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Altered Ultrasonic Vocalizations in a Tuberous Sclerosis Mouse Model of Autism

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Altered Ultrasonic Vocalizations in a Tuberous Sclerosis Mouse Model of Autism

by

David Matthew Young

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Acknowledgments

No matter how my project directions shifted throughout these years as a graduate student, one thing remained consistent: the unwavering support of Lily and my labmates. I would like to gratefully acknowledge a few of the key players from the lab and life.

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Lily Y. Jan directed and supervised the research that forms the basis of the dissertation. Katrin Schenk contributed to the experimental design and data analysis. Shi-Bing Yang and Yuh Nung Jan provided advice on the design and analysis. All the experiments have been designed, implemented, and analyzed by David Young; this work is comparable with a standard thesis awarded by the University of California, San Francisco.

Lily Y. Jan

Abstract

Tuberous sclerosis (TSC) is an autosomal dominant neurocutaneous disease notable for its high co-morbidity with autism in human patients. Studies of mouse models of tuberous sclerosis have found defects in cognition and learning, but thus far have not uncovered deficits in social behaviors relevant to autism. To explore social communication and interaction in TSC2 heterozygous mice, we recorded ultrasonic vocalizations (USV) and found that although both wild-type (WT) and heterozygous pups born to WT dams showed similar call rates and patterns, baseline vocalization rates were elevated in pups born to heterozygous dams. Further analysis revealed several robust features of maternal potentiation in all but WT pups born to heterozygous dams. This lack of potentiation is suggestive of defects in mother-pup social interaction during or prior to the reunion period between WT pups and heterozygous dams. Intriguingly, male pups of both genotypes born to heterozygous dams showed particularly heightened call rates and burst patterns. Because our maternal retrieval experiments revealed that $TSC2^{+/-}$ dams exhibited improved defensive reactions against intruders and highly efficient pup retrieval performance, the alterations in their pups' USVs and maternal potentiation do not appear to result from poor maternal care. These findings suggest that a pup's interaction with its mother strongly influences its vocal communication, revealing an intriguing dependence of this social behavior on TSC2 gene dosage of both parties involved. Our study of this mouse model thus uncovers social abnormalities that arise from TSC haploinsufficiency and are suggestive of autism.

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Altered Ultrasonic Vocalizations in a Tuberous Sclerosis Mouse Model of Autism

Introduction

Autism afflicts roughly 0.2% of people worldwide, often for unknown reasons and almost always without definitive or curative treatment (1). Although many cases are of completely unknown etiology, certain environmental or genetic factors are thought to dramatically increase the risk for autism. Tuberous sclerosis (TS) is an autosomal dominant neurocutaneous disorder that exhibits a high co-morbidity with autism. Roughly 20% of tuberous sclerosis patients fall ill to autism, posing anywhere from a 50- to a 100-fold increased risk of autism compared with the normal population (2).

The mTOR-TSC Pathway

TS was first discovered by dermatologists in the 19th century based on the characteristic pattern of facial angiofibromas, which resembled potatoes, or tubers, thus coining the name tuberous sclerosis (3). These skin abnormalities were soon found to be only the tip of a much larger iceberg, a multi-system disorder that includes numerous but benign tumor-like nodules of the heart and brain as well as neuropsychiatric involvement, leading to a classic diagnostic triad of seizures, mental retardation, and facial angiofibromas (4). Although benign, tubers develop in the kidneys, with risk for catastrophic hemorrhage or polycystic kidney disease; in the heart, frequently leading to arrhythmias or obstruction; and in the eyes, increasing risk for calcification. Most notably, tubers appear in wide variety and with high prevalence in the brain and are thought to contribute to the telltale neuropsychiatric signs of TSC: epilepsy, mental retardation, and autism. However, the presence of brain tubers is not necessary for

neuropsychiatric involvement, and no clear correlation has been found between tuber placement and cognitive dysfunction, pointing instead to profound defects in the underlying molecular pathways and neural connectivity (2, 3, 5, 6).

The genetic etiology of TSC has been traced to two molecules that form a complex to regulate the mammalian target of rapamycin (mTOR) pathway, a major player in transcriptional and translational regulation (Appendix 1). Normally, mTOR phosphorylates critical regulators of protein synthesis and promotes translation of a wide variety of proteins involved in cell growth and proliferation, including neuronal proteins that play key roles in axon pathfinding and synaptic plasticity (7). Two proteins, TSC1 and TSC2, combine to form a complex known as the tuberous sclerosis complex, a GTPase activating protein (GAP) that negatively regulates mTOR (8). TSC1/2 keeps mTOR in check by inhibiting the small G protein Rheb (Ras homolog enriched in the brain) from activating mTOR (9). The complex, however, is prone to mutation, and mutations in either TSC1 or TSC2 inactivate the complex, resulting in unchecked cellular proliferation and, eventually, tuber or tumor formation (3).

The target of rapamycin (TOR) and its mammalian homologue (mTOR) are major integrators of nutrient and hormonal signals that generally lead to increased protein translation and cellular growth. Perturbations along the mTOR pathway are implicated in a number of human clinical disorders in addition to TS, ranging from familial cardiac hypertrophy to Huntington disease to VHL disease (10). The surprising recent discovery that mTOR downregulates the protein expression of Kv1.1 in rat hippocampal neurons has opened new doors to our understanding of the mTOR pathway as a regulator of

protein synthesis (11). The finding that mTOR not only promotes protein synthesis but also inhibits expression of the neuronal ion channel protein Kv1.1 have functional implications that help explain the diverse clinical phenotypes of mTOR pathway diseases (11).

As its name implies, mTOR was originally discovered through use of the drug rapamycin. Rapamycin is a naturally occurring antifungal metabolite produced by the bacteria *Streptomyces hygroscopicus* found natively in the island Rapa Nui. Rapamycin complexed with FK506-binding-protein 12 (FKBP12) was found to bind TOR family proteins at a conserved 100 amino acid stretch and to prevent them from promoting cell growth (12). TOR proteins have been found to exhibit differential sensitivity to rapamycin. Biochemical purification in yeast revealed two distinct complexes of TOR, TOR complex 1 and TOR complex 2 (TORC1 and TORC2, respectively, and their mammalian counterparts, mTORC1 and mTORC2). TORC1 is selectively sensitive to rapamycin and is also responsive to growth factors, whereas TORC2 plays a major role in cystoskeletal dynamics (13). First approved as an immunosuppressant to prevent renal allograft rejection by preventing division of T cells (12), rapamycin has increasingly entered the spotlight as an anticancer agent. Franz and colleagues administered oral doses of rapamycin to TS patients with subependymal giant cell astrocytoma brain tumors, with significant regression of tumor size after several months of treatment (14).

Although both TSC1 and TSC2 are necessary for a functional tuberous sclerosis complex, the two proteins are not equal in pathophysiology. TSC2 has been shown to be prone to a wider variety of mutations, leading to a higher prevalence of TS patients with

TSC2 mutations. Moreover, patients with mutations in TSC2 show a more severe phenotype than those with TSC1 mutations (3). The reason for the increased severity is unclear, as both proteins are necessary for a functioning complex, but it should be noted that TSC2 bears the GAP domain responsible for inhibiting Rheb and its downstream effector.

As an autosomal dominant disorder, TS is completely penetrant even though it displays a wide range of expressivity. An ongoing debate has been whether TS functions along the two-hit or loss-of-heterozygosity (LOH) hypothesis, which postulates that whereas patients carry a single mutation, many patients only begin to express clear clinical signs of TS after a "second hit," a mutation in the remaining allele in cells that then proliferate to generate tubers, or along the haploinsufficiency hypothesis, in which loss of just one copy is enough to develop TS. In support of LOH, loss of function in both copies of either TSC1 or TSC2 have been found in high percentages of human samples of kidney (angiomyolipomas and renal cell carcinomas) and heart (rhabdomyomas) lesions (15). By contrast, LOH has not been demonstrated in human cortical tubers and has been difficult to find in other brain malformations, such as subependymal giant cell astrocytomas (15, 16), and TSC happloinsufficiency was found to be sufficient for renal cyst and tumor formation in rodents (17, 18). Although TSC most visibly manifests itself in the formation of nodules and tubers in the brain, previous studies of acute disruption of TSC1/2 in neuronal cultures reveal marked enlargement in soma and dendritic spines. $TSC1^{C/+}$ heterozygous neurons have reduced spine density and neuronal morphological defects (19). The cellular and molecular defects evident in these models of TSC

heterozygosity may thus provide a pathophysiological basis for the clinical disease seen in heterozygous patients.

TS and Susceptibility to Autism

The greatly increased risk for autism in TS patients positions this disease as a superb candidate for understanding the genetic basis and pathophysiology of autism. Verification of mouse models bearing mutations in the tuberous sclerosis complex as models for autism could yield important insights into the molecular pathway defects rendering increased susceptibility to autism and provide a testbed for exploring therapeutic options (20).

In particular, mouse models of TSC2 have been used to model the more severe mutations of the human disease and the impact of TSC2 gene dosage on mental retardation, epilepsy, and signs and symptoms reminiscent of autism. Previous studies of $TSC2^{+/-}$ mice revealed hippocampal-dependent learning deficits and alterations in late-phase long-term potentiation (L-LTP) (21) as well as abnormal retinogeniculate projections and inhibited growth cone collapse suggestive of defects in axon guidance (22). Remarkably, these impairments were not accompanied by brain tubers or epilepsy, providing further evidence that perturbations in the underlying neural networks are responsible for behavioral phenotypic changes in $TSC2^{+/-}$ heterozygous mutant mice. Studies of these mouse models thus raise the possibility that subtle defects in synaptic plasticity and neural network connectivity underlie the mental retardation and autism seen in TS patients.

Although the establishment of an “autistic mouse” may be difficult because of

limitations in the mouse social apparatus, behavioral assays have been designed to detect in mutant mice features of the three domains of autism in humans: sociability, communication, and repetitive behavior (23-25). Sociability assays are based on the finding that wild-type (WT) mice preferentially spend time with other mice rather than with inanimate objects (26). One standard sociability assay is the 3-chambered social preference test, in which a subject mouse is placed in a central chamber and allowed to roam freely two separate chambers on either side, one of which contains another mouse, while the other side contains either no mouse or an already familiar mouse. Typically, WT mice prefer the side containing a mouse rather than the side with no mouse, or a novel mouse rather than a familiar mouse, whereas mouse models of autism often show a diminished or absent preference (23, 27).

In communication, defects are often seen in olfactory and ultrasonic vocalizations (USV). To test for communication by olfactory means, olfactory cues from soiled cage wipes are often presented to mice to evaluate the level of responsiveness to social odors (24). USVs are the key vocal method by which male mice conduct mating routines and mouse pups incite their moms upon separation (28, 29). Neonatal pups are typically tested for vocalizations by removing pups from their nest to induce cries, which are recorded using a USV microphone for measurement of call rates (20) In repetitive behavior, autism mouse models repeatedly groom themselves and often spend an abnormally high percentage of time in a holeboard assay poking their noses in a single hole rather than exploring a wide range of holes (30, 31). Other assays test for autism-related symptoms, of which anxiety has been particularly well-characterized. Standard

assays of anxiety include the elevated plus maze, in which mice must choose between the closed and open arms of a plus-shaped maze, and an open field assay. Open spaces are thought to provoke anxiety in mice, resulting in less time in the open than in the closed arm of the maze, whereas anxiolytics have been shown to increase the amount of time mice spend in the open arm (32).

The mouse model of TSC2 heterozygosity leading to cognitive impairments in learning and memory tests and L-LTP is consistent with the mental retardation evident in tuberous sclerosis patients, but no defects were found in several behavioral assays designed to identify behaviors consistent with autism (21). In the 3-chambered sociability test, both WT and $TSC2^{+/-}$ mice demonstrated a preference for time spent exploring another mouse rather than an empty cup, although numbers of entries into each side were not reported. No differences between genotype were detected in both the open field and elevated plus maze; both WT and $TSC2^{+/-}$ mice showed similar levels of anxiety based on time spent in open areas. The absence of social phenotypes in $TSC2^{+/-}$ mice could either imply the lack of such autistic features in this mouse model, or simply reflect limitations in detecting mouse behavioral abnormalities.

Ultrasonic Vocalizations As a Tool for Studying Autism in $TSC2^{+/-}$ Mice

Crucial to the diagnosis of autism are impairments in communication, including delays in verbal language and stereotyped, repetitive language patterns (1). Hidden from normal human detection, mouse communicative strategies include numerous olfactory cues as well as ultrasonic vocalizations with frequencies that reach up to almost 10-fold above the upper limit for human hearing. Ultrasonic communication has been well-

documented in rats and more recently in mice, first through the use of modified bat detectors for transposition of ultrasonic to audible frequencies and later through sophisticated microphones that could record ultrasonic frequencies for signal processing and analysis via spectrograms on computers (33, 34). Mice begin vocalizing shortly after birth, peak in vocalization rates around postnatal day 8 (P8), and continue vocalizing albeit at reduced rates throughout adulthood (35). Two of the most characterized vocalization patterns are courtship calls, in which male mice vocalize to potential female mates (36), and isolation-induced calls, in which mouse pups vocalize in response to separation from their dams (35). The communicative role of USVs in mice has been highly debated over the years, but the demonstration that courtship calls are equivalent to songs and the ability of pup calls to induce maternal retrievals show that USVs play a key role in mouse-to-mouse communication (37).

As a key mediator of communication starting at birth, USVs provide insight into mouse social behavior. Many potential mouse models of autism bearing mutations in autism susceptibility genes have been reported to exhibit deficits in USVs. FOXP2 (Forkhead box 2), OXT (oxytocin neuropeptide), OXTR (oxytocin neuropeptide receptor), RELN (reelin), 5-$HT_{1A/B}$ (serotonin receptor subtypes HTR1A/B), and NLGN4 (neuroligin 4) (20, 38) vocalize at reduced rates compared with WT mice as pups, while MECP2 (methyl-CpG-binding protein 2) mice, a model of Rett syndrome, actually vocalize at higher rates (39). Thus either higher or lower vocalization rates compared with that of WT mice may reflect communication impairments. Recent advances in computational analysis of vocalizations in BTBR mice, an inbred mouse strain and

mouse model of autism that also emitted higher vocalizations rates, enabled classification of calls into ten distinct call types and revealed an alteration in the distribution of call types in BTBR mice, as well as changes in mean durations of individual call (40).

A particularly robust social feature of USVs is a phenomenon known as maternal potentiation (35, 38), in which pups vocalize when separated from their dam, become quiet after reunion with their dam, and vocalize at even higher rates when re-isolated. This effect is independent of factors such as temperature or pup gender and specific to reunion with the dam rather than with virgin females, siblings, or home cage materials, suggesting that the potentiation is dependent on the social interaction taking place during reunion. Maternal potentiation was originally discovered in the rat and, with slight modification to the paradigm, found to be robust in the mouse as well (38). Mice lacking the μ opioid receptor (Orpm), thought to be a mediator of natural rewards and infant attachment behavior, displayed deficits in maternal potentiation (41), as do mice lacking fibroblast growth factor 17 (Fgf17), implicated in rodent frontal cortex development and neural patterning (42). Vasopressin 1b receptor (Avpr1b) has been shown to be a key player in social and affiliative behaviors, and while knock-out mice display normal baseline vocalization rates, they do not express maternal potentiation (43). Together, these mutant mouse lines strongly suggest that deficits in USVs serve as a behavioral biomarker for social communication impairments.

$TSC2^{+/-}$ mice have thus far failed to show any detectable deficits in sociability by standard assays of social behavior. However, here we show that changes in ultrasonic vocalizations of mouse pups upon separation from their mothers is dependent on the

genotypes of not only the pup, but the mom as well. Thus we reveal for the first time abnormalities in the social communication of $TSC2^{+/-}$ mice.

