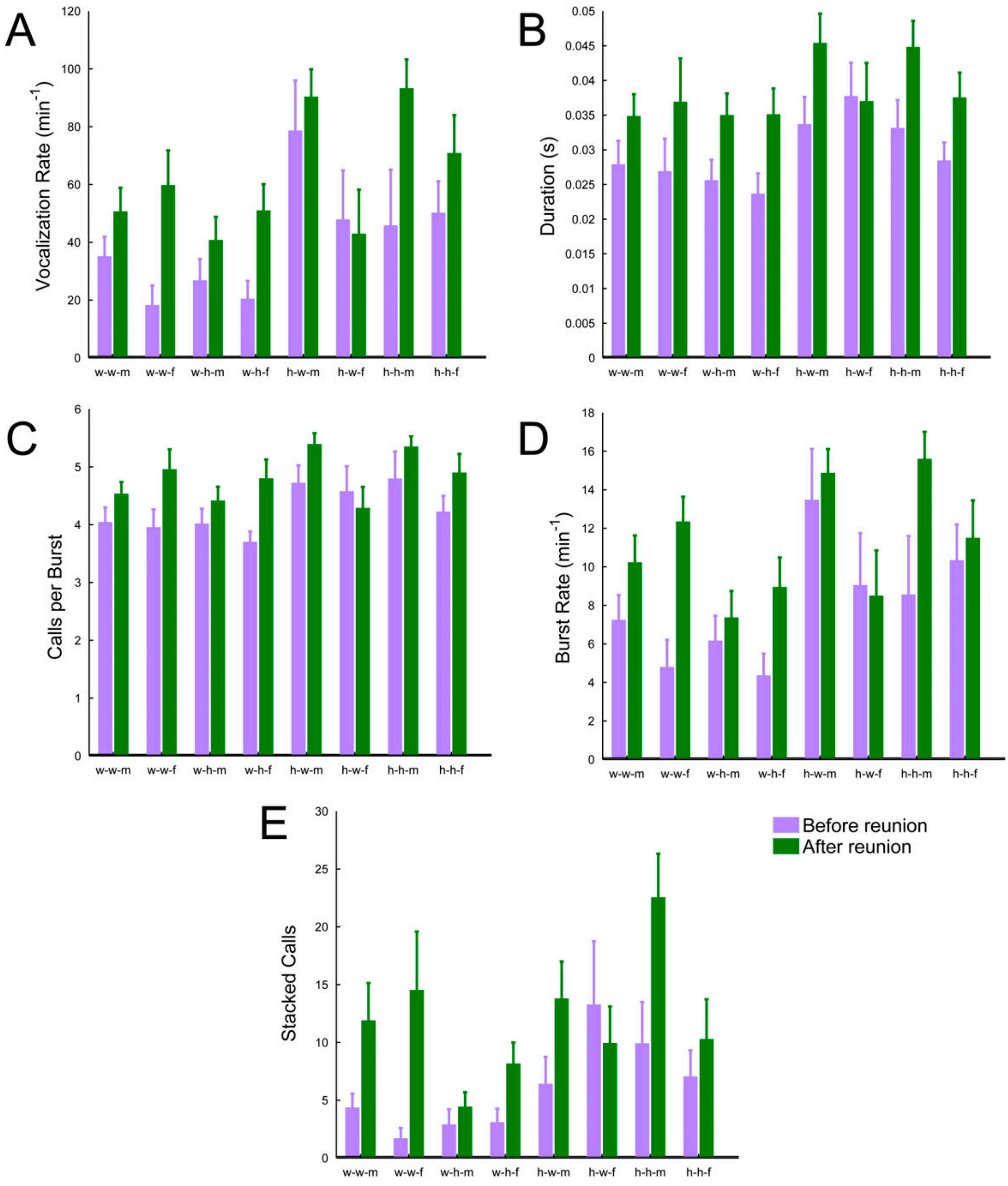

Fig. S4. WT females from heterozygous dams showed an unusual numerical decrease in a number of parameters following reunion. (*A*) When further split by pup gender, WT female pups from heterozygous dams were found to be the only pups that exhibited a numerical decrease in vocalization rate (*A*), call duration (*B*), calls per burst (*C*), burst rate (*D*), and stacked calls (*E*), while all other groups exhibited a numerical increase. In all graphs, the first letter refers to the dam genotype (w = WT, h = heterozygous); the second letter refers to the pup genotype; and the final letter refers to the pup gender (m = male, f = female). Note that the decreases depicted in these graphs are not statistically significant and are simply meant to highlight the female pups from heterozygous dams as the only group to undergo numerical decreases.