Materials and Methods

Animals. $TSC2^{+/-}$ mice that were generated as described previously (22) 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 one week after breeding pairs were set up, and tattooing on P3 by an AIMS tattoo machine, where 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 prior to 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 32-68%. 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-minute isolation period, vocalizations were recorded from an Avisoft UltraSoundGate CM16/CMPA microphone capable of accurately recording calls up to 180 kHz, digitized using a National Instruments PCI data acquisition board (PCI NIDAQ 6251) at a sampling rate of 366 kHz, and collected and written to disk using custom-built Matlab software. 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-minute reunion, that pup was re-isolated and recorded for an additional 5 minutes. 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 in Liu (2003) (44). 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 minute sound recording, two-second 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 [45kHz cut-off using a 512 pole Finite Impulse Response (FIR) filter]. After denoising we extracted calls by thresholding the sound file's amplitude envelope, calculated using a peak-tracing (with delay) algorithm (44). We observed that there were several call types that exhibited very short (< 40 ms) "gaps" between two or more frequency components. Following Liu (2003) 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 inter-

call-intervals were defined as the time between the end of one call and the start of the next. Inter-burst-intervals were determined by log transforming the distribution of inter-call-intervals and identifying the largest peak as within-burst intervals and the next smallest peak as inter-burst-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 set-up, 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. Calculate the spectrogram for a specific call and compare, 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, define the instantaneous frequencies as all frequencies where the relative loudness rose 3 standard deviations above the relative loudness of the mean noise for that recording. Then pool all of these frequencies over all time-slices for that call. Finally, define the median frequency as the median of the distribution of these pooled frequencies.

To classify calls, each call was outputted 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 6673 classified calls). The classification scheme employed is very similar to that proposed by Scattoni et al. (28), 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. For clarity, the "composite" call type by Scattoni et al. was renamed "harmonic" to emphasize the harmonic relationship of the two components, while "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, while "frequency steps" as used in this study are similar to two-syllable calls but with three or more syllables. To prevent confusion, "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. 9). All processing was conducted through custom-designed MATLAB 7.7 programs, and call data was stored in a SQLite 3 database and extracted through custom Python 2.5 scripts (Appendix 2). Calls were classified using custom built scoring software (On The Mark 1.0; Appendix 3).

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 picks 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 minutes 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 1.0 event scoring software.

Statistics. All effects are reported as significant at $p < 0.05$. Statistics were performed by PASW 18.0 (SPSS, Inc.) using general linear model (GLM) repeated measures analyses. Isolation periods were treated as within-subject effects, while 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, het pup; 13 female, 15 male), n =17 (het dam, het pup; 9 female, 8 male), n=25 (het dam, het pup; 17 female, 8 male); n=6 WT dam litters, n=5 heterozygous dam litters. 46,498 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 data set of these measures for each group. These fractions were then compared by GLM repeated measures analyses.

Results

Maternal Potentiation in TSC2$^{+/-}$ Pups. Pups were individually removed from the nest and placed directly in an isolation chamber, then returned directly to the nest before undergoing a second isolation period identical to the first (Fig. 1A). As expected, WT pups from WT dams underwent robustly potentiated vocalization responses following reunion with dam, with vocalization rates during the second isolation period double that of the first [$F(1, 92) = 13.95$, $p < 0.001$] (Fig. 1B). Similarly, heterozygous pups from both WT and heterozygous dams also potentiated [$F(1, 92) = 10.04$, $p = 0.002$ and $F(1, 92) = 16.34$, $p < 0.001$, respectively]. Only one group, WT pups born to heterozygous dams, failed to potentiate [$F(1, 92) = 0.11$, $p = 0.745$], with a correspondingly significant 3-way statistical interaction of the isolation period, dam genotype, and pup genotype [$F(1, 88) = 6.107$, $p = 0.015$]. Whereas heterozygous pups from heterozygous dams underwent the expected potentiation, all pups born to heterozygous dams exhibited dramatic increases (up to 2-fold more calls at baseline) in USV call rates both before and after reunion with their mothers. This increase yielded a significant main effect (the effect across all other independent variables) of dam genotype on call rate [$F(1, 88) = 13.899$, $p < 0.001$]. Thus the rate of pup vocalizations upon isolation varied with the genotype of the mother, whereas the extent of maternal potentiation seemed to be governed by the interaction between maternal and pup genotypes.

Maternal potentiation is usually quantified by call counts, typified by higher call rates in the re-isolation period. However, examination of additional parameters may

reveal other call features relevant to communication and maternal potentiation. To explore these features, we measured the impact of reunion on call duration, latency to call, and median sound frequency. Calls were segmented from noise by measuring and thresholding the intensity envelope. Call durations could then be precisely calculated from the identification of the start and end point of the segmented call.(Fig. 2A). Across all genotype groups, the mean call durations were found to be significantly higher in the second isolation period [$F(1, 86) = 40.344$, $p < 0.001$], indicating that potentiation is a matter not merely of increased call rate, but also increased average call duration (Fig. 2B). Although duration increased on average across all groups, there was a main effect of dam genotype, in which calls emitted by pups from heterozygous dams were longer on average [$F(1, 86) = 6.645$, $p = 0.012$]. WT pups from these heterozygous dams were also the only group that failed to significantly increase call duration during re-isolation. Thus the same group that did not undergo potentiation in terms of call rate also did not potentiate in call duration. Pups from heterozygous dams exhibited both higher call rates and longer call durations, meaning that these pups spent a longer total amount of time vocalizing than did pups born to WT dams.

Latency to call was measured as the time from the start of recording until the first call. Latencies decreased dramatically on average across all groups during the second isolation period [$F(1, 86) = 24.703$, $p < 0.001$], thus revealing yet another measure of maternal potentiation (Fig. 2C). No main effect of dam or pup genotype in latency was found; however, the WT pups from heterozygous dam were again notable as the only group that did not exhibit a decrease in call latency at a statistically significant level.

Changes in the pitch of crying babies have been shown to alter human adults' perception of distress (45). To assess individual sound frequencies and call type classification of pup USVs, we developed a technique for extracting calls by identifying instantaneous frequencies within a call throughout the ultrasonic range of 40-180 kHz This call extraction allowed us to accurately measure median frequencies even in calls with many different frequency components in the same time window. Median frequency probability density plots revealed a bimodal distribution of calls, with a population above and below 75 kHz. During the first isolation period, calls with median frequencies above 75kHz were predominant for pups born to both heterozygous and WT dams. However, during the second isolation period, pups born to heterozygous dams tended to equalize the number of calls with frequencies above and below 75kHz (Fig. 3). This selective increase in proportion of lower frequency calls by pups from heterozygous dams may convey a different level of pups' distress to their parents.

Increased Emission of Multi-Component Calls. Informed by Scattoni et al. (40), who classified individual pup calls into one of ten types, we categorized pup vocalizations based on properties of their sub-calls. At the broadest level, our categorization identifies simple calls, containing a single component, and multi-component calls. We further subdivided multi-component calls into multi-syllabic calls, where components are separated in time, and stacked calls, where components are separated in frequency space (Fig. 4A).

When we examined rates of simple calls alone, we saw no differences across isolation period or dam genotype [$F(1, 73)_{iso} = 0.831$, $p = 0.370$; $F(1, 73)_{dam} = 0.054$, $p =$

0.817], suggesting that any increase in calls mostly derived from an increase in multi-component calls (Fig. 4B). Indeed, the fraction of emitted multi-component calls increased (potentiated) after reunion in pups from WT dams [$F(1, 77)_{\text{WT dam/WT pup}} = 26.04$, $p < 0.001$; $F(1, 77)_{\text{WT dam/het pup}} = 6.28$, $p = 0.014$] (Fig. 4C). $TSC2^{+/-}$ pups from $TSC2^{+/-}$ dams also potentiated in terms of multi-component calls [$F(1, 77) = 12.14$, $p = 0.001$]. Again, WT pups from $TSC2^{+/-}$ dams were the only group that failed to potentiate in terms of this call type [$F(1, 77) = 1.39$, $p = 0.242$]. Interestingly, all pups born to heterozygous dams demonstrated a strong preferences for multi-component calls [$F(1, 73) = 7.751$, $p = 0.007$], indicating that the higher vocalization rate in pups born to heterozygous dams also results from a selective preference for multi-component calls.

USV Burst Patterns. Having seen changes in individual call parameters, we were curious whether TSC2 heterozygosity altered the temporal organization of calls. Inspection of inter-call-interval (ICI) distributions revealed two peaks: a larger peak at shorter ICIs corresponding to intra-burst calls, followed by a much smaller peak at longer ICIs that corresponded to inter-burst-intervals (Fig. 5A). To automatically detect bursts in the record of call times we set a burst threshold by finding the minimum of the ICI distribution between these two peaks. We were then able to measure mean burst durations as well as call counts per burst and burst rates. Similar to call potentiation, bursts showed potentiation (increases) in duration and burst rate as well as an increased number of calls per burst following reunion (Fig. 5B-D). Again, there was a main effect of dam genotype, where pups born to heterozygous dams emitted longer bursts at faster rates and with more calls per burst [$F(1, 73) = 5.526$, $p = 0.021$ for duration; $F(1, 73) = 8.737$, $p = 0.004$ for

rate; and $F(1, 73) = 5.913$, $p = 0.017$ for note counts].

Influence of Gender on USV Call Intensity. To examine possible gender-dependent vocalization differences in $TSC2^{+/-}$ mutant mice, all groups were split along dam genotype and pup gender (pooling across pup genotype), revealing a 3-way statistical interaction of isolation period, dam genotype, and pup gender for call rates [$F(1, 88) = 8.267$, $p = 0.005$] (Fig. 5A). Males from heterozygous dams were found to express particularly heightened rates of both individual calls as well as call bursts, regardless of pup genotype (Fig. 5B-D). This response is suggestive of heightened anxiety that is specific to male pups from heterozygous dams. Though not statistically different from other groups by post-hoc analysis, WT females from heterozygous dams were the only pups to actually decrease in absolute value of call rates, mean call duration, mean burst note count, and burst rate (Fig. 7). Thus pup vocalization patterns vary not only with dam genotype, but also with dam genotype combined with pup genotype or gender.

Measures of Maternal Care. Dam genotype exhibits a strong main effect on maternal potentiation in both call rates and call duration, raising the question of whether differences in maternal care during development, the reunion, or both impact pups' behavior. To further dissect the impact of maternal care on pup behavior, we measured latency to retrieve pups from a scattered nest (Fig. 8A). Both WT and $TSC2^{+/-}$ dams retrieved their first pup with no trouble and at equal latencies. Retrieval latency was similar for the second pup but differed dramatically during retrieval of the third and final pup (Fig. 8B). While WT dams often deposited pups away from the nest or even "de-retrieved" pups by taking them out of the nest, heterozygous dams methodically and

consistently retrieved and returned all three pups back to their nest ($p = 0.012$ for mis-retrievals; Fig. 8D). Overall, heterozygous dams responded to pups more quickly [$F(1, 16) = 10.094$, $p = 0.006$].

As another measure of maternal care, male intruders were placed into dams' otherwise undisturbed home cages. Dams typically defend their nest by sniffing and nipping at the intruder and spending time hovering over the nest. While both WT and heterozygous dams sniffed and bit intruders equally well ($p = 0.528$ for sniff duration, $p = 0.912$ for sniff count), heterozygous dams showed a trend toward increased duration above their nest and a significantly higher number of entries into their nest ($p = 0.003$; Fig. 8E-F). The increased number of nest entries as well as the shorter pup retrieval latencies suggest that heterozygous dams show greater attentiveness to caring for and protecting their pups.

Discussion

TSC2 heterozygosity has been shown to impact learning and memory, but previously no changes in social behavior have been reported. Here we show that TSC2 heterozygosity is sufficient to alter ultrasonic vocalizations in a behavioral assay of social interaction between mother and pup. Advances in computational analysis of these vocalizations have allowed us to explore a wide variety of parameters of maternal potentiation and show the impact of maternal genotype as well as the interaction of maternal and pup genotype on pup vocalizations. To explore possible effects of TSC2 mutation on dams' ability to care for their pups, we examined their reactions to an intruder and ability to retrieve pups, revealing an improvement rather than a deficit in maternal behavior.

USV recordings in $TSC2^{+/-}$ pups and their WT siblings from both $TSC2^{+/-}$ and WT dams show that TSC2 heterozygosity is sufficient to induce changes in social communication. Isolation-induced vocalizations have long been thought to serve a communicative role by eliciting search-and-retrieval behavior in lactating dams. Indeed, vocalizations produced from isolated speakers induce similar maternal retrieval behaviors as do isolated vocalizing pups (46). The baseline rate of isolation-induced vocalizations in pups may serve as an early biomarker of communicative function in mice, and a large number of mouse lines with mutations in autism candidate genes show changes in baseline vocalization rates (47). Although both WT and $TSC2^{+/-}$ pups born to WT dams expressed no difference in baseline rates compared with each other or C57BL/6J mice in other studies (40, 43), pups born to $TSC2^{+/-}$ dams showed baseline rates roughly 3-fold

that of their WT-dam counterparts. This dramatic increase in overall vocalizations may reflect a particularly heightened anxiety state, as vocalization rates are known to be susceptible to anxiolytic drugs, while anxiogenics increase call number and amplitude (48).

Although some autistic infants make unusually few sounds, others are characterized by atypical vocalizations and crying for protracted periods of time, humming, grunting, or squealing instead of calmly cooing or babbling (49). Consistent with this observation, several mouse models of autism with known vocalization defects express decreased vocalization rates, which may mimic the decrease in verbosity and social gestures in human autistic patients. Others, however, vocalize at higher than normal rates, which may correspond to an increase in anxiety or disturbances in the use of language. The MECP2 autism candidate mice, for example, emit over 2-fold more vocalizations compared to WT siblings (39), as does an autism mouse model of human 15q11-13 duplication (50). The BTBR inbred line and mouse model of autism simultaneously demonstrate an increase in baseline vocalizations and a restriction in vocalization call types, hinting at the stereotyped or idiosyncratic language patterns of autistic patients (40). The almost 2-fold increase in baseline vocalization rates we observed in pups born to $TSC2^{+/-}$ dams is similar to the increased rates in MECP2 and BTBR mice. Interestingly, there was no difference in baseline rates between WT and heterozygous pups born to heterozygous dam, suggesting that maternal genotype plays a dominant role in influencing baseline call rates in the TSC2 mouse line. Environmental stimuli are known to influence vocalization rates, including changes in handling, litter

size, and fostering (51, 52), and differences in maternal care might impact the environment in which pups are raised and subsequently influence their vocalization rates. For example, mice born to dams known for exceptional maternal responsiveness vocalize at reduced rates (53), and rat pups licked more frequently by their dams likewise vocalized less (54). As vocalization experiments are typically set up with solely heterozygous mating pairs or solely WT dams, it would be intriguing to compare baseline vocalization rates in these lines from WT, heterozygous, and homozygous mutant dam genotypes, when available.

While baseline vocalizations provide insight into the pups' immediate response to isolation, maternal potentiation highlights the influence of mother-pup interaction on vocalization as social communication. Baseline vocalizations have typically been interpreted as distress calls, susceptible to changes in temperature and other environmental cues, whereas maternal potentiation is robust in rats and mice, independent of temperature and specific to reunion with a familiar parent (43). During the reunion period, the pup may associate maternal retrieval as a reward for cries, and thus on a subsequent isolation, the pup cries out even more vehemently. Indeed, mice lacking the μ-opioid receptor gene, which has been shown to mediate reward pathways, are deficient in maternal potentiation (41), suggesting that potentiation is dependent on a learning process by which the pup realizes that its cries communicate a message to its dam (27). $TSC2^{+/-}$ mice were recently shown to exhibit cognitive and learning deficits in spatial memory tasks (21), which may also impinge on social learning and communication.

In our experiments, pups of both genotypes born to WT dams expressed robust

potentiation, indicating that TSC2 haploinsufficiency of the pup does not by itself preclude potentiation. Heterozygous pups born to heterozygous dams likewise expressed potentiation, and called at higher rates in both periods compared with their WT dam counterparts. One group, however, failed to undergo potentiation: WT pups born to heterozygous dams. Although pups born to heterozygous dams vocalized at similar rates at baseline regardless of pup genotype, WT pup vocalization rates did not rise during the second isolation period. This failure to potentiate could arise from a deficit in reward pathways in the pup, the reward itself as provided by the dam, or both—the mother and pup's mutual response to one another. An inborn deficit of the pup is an unlikely explanation because those pups that show no potentiation have no TSC2 mutation, and WT pups from WT dams potentiate normally. Maternal care per se also cannot account for our findings given the faster pup retrieval times and improved defense against resident intruders by $TSC2^{+/-}$ dams. It thus appears more likely that the USV phenotypes reflect differences in mother-pup interaction. In the theory of co-adaptation, offspring who can best solicit parental investment, and parents who best respond to those offspring, are most likely to survive and carry on the family lineage. Thus offspring solicitation and parental provisioning become genetically correlated—in other words, the offspring trait becomes co-adapted with the maternal response for the trait (55-57). Maternal potentiation may reflect a positive maternal provisioning to pups' vocal solicitation, in which the genetic correlation between $TSC2^{+/-}$ dams and $TSC2^{+/-}$ pups enhances the potentiation response, whereas the genetic dichotomy between $TSC2^{+/-}$ dams and WT pups diminishes the response.

Typically, maternal potentiation is measured as an increase in rates of call. In addition to rates of call, we found that maternal potentiation is also expressed as an increase in call duration and decrease in latency to call. Together, the increased call rate, increased duration of individual calls, and reduced latency to begin calling constitute a dramatic increase in total time of calling during maternal potentiation as well as a much earlier vocalization response. Intriguingly, the only genotype combination that did not increase in rate of call also failed to undergo a statistically significant alteration in mean call duration or latency to call. Thus these three parameters appear to correlate strongly with one another, and WT pups born to heterozygous dams appear to be robustly deficient in maternal potentiation by all criteria.

The pitch of infants' cries has been shown to alter adults' perception of distress. Esposito & Venuti (45, 58) showed that when cries were artificially manipulated to change their pitch, inter-call-interval, or speed, both higher and lower pitched cries as well as cries with shorter pauses increased the perception of distress in both parents and non-parents. Subjects who were later diagnosed to have autism emitted higher pitched cries than those subjects with typical or delayed development, and autistic children's cries elicited greater negative states in their listeners. When we examined the sound frequencies emitted by $TSC2^{+/-}$ mice, we found an altered distribution of high- versus low-frequency calls after reunion in pups born to heterozygous dams, which may correspond to the altered pitch of cries in autistic children. The altered pitch may have conveyed a heightened level of distress to the dams and contributed to the increased maternal response rates during retrievals and defense against intruders.

While total time of calling reflects the total quantity of calls, we also explored the organizational structure of USVs into call patterns and types. Burst analysis revealed that calls were not a series of isolated events, but rather clustered into discrete bouts. Consistent with the individual call parameters, the burst durations, note counts, and rates were all increased in pups born to heterozygous dams. The increase in call and burst durations as well as notes per burst during the second isolation may convey a different message from the pup to the dam, perhaps one of greater anxiety or urgency. It would be interesting to see whether speaker playback of preferentially longer calls or bursts solicits faster retrieval times by dams.

Preverbal autistic children have been shown to produce a higher proportion of atypical vocalizations, which include squeals, growls, and yells (59). The BTBR mouse model of autism likewise emits a higher proportion of multi-component calls. When we examined the numbers of simple and multi-component calls separately, we found that at baseline, pups born to WT dams emitted an approximately 2:1 ratio of simple calls to multi-component calls, suggesting that multi-component calls are "atypical" vocalizations. Both the increased calls during potentiation and the higher rates of calls from pups born to heterozygous dams are due to a selective increase in multi-component calls. Thus pups from heterozygous dams exhibit a higher proportion of multi-component or atypical vocalizations consistent with that seen in autistic infants, and the higher baseline calls may reflect a chronically potentiated, anxious state. Further acoustical analysis of infants' crying may identify prognosticators of language development and early biomarkers of communication impairments.

Autism shows a strong genetic preference for males, exhibiting a 4:1 ratio of male to female in the human population, although autism in TS is closer to 1:1 and has been reported to show no differences in behavioral features between genders (60). Males from heterozygous dams vocalized at much higher rates both at baseline and following reunion compared with WT dam groups at either period, both in individual calls and several burst parameters, suggestive of a particularly heightened anxiety in males and consistent with the higher incidence of autism seen in males. Independently of pup genotype, male gender thus appears to lend susceptibility to autistic-like features in pups from heterozygous dams. The differences seen here based on pup gender raise the question of whether—despite the report that autism in human TS patients are closer to parity between males and females—different genders may display features of autism in different ways. While the end diagnoses might be the same, it may be worthwhile to look for gender-dependent qualitative differences in expression of autism and other co-morbid behavioral disorders with TS to see whether this approach might help improve treatment plans for TS patients of different genders.

The possible maternal impacts on vocalization may be broadly classified into prenatal versus postnatal effects. Prenatal effects include possibilities as diverse as imprinting of genes inherited maternally, metabolic defects that affect gestational development, or immune responses to the pups during pregnancy (61). To explore the impact of postnatal effects, we assayed pup retrieval performance and maternal defense against a resident intruder as a measure of responsivity to both internal and external cues. Remarkably, $TSC2^{+/-}$ dams demonstrated striking improvements in pup retrieval

performance and entries to their nest. While WT dams more often failed to drop off their pups in the proper location or even withdrew pups from the protection of their nests, heterozygous dams completed each retrieval as quickly as the one before. Similarly, heterozygous dams paid extra attention to their pups in the presence of a male intruder by entering the nest more often than did their WT dam counterparts. These behaviors may be reminiscent of the repetitive, idiosyncratic behavior evident in autistic patients, who are often extremely focused on and perhaps extremely capable of performing a particular task.

Taken together, our findings reveal that TSC2 haploinsufficiency impinges on social communication in a manner dependent on the interaction of genotype of both dam and pup. Further experiments to dissect the molecular underpinnings of this complex relationship may identify new targets for intervention in TS patients, whether pharmaceutical or behavioral.

Figures

Figure 1. (a) Offspring from both wild type (WT) and heterozygous (het) dams (blue and red, respectively) were isolated, reunited, and re-isolated for 5 minutes per session. Although WT and heterozygous pups from WT dams did not differ significantly in call rate or temporal pattern, pups from heterozygous dams were more vocal than pups from WT dams. (b) All groups but WT pups born from heterozygous dams exhibited maternal potentiation. Note the overall increase in calls from the heterozygous dam group ($p < 0.001$), and a three-way statistical interaction among isolation period x dam genotype x pup genotype ($p = 0.015$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

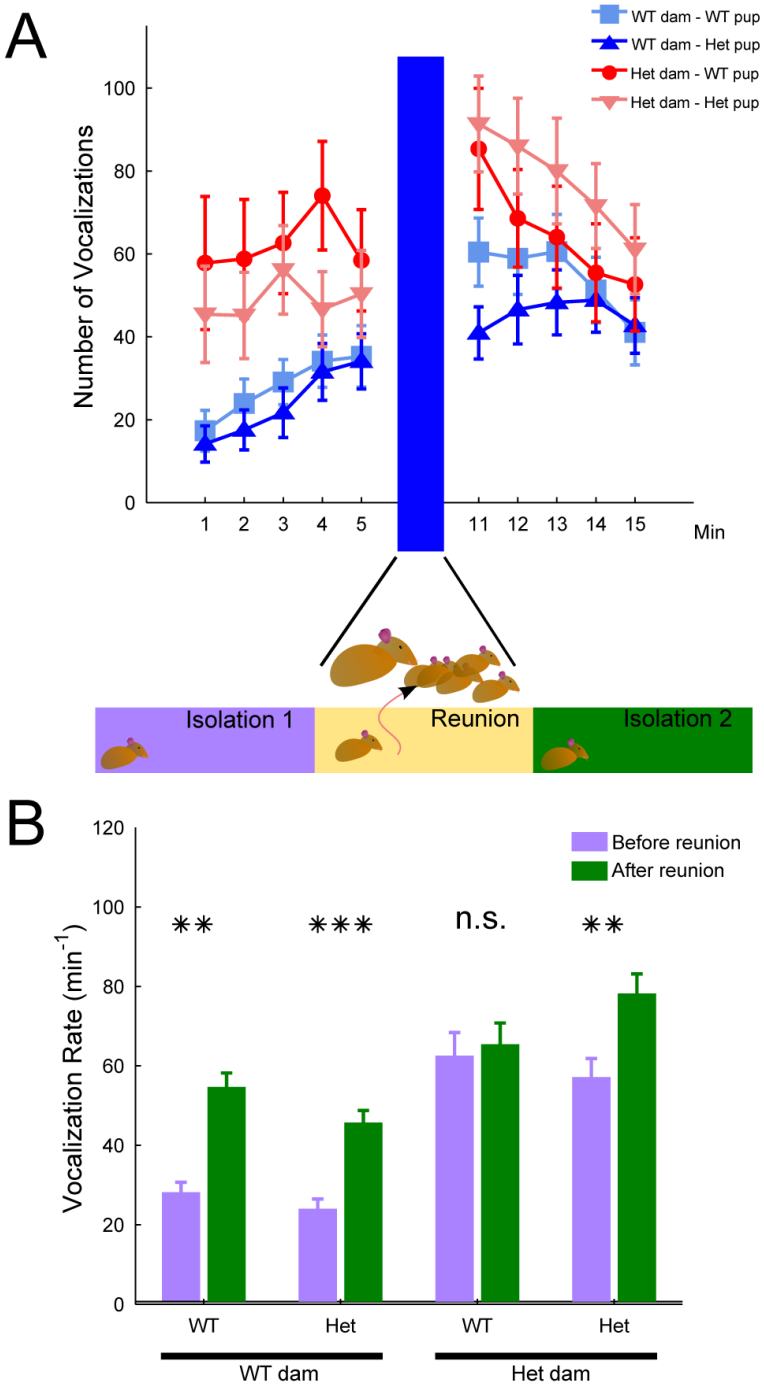

A
WT dam - WT pup
WT dam - Het pup
Het dam - WT pup
Het dam - Het pup
Number of Vocalizations
0
20
40
60
80
100
1
2
3
4
5
11
12
13
14
15
Min
Isolation 1
Reunion
Isolation 2
B
Before reunion
After reunion
Vocalization Rate (min-1)
0
20
40
60
80
100
120
**
***
n.s.
**
WT
Het
WT
Het
WT dam
Het dam

Figure 2. (a) Representative spectrogram of a multi-component call and its segmentation. The left panel shows the call with segment lines, calculated from the amplitude envelope shown in overlay. The extracted instantaneous frequencies are shown in the middle panel, with a histogram of frequency counts in the right panel. (b) Mean duration of each call rose on average from isolation 1 to isolation 2 ($p < 0.001$), and call durations were longer on average across isolation periods from pups born to heterozygous dams ($p=0.012$). One group—$TSC2^{+/-}$ dam, WT pup—stood out as the only group whose post-reunion calls were not statistically significantly longer in duration. (d) Call latency strongly decreased following reunion on average across all genotypes ($p<0.001$). Only the WT pup, heterozygous dam group did not statistically significantly decrease in latency, owing to shorter pre-reunion and longer post-reunion latencies.

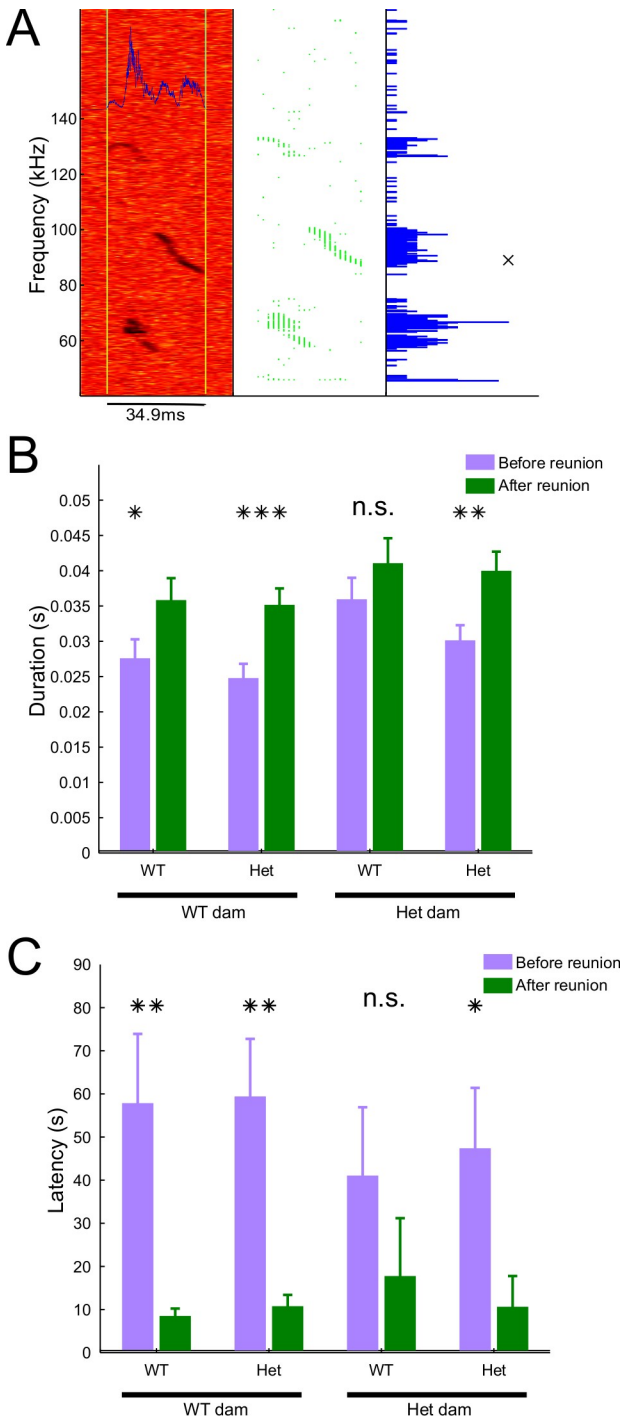

A
Frequency (kHz)
140
120
100
80
60
34.9ms
B
Before reunion
After reunion
Duration (s)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
*
***
n.s.
**
WT
Het
WT
Het
WT dam
Het dam
C
Before reunion
After reunion
Latency (s)
90
80
70
60
50
40
30
20
10
0
**
**
n.s.
*
WT
Het
WT
Het
WT dam
Het dam

Figure 3. (a) Probability density plots of median sound frequencies of individual calls overlaid from before and after reunion (purple and green, respectively) reveal a bimodal distribution of call frequencies. (b) The fraction of calls above and below 75 kHz became almost equal to one another in pups from heterozygous dams after reunion. These pups' fraction of high frequency calls was significantly lower than that of pups from WT dams across isolation period ($p < 0.001$).