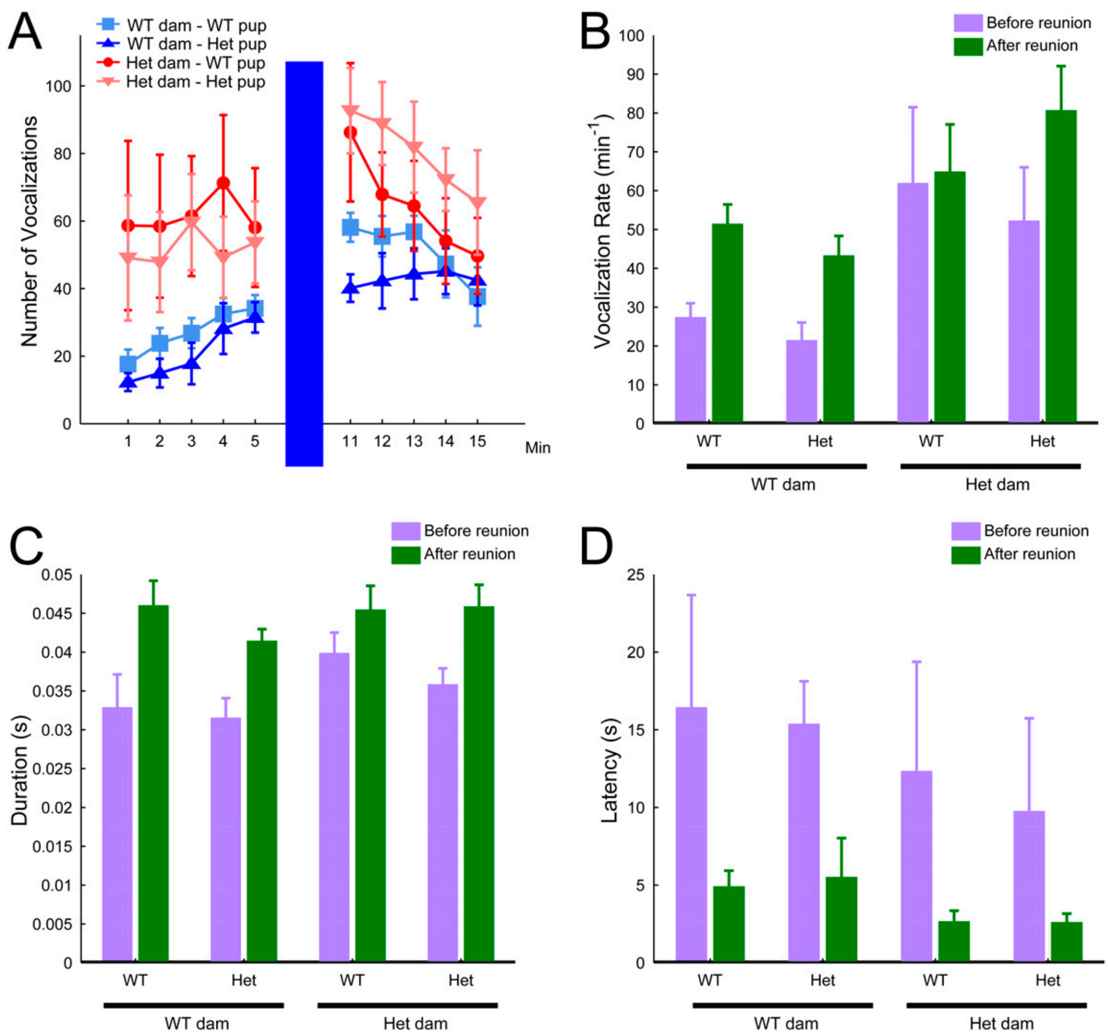

Fig. S5. USV maternal potentiation averaged across litters reveals no litter effects. (*A*) Comparison of call rate (*B*), mean duration (*C*), and latency (*D*) by litter and by individual pups (Fig. 2) reveal similar trends.

Movie S1. Representative video recording of a WT dam retrieving three of her pups from the corners of her cage back to the nest in 80 s. Note the retrieval failure on the third pup.

Movie S1

Movie S2. Representative heterozygous dam and sibling of the WT dam in Movie S1 retrieves three of her pups in 26 s. Note the absence of retrieval failures.

Movie S2