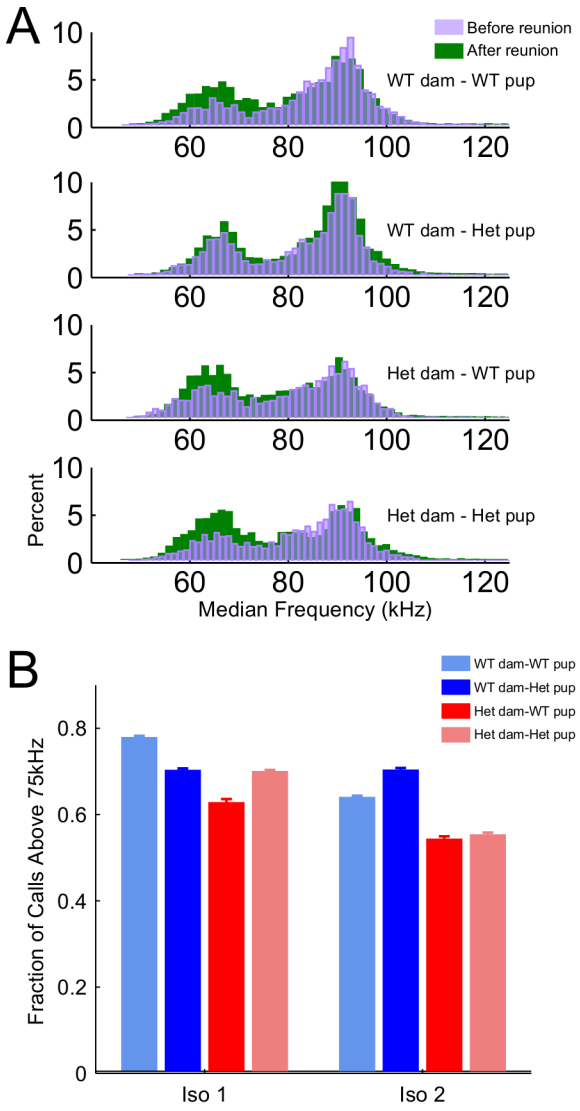

A
Before reunion
After reunion
WT dam - WT pup
WT dam - Het pup
Het dam - WT pup
Het dam - Het pup
Percent
Median Frequency (kHz)
B
WT dam-WT pup
WT dam-Het pup
Het dam-WT pup
Het dam-Het pup
Fraction of Calls Above 75kHz
Iso 1
Iso 2

Figure 4. (a) Calls can be classified based on the number of components within a call as well as the relationship of these components to one another. (b) There were no significant differences in rates of simple calls across isolation period or genotype group. (c) Multi-component call rates increased after reunion in both WT ($p < 0.001$) and $TSC2^{+/-}$ ($p = 0.014$) pups from WT dams as well as $TSC2^{+/-}$ pups from $TSC2^{+/-}$ dams ($p = 0.001$), but not from WT pups born to $TSC2^{+/-}$ dams ($p = 0.242$). Pups from heterozygous dam emitted a significantly higher fraction of multi-component calls across both periods ($p = 0.007$). (c) The higher rates of multi-component calls in pups from $TSC2^{+/-}$ dams is partially explained by the higher rates of multi-syllabic calls ($p = 0.012$ for dam effect). (d) The higher rates of multi-component calls is more predominant in the stacked subset of calls ($p = 0.001$ for dam effect).

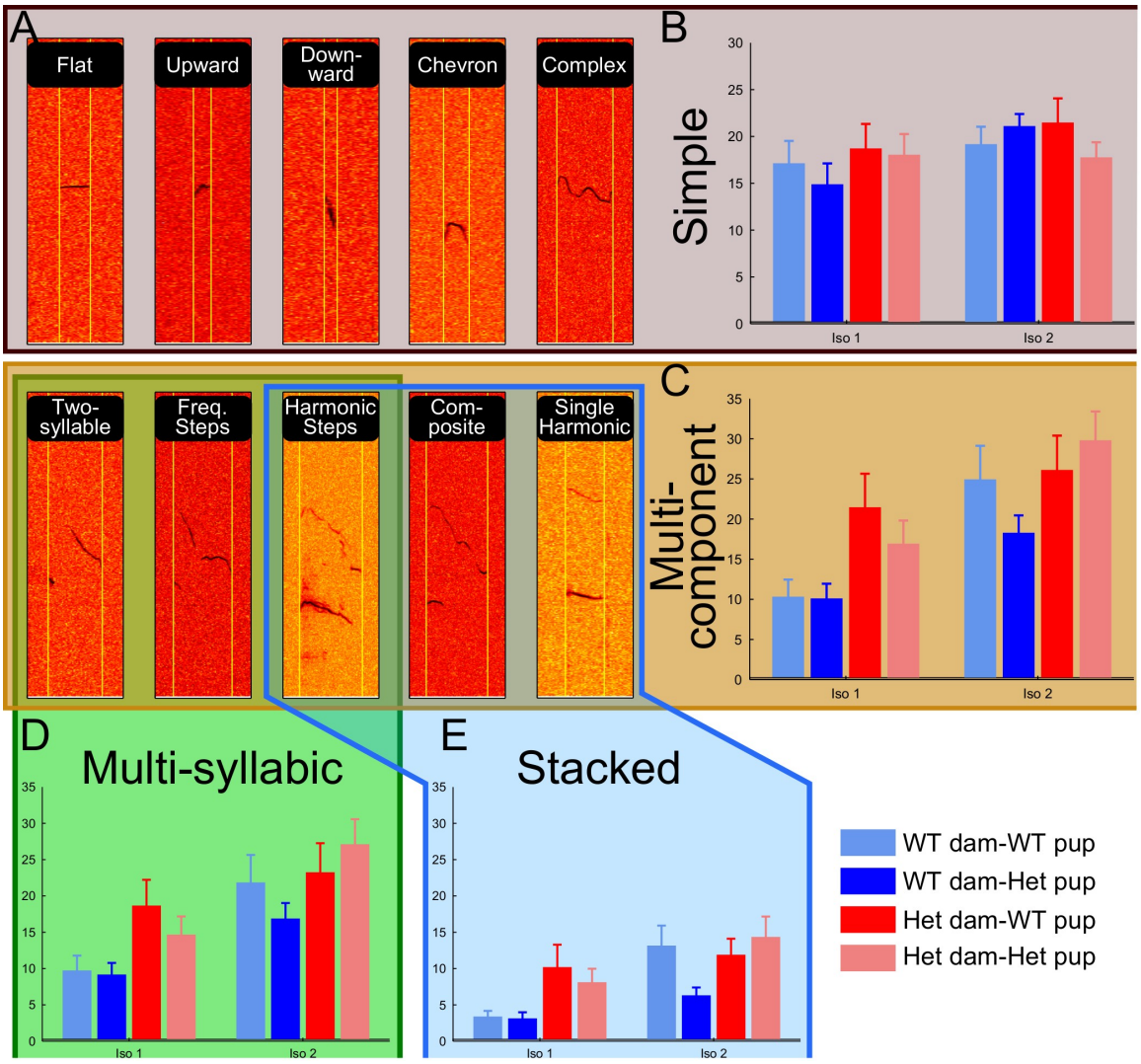

A
Flat
Upward
Down-
ward
Chevron
Complex
B
Simple
30
25
20
15
10
5
0
Iso 1
Iso 2
Two-
syllable
Freq.
Steps
Harmonic
Steps
Com-
posite
Single
Harmonic
C
Multi-
component
35
30
25
20
15
10
5
0
Iso 1
Iso 2
D
Multi-syllabic
35
30
25
20
15
10
5
0
Iso 1
Iso 2
E
Stacked
35
30
25
20
15
10
5
0
Iso 1
Iso 2
WT dam-WT pup
WT dam-Het pup
Het dam-WT pup
Het dam-Het pup

Figure 5. (a) Histograms of the natural log of the inter-call-intervals reveal a larger peak followed by a smaller peak. The larger peak corresponds to intra-burst-intervals, whereas the smaller peak corresponds to inter-burst-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 the potentiation in call numbers as well as the shift in inter-burst-interval in pups born to heterozygous dams. The asterisks marked by red boxes mark the calculated IBI. (b-d) The mean burst duration (b), note count (c), and rate (d) mimicked individual call parameters by increasing following reunion and showing significantly higher rates in pups born to heterozygous dams.

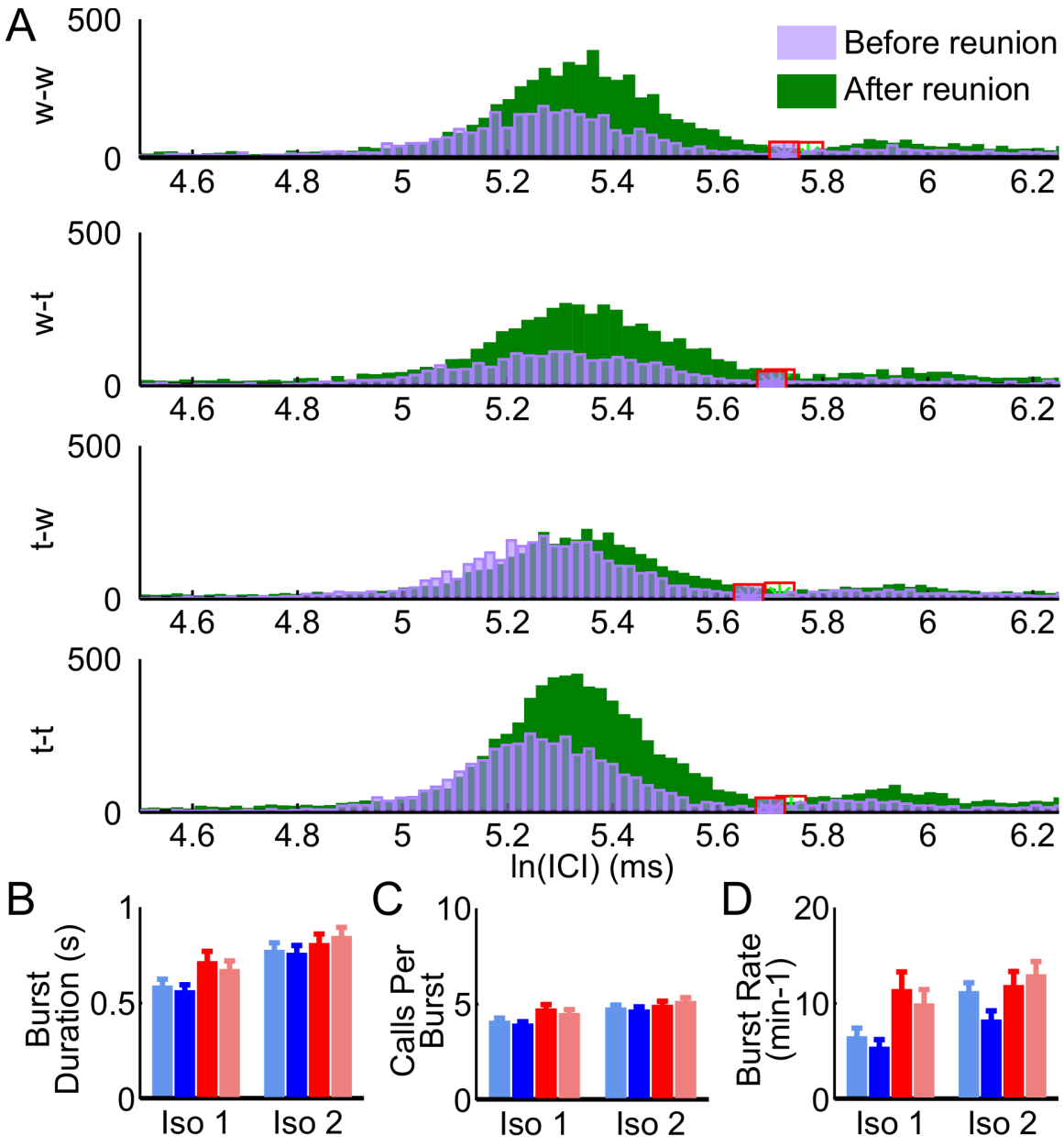

A
Before reunion
After reunion
w-w
w-t
t-w
t-t
500
0
4.6
4.8
5
5.2
5.4
5.6
5.8
6
6.2
ln(ICI) (ms)
B
Burst Duration (s)
1
0.5
0
Iso 1
Iso 2
C
Calls Per Burst
10
5
0
Iso 1
Iso 2
D
Burst Rate (min-1)
20
10
0
Iso 1
Iso 2

Figure 6. (a) Male pups born to heterozygous dams were found to vocalize at significantly higher rates than either male ($p = 0.002$) or female pups ($p = 0.003$) born to WT dams. (b-d) Burst analysis revealed that this same group, male pups from heterozygous dams, vocalized in longer burst duration (b), higher note counts (c), and higher rates than did males pups from WT dams ($p = 0.035$, $p = 0.022$, $p = 0.023$, respectively) and higher rates than did female pups from WT dams ($p = 0.021$) (d).

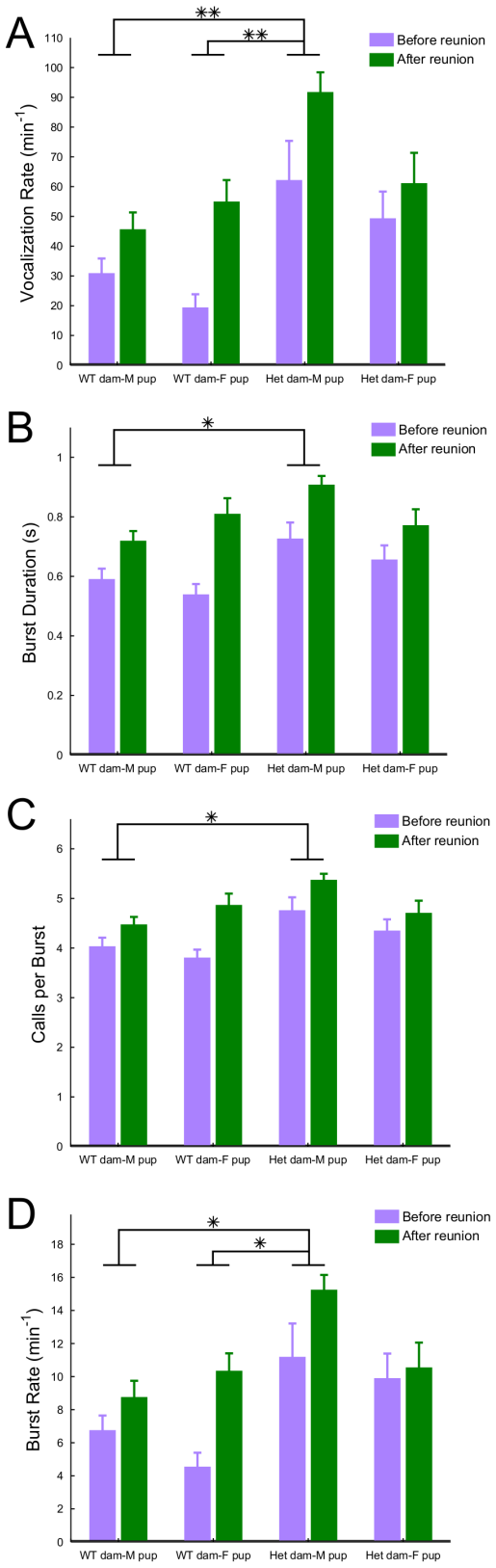

A
Vocalization Rate (min-1)
Before reunion
After reunion
WT dam-M pup
WT dam-F pup
Het dam-M pup
Het dam-F pup
B
Burst Duration (s)
C
Calls per Burst
D
Burst Rate (min-1)

Figure 7. (a) When split by pup gender, male and female WT pups from the $TSC2^{+/-}$ dam group showed vocalizations at opposite extremes, where males vocalized at an increased rate, and neither males nor females showed maternal potentiation. The interaction of iso x dam genotype x pup gender was significant ($p=0.005$), reflecting the particularly high rates of calls in heterozygous dam, male pup groups and lack of maternal potentiation in both male and female groups specific to this genotype group. (b) WT females born to heterozygous dams exhibited a number of other failures to potentiate (before reunion in blue, after reunion in green) when most other groups did potentiate, including mean call duration, where this group was the only group to undergo a decrease on average. (c) Similarly, this group was the only group to exhibit a decrease on average in mean burst note counts. (d) Again, the group was the only one to decrease in burst rate. (e) This group stands out for its high baseline fraction of stacked calls and negligible increase after reunion.

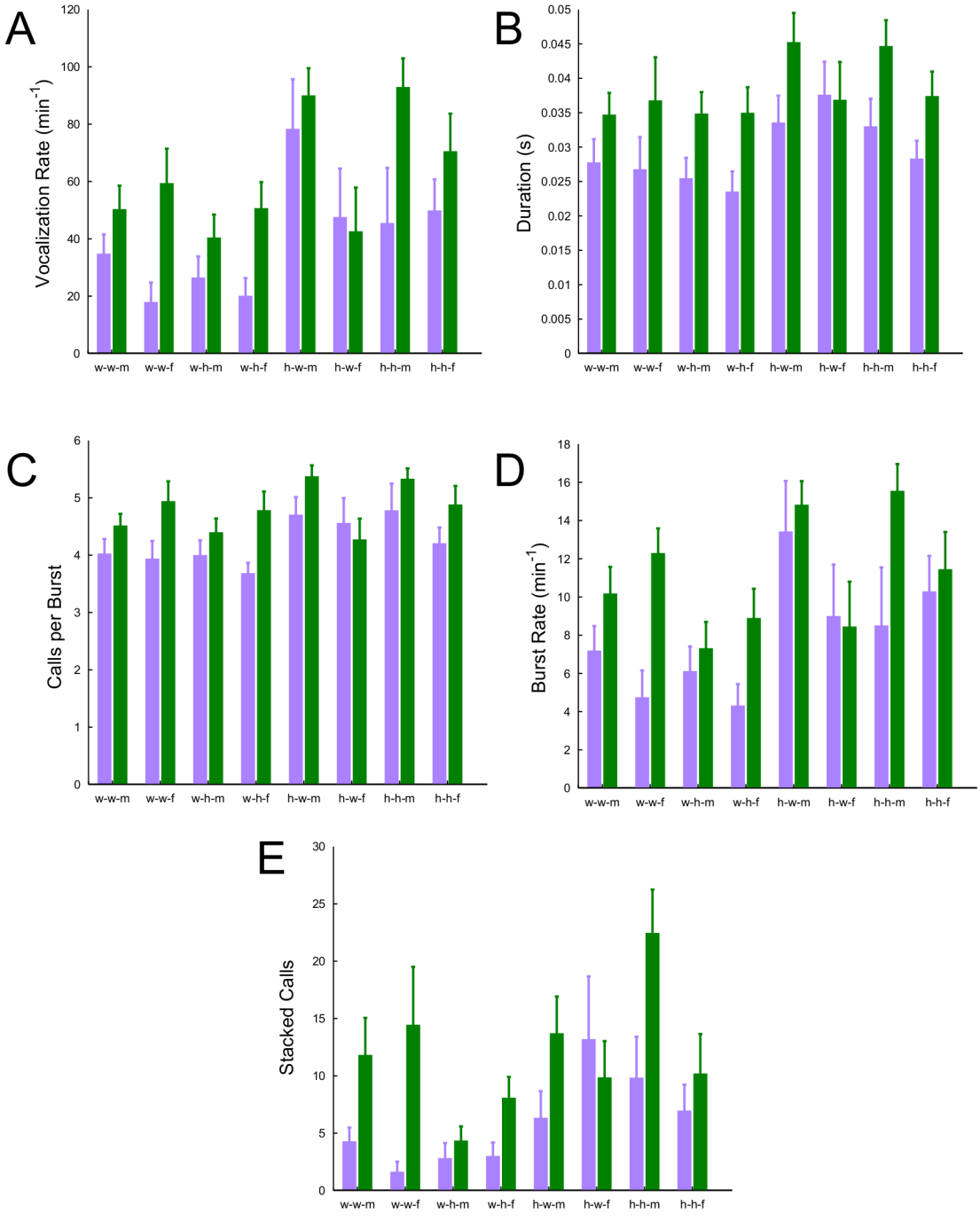

A
Vocalization Rate (min-1)
120
100
80
60
40
20
0
w-w-m
w-w-f
w-h-m
w-h-f
h-w-m
h-w-f
h-h-m
h-h-f
B
Duration (s)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
C
Calls per Burst
6
5
4
3
2
1
0
D
Burst Rate (min-1)
18
16
14
12
10
8
6
4
2
0
E
Stacked Calls
30
25
20
15
10
5
0

Figure 8. (a) Pup retrievals were measured by the latency to contact each pup situated in a corner of the home cage. (b) Heterozygous dams demonstrated more efficient retrieval scores, bringing all three pups back to the nest in less than half the time as WT dams ($p = 0.006$ for dam effect). (c) The total time for final collection of all pups back to the nest was significantly lower for $TSC2^{+/-}$ dams ($p = 0.011$). (d) The non-linearity of retrieval latencies by WT dams is partially explained by a significantly higher failure rate to deposit the pup in the nest or to keep the pup there ($p = 0.012$). (d) Resident intruder tests showed no significant difference in total duration of sniffs or attacks and time over the nest. (e) While numbers of sniffs or attacks did not differ between dam groups, heterozygous dams entered the nest significantly more often ($p = 0.003$).

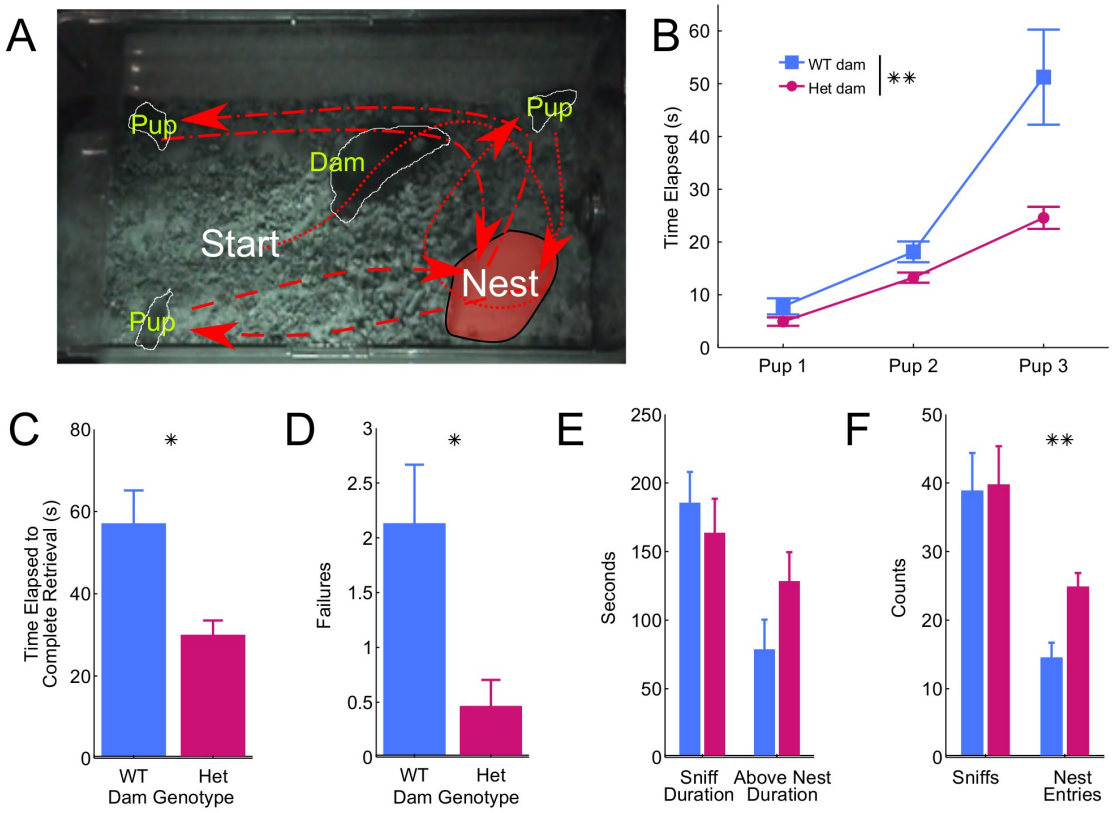
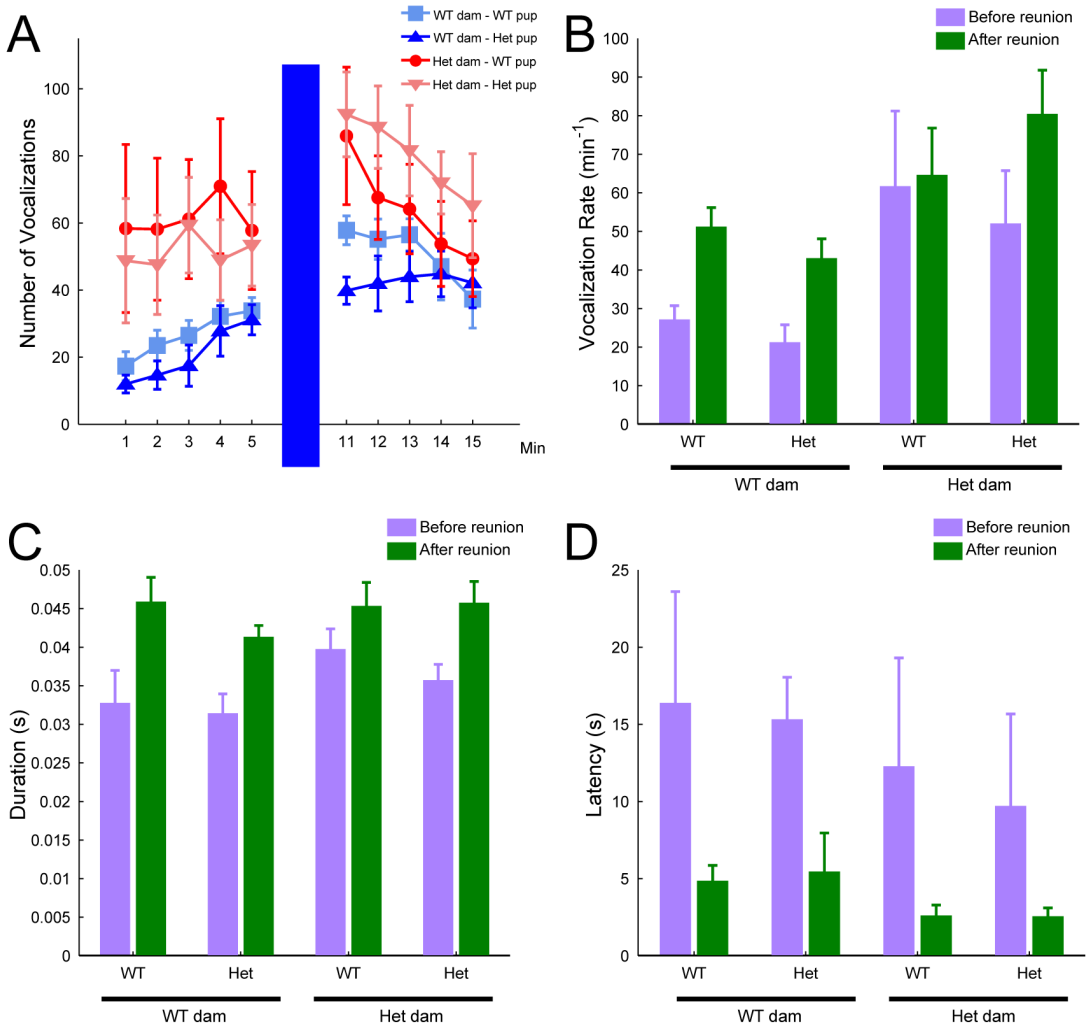

A
Pup
Pup
Dam
Start
Nest
Pup
B
WT dam
Het dam
**
Time Elapsed (s)
0
10
20
30
40
50
60
Pup 1
Pup 2
Pup 3
C
*
Time Elapsed to Complete Retrieval (s)
0
20
40
60
80
WT
Het
Dam Genotype
D
*
Failures
0
0.5
1
1.5
2
2.5
3
WT
Het
Dam Genotype
E
Seconds
0
50
100
150
200
250
Sniff Duration
Above Nest Duration
F
**
Counts
0
10
20
30
40
50
Sniffs
Nest Entries

Figure 9. (a) USV maternal potentiation averaged across litters reveals no litter effects. Comparison of call rate (b), mean duration (c), and latency (d) by litter and by individual pups (Fig. 2) reveal similar trends. Groups from left to right: het dam, het pups; het dam, WT pups; WT dam, het pups; WT dam, WT pups.


A
WT dam - WT pup
WT dam - Het pup
Het dam - WT pup
Het dam - Het pup
Number of Vocalizations
0
20
40
60
80
100
1
2
3
4
5
11
12
13
14
15
Min
B
Before reunion
After reunion
Vocalization Rate (min-1)
0
10
20
30
40
50
60
70
80
90
100
WT
Het
WT
Het
WT dam
Het dam
C
Before reunion
After reunion
Duration (s)
0
0.005
0.01
0.015
0.02
0.025
0.03
0.035
0.04
0.045
0.05
WT
Het
WT
Het
WT dam
Het dam
D
Before reunion
After reunion
Latency (s)
0
5
10
15
20
25
WT
Het
WT
Het
WT dam
Het dam

Appendices

Appendix 1: Molecular map of the TSC-mTOR pathway

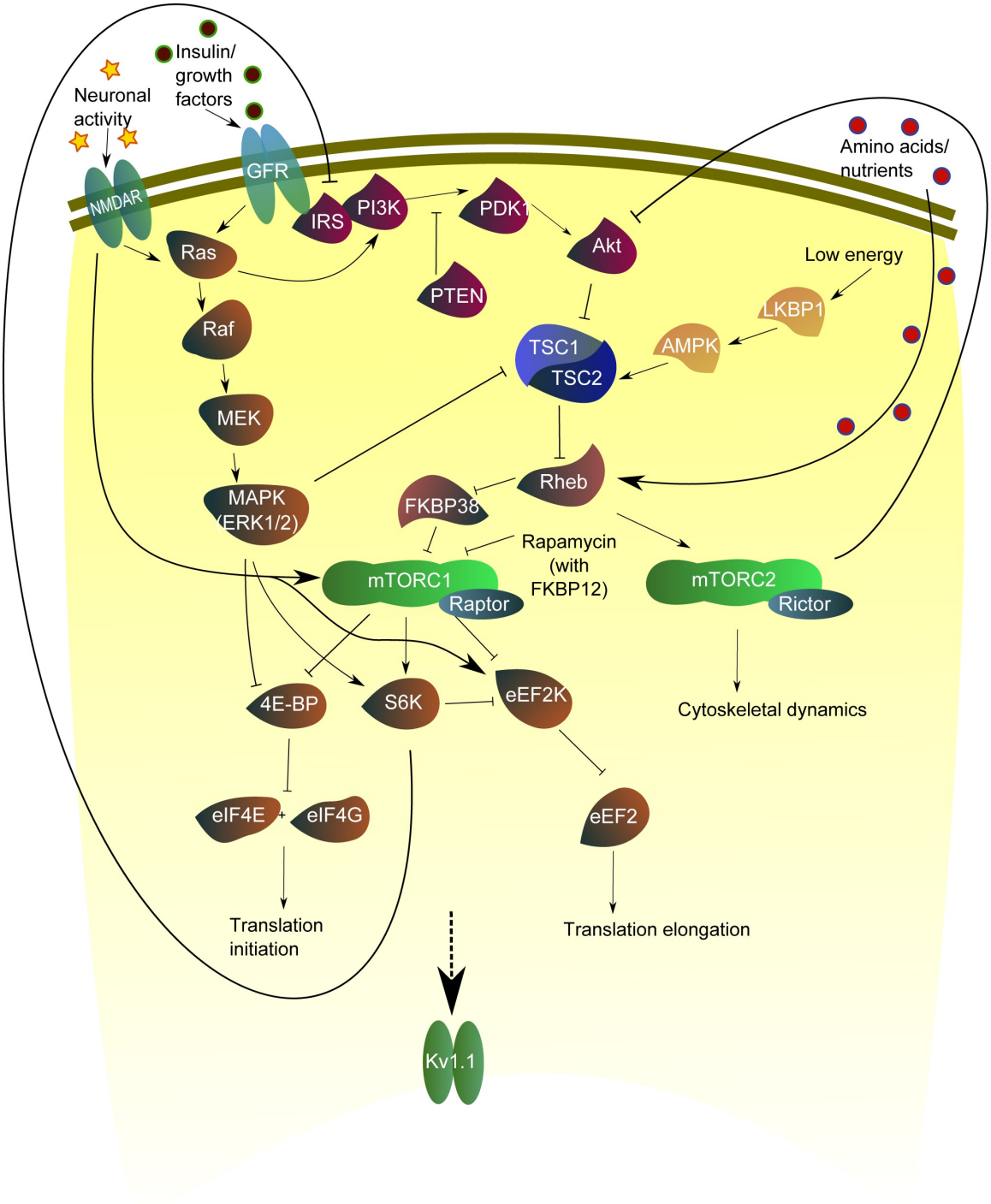
mTOR is at the crossroads of signaling pathways both upstream and downstream (Figure A1). Upstream of mTOR, insulin and growth factors bind receptors that activate the PI3K-Akt pathway. Akt inhibits the tuberous sclerosis complex, which consists of two molecules, hamartin (TSC1) and tuberin (TSC2), that assemble to form a complex that inhibits mTOR. TSC2 is a GTPase-activating protein (GAP) that targets Rheb, a small GTPase. When bound to GTP, Rheb binds and antagonizes FKBP38, an endogenous inhibitor of mTOR that binds mTOR at a region overlapping with the rapamycin-FKBP12 binding site. TSC prevents mTOR activation and cell growth by promoting hydrolysis of GTP in Rheb, thereby reducing its binding affinity for FKBP38 (9). Growth factors activate Akt, which inhibits TSC and permits mTOR-mediated cell growth. Also upstream of mTOR, AMPK acts as an energy sensor feeding into the TSC-mTOR pathway. Low energy stores increases the ratio of AMP to ATP, thus activating AMP kinase (AMPK), which in turn activates TSC to inhibit mTOR (62). Nutrients such as amino acids promote binding of Rheb to mTOR independently of TSC (63).

Downstream of mTORC1 are multiple pathways leading to cell growth through regulation of both protein transcription and translation. mTOR-mediated transcriptional regulation remains poorly understood but is thought to act through phosphorylation of unconventional prefoldin RPB5 interactor (URI), which interacts with all three RNA polymerases. mTOR also regulates rDNA transcription for ribosome biogenesis through the transcription factors UBF and TIF1A (8).

mTOR-mediated translational regulation is better understood and involves both

initiation and elongation of translation. eIF4E cooperates with the scaffold protein eIF4G to bind mRNA 5' caps and recruit 40S subunits to mRNA to initiate translation (63). eIF4E-binding proteins (4E-BPs) normally bind and prevent eIF4E from forming this initiation complex, but mTORC1 phosphorylates 4E-BP1 to prevent its binding with eIF4E (8, 64). mTOR and mitogen-activated protein kinase (MAPK) signaling pathways, both activated by NMDA receptors, merge and cooperate to activate eIF4E-mediated protein translation (64). mTORC1 also promotes translation elongation by activating eukaryotic elongation factor 2 (eEF2). eEF2 is normally phosphorylated and inhibited by eEF2 kinase, but mTORC1 phosphorylates at least three inhibitory sites on eEF2 kinase, freeing eEF2 to facilitate translocation of ribosomes from one codon to the next. mTORC1 also activates S6K1, an AGC family protein kinase. Phosphorylation at Thr389 in S6K1 by mTORC1 leads to activation of S6K1, which in turn phosphorylates the 40S ribosomal protein S6. S6 was thought to increase translation of 5' tract of oligopyrimidine (TOP) mRNA, but recent studies have shown 5' TOP mRNA translation to be independent of S6K or S6 phosphorylation. S6K does promote elongation through eEF2 by phosphorylating one of the inhibitory sites on eEF2 kinase (65, 63), but it remains unclear how else S6K controls translation (8).

Figure A1.


Insulin/
growth
factors
Neuronal
activity
GFR
NMDAR
IRS
PI3K
PDK1
Akt
Ras
PTEN
Amino acids/
nutrients
Low energy
LKBP1
AMPK
Raf
TSC1
TSC2
MEK
MAPK
(ERK1/2)
Rheb
FKBP38
Rapamycin
(with
FKBP12)
mTORC1
Raptor
mTORC2
Rictor
4E-BP
S6K
eEF2K
Cytoskeletal dynamics
eIF4E
eIF4G
eEF2
Translation
initiation
Translation elongation
Kv1.1

Appendix 2: Awe-Song, a vocalization analysis database

Mouse songs have many moving parts that can be difficult to keep track of. To organize dams, pups, and the many features of their calls, the Awe-Song vocalization analysis database was born. Awe-Song is built as a web application, with a browser-based graphical interface connected to a SQLite database back-end through Python scripts, AJAX, and Javascript technologies. Users can view database contents through a browser, selecting calls from individual pups or groups that match selected criteria, or extract database information directly to file through the command-line for further processing. Individual calls along as well as burst groupings were stored in the database, allowing for aggregation and comparison spanning a wide variety of data parameters. Both maternal potentiation and developmental recordings were accommodated in the database.

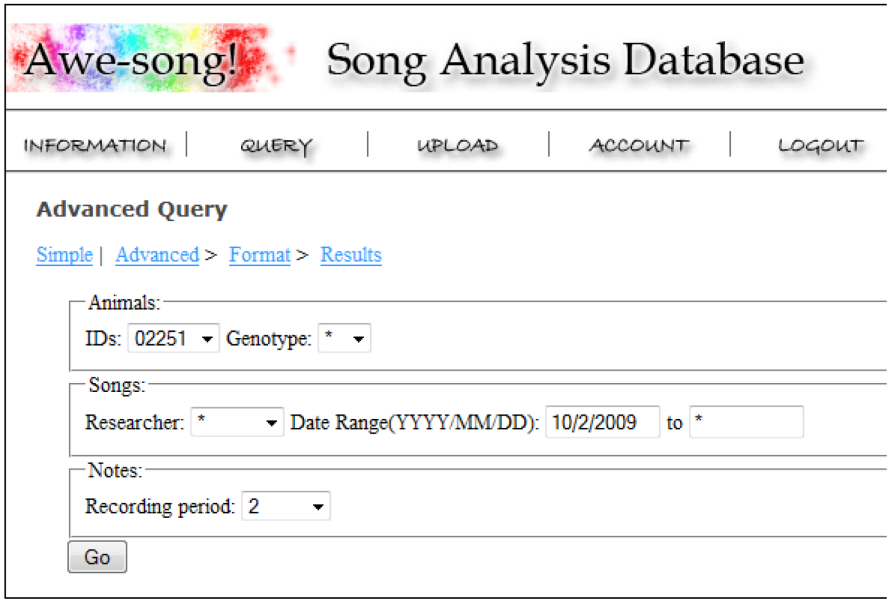
At the heart of Awe-Song is the SQLite database. The schema was initially designed during a bioinformatics course to fit both birdsong and mouse vocalizations and subsequently tailored to the current mouse experimental specifications. Animal data is stored for comparison across genotypes and to control for housing conditions, body weight, temperature, or other indicators of animal health and physiology. Vocalizations are organized by experiment, and each vocalization contains a number of parameters, such as call duration and latency. Sub-calls for each vocalization can also be stored in the database should the need arise.

The database can be accessed either by exporting the data to spreadsheets or through a browser. To aggregate vocalization parameters for statistical processing in

software packages such as PASW, command-line-based Python scripts were generated to access data, organize it into variables readable by PASW, and write to plain text file. For example, export scripts were used to convert data from individual calls into means per pup, including total calls for each minute based on call onset. Browser-based access allows the user to quickly check vocalizations for individual pups or those grouped by selected criteria, whether to ensure that the data was properly loaded into the database or as a way a way to double-check statistics (Figure A2). Although animal and experimental data, individual calls, and call bursts were all stored and processed separately, they were aggregated and reconnected for statistical analysis through Awe-Song.

Figure A2. (a) Vocalization data can be imported through the Upload tab or searched through the Query fields. The Simple Query option allows for quick access based on specific information such as the animal ID, while the Advanced Query allows for selection based on a number of additional criteria. In this example, only those vocalizations from isolation 2 of animal 02251 have been selected. (b) The findings from the query populate a table displayed under the results section. Currently, a default set of parameters are displayed, while further development will focus on allowing the user to customize the display of those parameters through the Format option.

A



B

d > Format > Results

animal_id	animal_genotype	dob	animal_sex	birth_dam_id	birth_dam_genotype	experiment	age	note_iso	note_onset	note_duration	note_median_freq	song_latency
02251	t	9/1/2009	F	01682	w	UV4(H)b	P10	2	7.169	0.00513	89.48864	7.16899
02251	t	9/1/2009	F	01682	w	UV4(H)b	P10	2	7.385	0.00612	91.2642	7.16899
02251	t	9/1/2009	F	01682	w	UV4(H)b	P10	2	8.1112	0.05003	88.77841	7.16899
02251	t	9/1/2009	F	01682	w	UV4(H)b	P10	2	8.3066	0.05401	88.06818	7.16899
02251	t	9/1/2009	F	01682	w	UV4(H)b	P10	2	8.5094	0.07438	89.13352	7.16899

Appendix 3: On The Mark custom-built event scoring software

To score videos and images, event scoring software such as Noldus Observer must often be used to track animal behavioral activities. Few if any open-source event scoring software exists. As an alternative to Noldus Observer, I developed On The Mark as an open-source, web-based tool for tracking mouse behavioral movements as well as classifying vocalization images. On The Mark is designed to be a user-friendly, cross-platform, freely available tool that can be used for behavioral analysis, scientific or otherwise. The software is written in the JavaFX language and runs via Java Web Start for launching through a web browser.

On The Mark in video mode displays the video alongside clickable scoring units (Figure A3-1A). The video can be expanded or contracted by adjusting the size of the application window, and playback is controlled through the various rate, volume, and position sliders as well as fast-forward and rewind buttons. Scoring units can be added or removed through the plus and minus bars, respectively. Each scoring unit contains a field for entering the name of the event and another field for the user-defined shortcut. During video playback, the user can press on the appropriate scoring unit to mark the start of the event, and then again to mark its end. Different events can overlap one another, such as a sniff that occurs while the animal is over the nest. Alternatively, the user can press the appropriate shortcut to control the scoring units. The arrow keys and page up/down buttons can also be used to fast-forward and reverse the video without leaving the keyboard.

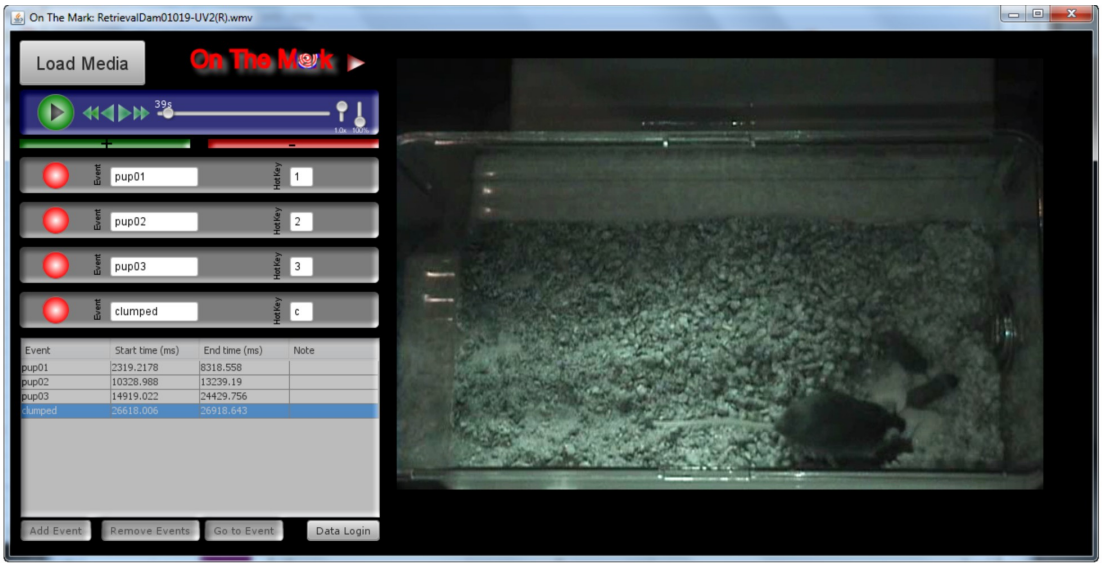
To score static images, On The Mark automatically switches to image mode whenever image media are loaded (Figure A3-1B). Images can either be loaded from a directory or through a .csv file that contains the names of the files to be scored, such as a listing of all vocalizations in the first bursts of each isolation period. During call classification, numbers were used as shortcuts to allow efficient scoring with one hand on the numeric keypad and the other hand on the arrow buttons.

On The Mark can be switched to compact view using the arrow in the upper right corner, in which the video fits above the playback and scoring controls (Figure A3-2A). This "vertical integration" allows multiple instances of On The Mark showing various videos (or even different positions of the same video) to be viewed and compared. Compact view works for both video and image modes and can be used anytime desktop space is at a premium.

Scored events are stored in a table underneath the scoring units. The entire set of events can be copy-and-pasted into a spreadsheet program or text editor through standard select all (ctrl-a), copy (ctrl-c), and paste (ctrl-p) functions. Alternatively, data can be exported directly to online Google spreadsheets through the Google Data API (Figure A3-2B). By plugging into Google spreadsheets, On The Mark can seamlessly transfer data "to the cloud" for ubiquitous access from any computer with connected to Google Docs. Data export also uploads scoring unit configuration data to the given spreadsheet. When the user re-opens the program, the user can select the exported worksheet to re-import all configuration content (Figure A3-2C).

Figure A3-1. (a) In video mode, On The Mark displays the video alongside playback controls and scoring units. Underneath the score units is the event table. (b) In image mode, On The Mark playback buttons and sliders double as image browsing controls. While scoring units act as on-off switches in video mode to record event durations, a single click on a scoring unit in image mode records the event.

A


On The Mark: RetrievalDam01019-UV2(R).wmv
Load Media
On The Mark
pup01
pup02
pup03
clumped
Event
Start time (ms)
End time (ms)
Note
Add Event
Remove Events
Go to Event
Data Login

B

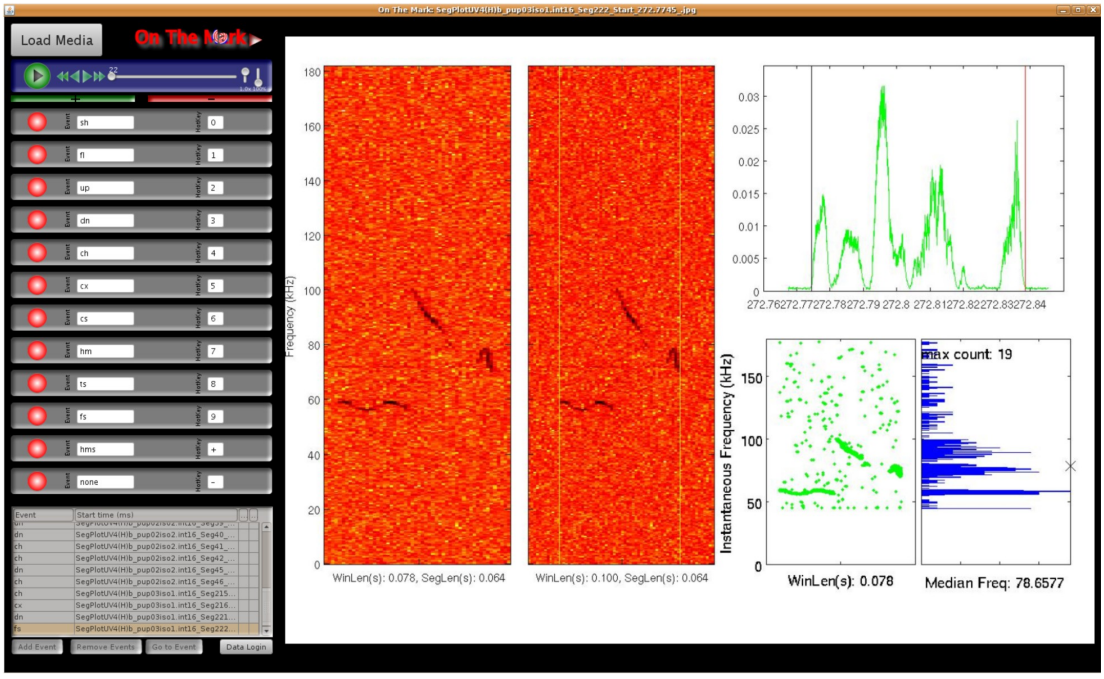
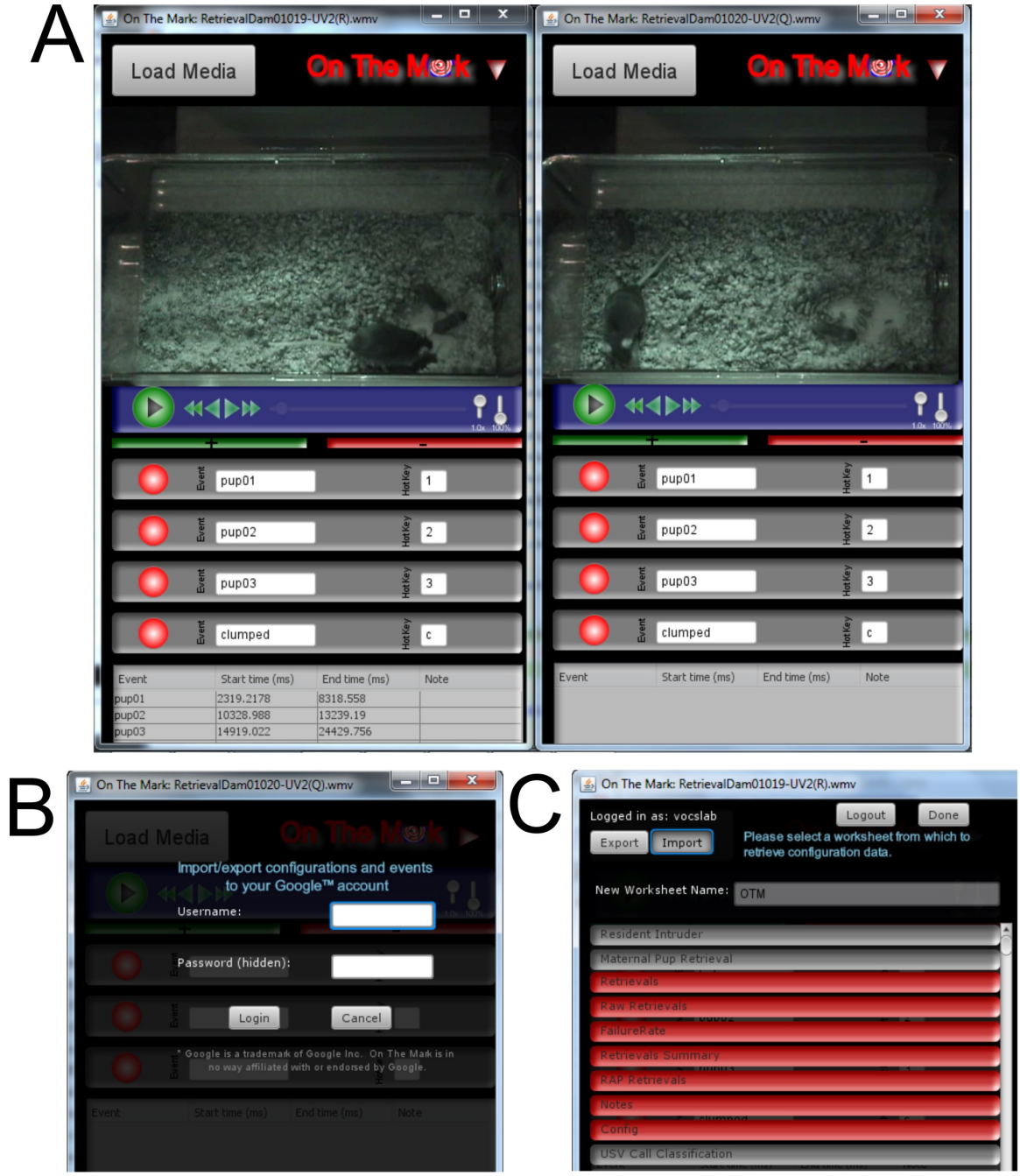

Load Media
On The Mark
Frequency (kHz)
WinLen(s): 0.078, SegLen(s): 0.064
WinLen(s): 0.100, SegLen(s): 0.064
Instantaneous Frequency (kHz)
max count: 19
WinLen(s): 0.078
Median Freq: 78.6577

Figure A3-2. (a) Compact mode allows for side-by-side viewing of multiple videos simultaneously. Controls and the event table remain accessible below the video. (b) Through the Google Data API, event and configuration data can be exported to online spreadsheets. (c) Rather than re-entering scoring unit configurations each time the program is launched, users can export configuration data into Google spreadsheets and then re-import them after the next program launch.


A
On The Mark: RetrievalDam01019-UV2(R).wmv
Load Media
On The Mark
pup01
pup02
pup03
clumped
Event
HotKey
Start time (ms)
End time (ms)
Note
pup01 2319.2178 8318.558
pup02 10328.988 13239.19
pup03 14919.022 24429.756
On The Mark: RetrievalDam01020-UV2(Q).wmv
B
Import/export configurations and events to your Google™ account
Username:
Password (hidden):
Login
Cancel
* Google is a trademark of Google Inc. On The Mark is in no way affiliated with or endorsed by Google.
C
Logged in as: vocslab
Logout
Done
Export
Import
Please select a worksheet from which to retrieve configuration data.
New Worksheet Name:
OTM
Resident Intruder
Maternal Pup Retrieval
Retrievals
Raw Retrievals
FailureRate
Retrievals Summary
RAP Retrievals
Notes
Config
USV Call Classification

Appendix 4: Spreadshift, a tool for aggregating online spreadsheet data

The typical experiment workflow involves recording pups to file while storing animal and experiment parameters in online spreadsheets, processing the recording files, integrating data from the online and processed files for importation into a database, and exporting complete data sets for analysis in statistical software packages. The experimenter can take advantage of the graphical interfaces provided by the online spreadsheet and On The Mark (Appendix 3) to enter data, while the analyst can use Awe-Song (Appendix 2) to retrieve complete data sets from the database. The connector to integrate the various data sources into the database is provided by Spreadshift, a tool for sorting, selecting, and shifting online spreadsheets.

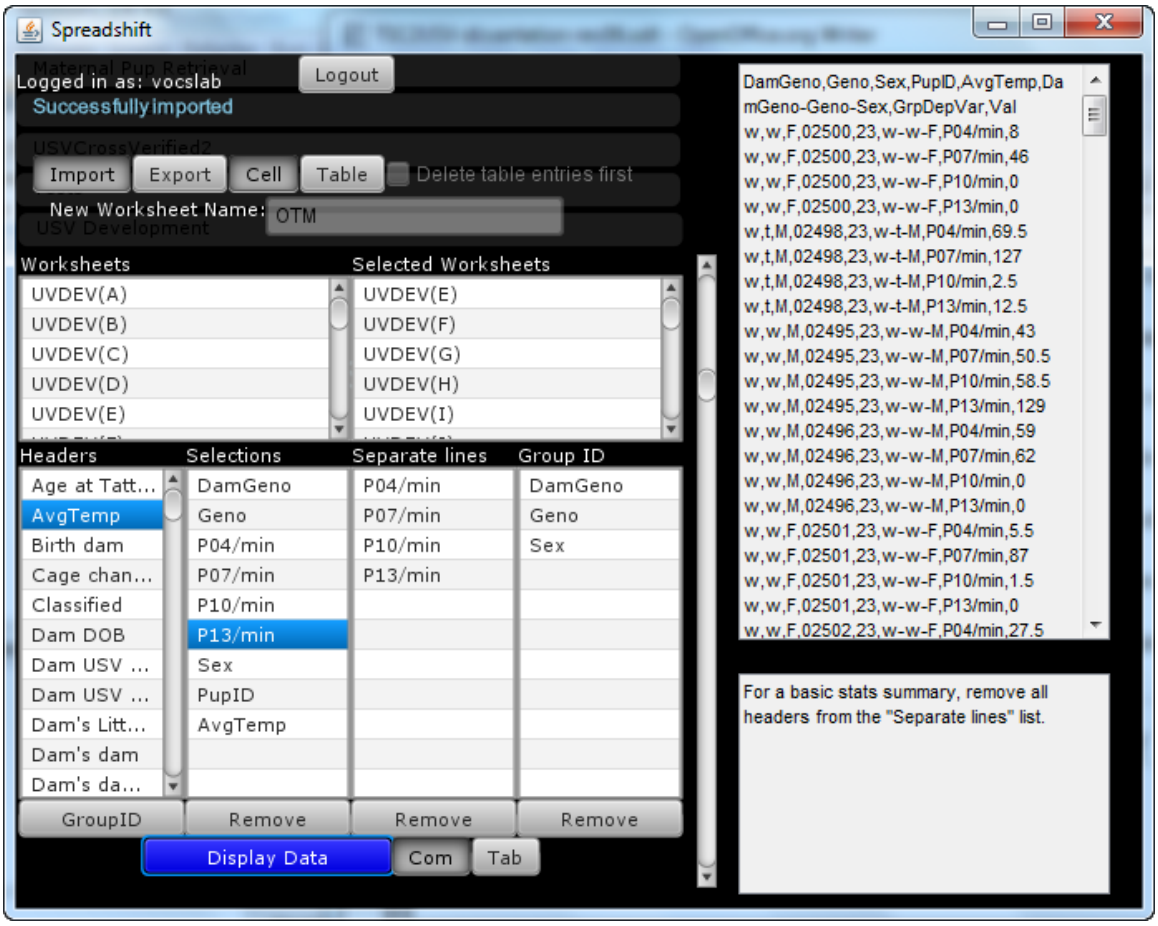
Online spreadsheets are often organized for ease of data entry, but their structure may preclude straightforward entry entry into a relational database. Typically, each experiment has its own spreadsheet, which is subdivided into a separate worksheet for each trial. Each worksheet consists of a set of columns corresponding to experimental parameters, while each row contains a separate animal. To aggregate the data into a database, select columns from each worksheet would need to be copy-and-pasted into a separate spreadsheet for importation, a potentially tedious and error-prone task. Alternatively, Spreadshift can be used to select the worksheets and columns for immediate aggregation (Figure A4). Selected data columns across all chosen worksheets are integrated into a single, new worksheet for import into a database and statistical

processing. New variables can also be computed from several current variables.

Statistical software packages often require data to be given as one row per subject, where all parameters for a given subject are stored in separate columns, or one row per parameter, where one column specifies the parameter, and another column holds the value for that parameter, depending on the type of analysis. In Spreadshift, data can be output in either format by specifying the parameters for grouping. For example, each pup can be given a separate line that includes separate columns for rate of calls during isolation 1, rate of calls during isolation 2, call duration during isolation 1, and call duration during isolation 2 for subsequent analysis by general linear models in PASW. For linear mixed models analysis, each pup can be given a separate line for each isolation period, with separate columns for the period number, the call rate for that period, and the call duration for that same period.

As with On The Mark, Spreadshift is cross-platform and open-source, meaning that it can run on Windows, MacOS, and Linux while also remaining free of charge and open for development. Spreadshift and On The Mark share a similar code base and are hosted together in the same open-source code repository.

Figure A4.


Spreadshift
Logged in as: vocslab
Logout
Successfully imported
Import
Export
Cell
Table
Delete table entries first
New Worksheet Name:
OTM
Worksheets
Selected Worksheets
UVDEV(A)
UVDEV(B)
UVDEV(C)
UVDEV(D)
UVDEV(E)
UVDEV(E)
UVDEV(F)
UVDEV(G)
UVDEV(H)
UVDEV(I)
Headers
Selections
Separate lines
Group ID
Age at Tatt...
AvgTemp
Birth dam
Cage chan...
Classified
Dam DOB
Dam USV ...
Dam USV ...
Dam's Litt...
Dam's dam
Dam's da...
DamGeno
Geno
P04/min
P07/min
P10/min
P13/min
Sex
PupID
AvgTemp
P04/min
P07/min
P10/min
P13/min
DamGeno
Geno
Sex
GroupID
Remove
Remove
Remove
Display Data
Com
Tab
DamGeno,Geno,Sex,PupID,AvgTemp,Da
mGeno-Geno-Sex,GrpDepVar,Val
w,w,F,02500,23,w-w-F,P04/min,8
w,w,F,02500,23,w-w-F,P07/min,46
w,w,F,02500,23,w-w-F,P10/min,0
w,w,F,02500,23,w-w-F,P13/min,0
w,t,M,02498,23,w-t-M,P04/min,69.5
w,t,M,02498,23,w-t-M,P07/min,127
w,t,M,02498,23,w-t-M,P10/min,2.5
w,t,M,02498,23,w-t-M,P13/min,12.5
w,w,M,02495,23,w-w-M,P04/min,43
w,w,M,02495,23,w-w-M,P07/min,50.5
w,w,M,02495,23,w-w-M,P10/min,58.5
w,w,M,02495,23,w-w-M,P13/min,129
w,w,M,02496,23,w-w-M,P04/min,59
w,w,M,02496,23,w-w-M,P07/min,62
w,w,M,02496,23,w-w-M,P10/min,0
w,w,M,02496,23,w-w-M,P13/min,0
w,w,F,02501,23,w-w-F,P04/min,5.5
w,w,F,02501,23,w-w-F,P07/min,87
w,w,F,02501,23,w-w-F,P10/min,1.5
w,w,F,02501,23,w-w-F,P13/min,0
w,w,F,02502,23,w-w-F,P04/min,27.5
For a basic stats summary, remove all headers from the "Separate lines" list.

Appendix 5: Serial time course of TSC2 vocalizations

Pups were recorded repeatedly on each of developmental days P04, P07, P10, and P13. During each recording, pups were isolated one-by-one for two minutes and returned to their home cage as soon as the recording was complete. Pups were picked in random order each day to reduce the possibility of order effects on vocalization rates. By employing the same tools for vocalization analysis as for the maternal potentiation experiments, we could compare the vocalization rates against known rates across development of C57BL6/J mice from other studies and determine when the vocalization abnormalities involving TSC2 heterozygosity became apparent.

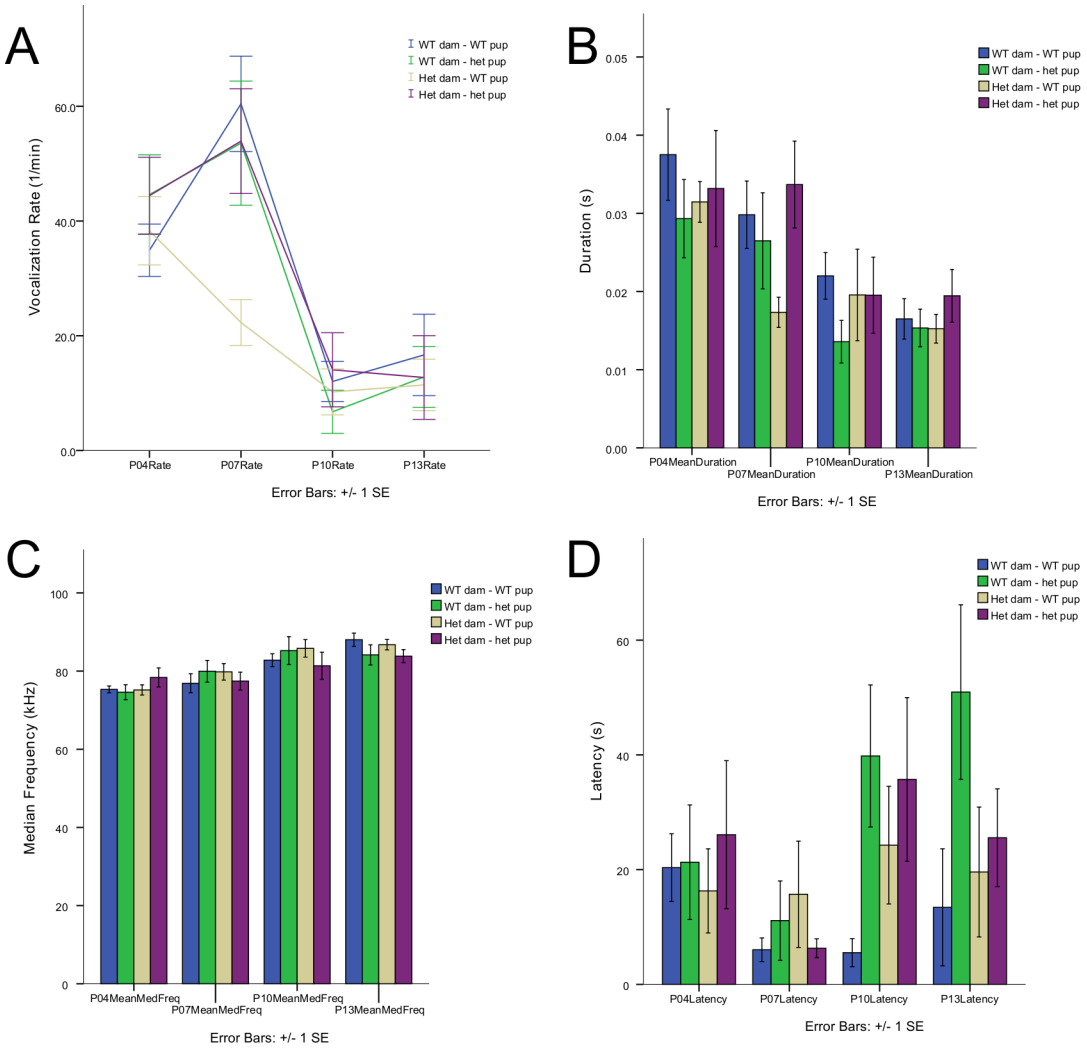
Developmental day had a strong overall effect on vocalization rate [$F(3, 80) = 29.705$, $p < 0.001$], consistent with serial recordings from C57BL6/J mice in a previous study (Figure A5A) (40). While rates were similar across genotype groups for most days, one group on one day stands out for a particularly low vocalization rate: WT pups born to heterozygous dams on P07. These pups vocalized at less than half the rate of the other groups and showed a significant simple effect of genotype group on that day [$F(3, 86) = 4.47$, $p = 0.006$ by multiple imputations because of missing values from pups that did not survive throughout recording days]. Thus the same group that failed to potentiate also failed to increase in call rate between successive developmental days of recording.

Development day also had a significant effect on duration of calls [$F(3, 80) = 22.262$, $p < 0.001$ by multiple imputations], revealing an overall decrease in duration during development or through habituation (Figure A5B). Again, the WT pups born to

heterozygous dams stand out for particularly short duration of calls on one specific day, P07 [$F(3, 86) = 3.58$, $p = 0.017$ by multiple imputations]. Thus the decreased call rate is accompanied by a shorter duration of calls, similar to the failure to potentiate accompanied by the failure to increase call duration for this same genotype group during potentiation experiments. Although median sound frequencies did not differ within developmental days across genotypes, the overall frequencies rose across developmental day [$F(3, 26) = 11.747$, $p < 0.001$] (Figure A5C). There were various significant statistical interactions among day, dam genotype, pup genotype, and pup gender, but no clear interpretation for individual genotype groups emerged. Latency to call showed complex trends in which latencies tended to decrease on P07, jump up on P10, and then, for all but one group, retreat again on P13. Developmental day had a significant effect on latencies [$F(3, 26) = 4.987$, $p = 0.007$], and various small statistical interactions emerged but without clear interpretation for particular genotype groups (Figure A5D).

The lack of dam effect on vocalization rate on the first day of recording suggests that the dam effect on baseline rates in the maternal potentiation experiments might not be seen until sometime between P04 and P10, the day when potentiation experiments were conducted. Although we did not see this effect on day P10 of these serial vocalization measurements, it is possible that the pups habituated to the isolation through the course of repeated recordings. It would be interesting to see the effect of developmental day on recording from separate batches of pups recording only once, on the given day of development.

Figure A5. (a) Vocalization rates across developmental days for each genotype group revealed a strong overall effect of developmental day ($p < 0.001$), with a peak in call rates for most groups at P07, followed by a decline by P10. WT pups born to heterozygous dams stood out as the only group to differ from this pattern, instead exhibiting a significantly depressed call rate on day P07 ($p = 0.006$). (b) Mean duration of calls likewise showed an effect of developmental day ($p < 0.001$). WT pups from heterozygous dams underwent a particularly strong depression in call duration, again on day P07 ($p = 0.017$). (c) Although increasing developmental day also correlated with median frequency ($p < 0.001$), no clear differences emerged among genotype group. (d) Similarly, latencies showed a strong effect of developmental day ($p = 0.007$), but no clear differences by genotype group. The original data (non-imputed) are shown for all graphs.


A
WT dam - WT pup
WT dam - het pup
Het dam - WT pup
Het dam - het pup
Vocalization Rate (1/min)
60.0
40.0
20.0
0.0
P04Rate
P07Rate
P10Rate
P13Rate
Error Bars: +/- 1 SE
B
WT dam - WT pup
WT dam - het pup
Het dam - WT pup
Het dam - het pup
Duration (s)
0.05
0.04
0.03
0.02
0.01
0.00
P04MeanDuration
P07MeanDuration
P10MeanDuration
P13MeanDuration
Error Bars: +/- 1 SE
C
WT dam - WT pup
WT dam - het pup
Het dam - WT pup
Het dam - het pup
Median Frequency (kHz)
100
80
60
40
20
0
P04MeanMedFreq
P07MeanMedFreq
P10MeanMedFreq
P13MeanMedFreq
Error Bars: +/- 1 SE
D
WT dam - WT pup
WT dam - het pup
Het dam - WT pup
Het dam - het pup
Latency (s)
60
40
20
0
P04Latency
P07Latency
P10Latency
P13Latency
Error Bars: +/- 1 SE

References

1. Levy SE, Mandell DS, Schultz RT (2009) Autism. *Lancet* 374:1627-1638.
2. Curatolo P, Porfirio MC, Manzi B, Seri S (2004) Autism in tuberous sclerosis. *Eur. J. Paediatr. Neurol* 8:327-332.
3. Holmes GL, Stafstrom CE (2007) Tuberous sclerosis complex and epilepsy: recent developments and future challenges. *Epilepsia* 48:617-630.
4. Schwartz RA, Fernández G, Kotulska K, Jóźwiak S (2007) Tuberous sclerosis complex: advances in diagnosis, genetics, and management. *J. Am. Acad. Dermatol* 57:189-202.
5. Wong V, Khong P (2006) Tuberous sclerosis complex: correlation of magnetic resonance imaging (MRI) findings with comorbidities. *J. Child Neurol* 21:99-105.
6. Wong M (2008) Mechanisms of epileptogenesis in tuberous sclerosis complex and related malformations of cortical development with abnormal glioneuronal proliferation. *Epilepsia* 49:8-21.
7. Jaworski J, Sheng M (2006) The growing role of mTOR in neuronal development and plasticity. *Mol. Neurobiol* 34:205-219.
8. Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* 124:471-484.
9. Bai X et al. (2007) Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. *Science* 318:977-980.
10. Inoki K, Corradetti MN, Guan K (2005) Dysregulation of the TSC-mTOR pathway in

human disease. *Nat. Genet* 37:19-24.

11. Raab-Graham KF, Haddick PCG, Jan YN, Jan LY (2006) Activity- and mTOR-dependent suppression of Kv1.1 channel mRNA translation in dendrites. *Science* 314:144-148.
12. Hartford CM, Ratain MJ (2007) Rapamycin: something old, something new, sometimes borrowed and now renewed. *Clin. Pharmacol. Ther* 82:381-388.
13. Yang Q, Guan K (2007) Expanding mTOR signaling. *Cell Res* 17:666-681.
14. Franz DN et al. (2006) Rapamycin causes regression of astrocytomas in tuberous sclerosis complex. *Ann. Neurol* 59:490-498.
15. Henske EP et al. (1996) Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions. *Am. J. Hum. Genet* 59:400-406.
16. Ess KC (2010) Tuberous sclerosis complex: a brave new world? *Curr. Opin. Neurol* 23:189-193.
17. Habib SL, Simone S, Barnes JJ, Abboud HE (2008) Tuberin haploinsufficiency is associated with the loss of OGG1 in rat kidney tumors. *Mol. Cancer* 7:10.
18. Wilson C et al. (2006) Tsc1 haploinsufficiency without mammalian target of rapamycin activation is sufficient for renal cyst formation in Tsc1+/- mice. *Cancer Res* 66:7934-7938.
19. Tavazoie SF, Alvarez VA, Ridenour DA, Kwiatkowski DJ, Sabatini BL (2005) Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. *Nat. Neurosci* 8:1727-1734.
20. Moy SS, Nadler JJ (2008) Advances in behavioral genetics: mouse models of autism.

Mol. Psychiatry 13:4-26.

21. Ehninger D et al. (2008) Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. *Nat. Med* 14:843-848.
22. Nie D et al. (2010) Tsc2-Rheb signaling regulates EphA-mediated axon guidance. *Nat. Neurosci* 13:163-172.
23. Crawley JN (2004) Designing mouse behavioral tasks relevant to autistic-like behaviors. *Ment Retard Dev Disabil Res Rev* 10:248-258.
24. Crawley JN (2007) Mouse behavioral assays relevant to the symptoms of autism. *Brain Pathol* 17:448-459.
25. Crawley JN (2008) Behavioral phenotyping strategies for mutant mice. *Neuron* 57:809-818.
26. Moy SS et al. (2009) Social approach in genetically engineered mouse lines relevant to autism. *Genes Brain Behav* 8:129-142.
27. Ricceri L, Moles A, Crawley J (2007) Behavioral phenotyping of mouse models of neurodevelopmental disorders: relevant social behavior patterns across the life span. *Behav. Brain Res* 176:40-52.
28. 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.
29. Ryan BC, Young NB, Moy SS, Crawley JN (2008) Olfactory cues are sufficient to elicit social approach behaviors but not social transmission of food preference in C57BL/6J mice. *Behav. Brain Res* 193:235-242.
30. Moy SS et al. (2008) Social approach and repetitive behavior in eleven inbred mouse

strains. *Behav. Brain Res* 191:118-129.

31. Silverman JL, Tolu SS, Barkan CL, Crawley JN (2010) Repetitive self-grooming behavior in the BTBR mouse model of autism is blocked by the mGluR5 antagonist MPEP. *Neuropsychopharmacology* 35:976-989.
32. Pellow S, Chopin P, File SE, Briley M (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* 14:149-167.
33. Hofer MA, Shair HN, Brunelli SA (2002) Ultrasonic vocalizations in rat and mouse pups. *Curr Protoc Neurosci* Chapter 8:Unit 8.14.
34. Ehret G (2005) Infant rodent ultrasounds -- a gate to the understanding of sound communication. *Behav. Genet* 35:19-29.
35. Shair HN (2007) Acquisition and expression of a socially mediated separation response. *Behav. Brain Res* 182:180-192.
36. Holy TE, Guo Z (2005) Ultrasonic songs of male mice. *PLoS Biol* 3:e386.
37. Bailey KR, Rustay NR, Crawley JN (2006) Behavioral phenotyping of transgenic and knockout mice: practical concerns and potential pitfalls. *ILAR J* 47:124-131.
38. Scattoni ML, Crawley J, Ricceri L (2009) Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. *Neurosci Biobehav Rev* 33:508-515.
39. Picker JD, Yang R, Ricceri L, Berger-Sweeney J (2006) An altered neonatal behavioral phenotype in Mecp2 mutant mice. *Neuroreport* 17:541-544.
40. 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.

41. Moles A, Kieffer BL, D'Amato FR (2004) Deficit in attachment behavior in mice lacking the mu-opioid receptor gene. *Science* 304:1983-1986.
42. Scearce-Levie K et al. (2008) Abnormal social behaviors in mice lacking Fgf17. *Genes Brain Behav* 7:344-354.
43. Scattoni ML et al. (2008) Reduced ultrasonic vocalizations in vasopressin 1b knockout mice. *Behav Brain Res* 187:371-8.
44. Liu RC, Miller KD, Merzenich MM, Schreiner CE (2003) Acoustic variability and distinguishability among mouse ultrasound vocalizations. *J. Acoust. Soc. Am* 114:3412-22.
45. Esposito G, Venuti P (2010) Understanding early communication signals in autism: a study of the perception of infants' cry. *J Intellect Disabil Res*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20136681 [Accessed February 9, 2010].
46. Uematsu A et al. (2007) Maternal approaches to pup ultrasonic vocalizations produced by a nanocrystalline silicon thermo-acoustic emitter. *Brain Res* 1163:91-99.
47. Moy SS, Nadler JJ (2008) Advances in behavioral genetics: mouse models of autism. *Mol Psychiatry* 13:4-26.
48. Insel TR, Hill JL, Mayor RB (1986) Rat pup ultrasonic isolation calls: possible mediation by the benzodiazepine receptor complex. *Pharmacol. Biochem. Behav* 24:1263-1267.
49. Johnson CP (2008) Recognition of autism before age 2 years. *Pediatr Rev* 29:86-96.
50. Nakatani J et al. (2009) Abnormal behavior in a chromosome-engineered mouse

model for human 15q11-13 duplication seen in autism. *Cell* 137:1235-1246.

51. Schwarting RKW, Jegan N, Wöhr M (2007) Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats. *Behav. Brain Res* 182:208-22.
52. Wöhr M, Houx B, Schwarting RKW, Spruijt B (2008) Effects of experience and context on 50-kHz vocalizations in rats. *Physiol. Behav* 93:766-76.
53. D'Amato FR, Scalera E, Sarli C, Moles A (2005) Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? *Behav. Genet* 35:103-112.
54. Wöhr M, Schwarting RKW (2008) Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. *Behav. Neurosci* 122:310-330.
55. Kölliker M, Brodie III ED, Moore AJ (2005) The Coadaptation of Parental Supply and Offspring Demand. *The American Naturalist* 166:506-516.
56. Kölliker M, Ridenhour BJ, Gaba S (2010) Antagonistic Parent-Offspring Co-Adaptation. *PLoS ONE* 5:e8606.
57. Smiseth PT, Wright J, Kölliker M (2008) Parent-offspring conflict and co-adaptation: behavioural ecology meets quantitative genetics. *Proc. Biol. Sci* 275:1823-1830.
58. Esposito G, Venuti P How is crying perceived in children with Autistic Spectrum Disorder. *Research in Autism Spectrum Disorders* 2:371-384.
59. Sheinkopf SJ, Mundy P, Oller DK, Steffens M (2000) Vocal atypicalities of preverbal autistic children. *J Autism Dev Disord* 30:345-354.

60. de Vries PJ, Hunt A, Bolton PF (2007) The psychopathologies of children and adolescents with tuberous sclerosis complex (TSC): a postal survey of UK families. *Eur Child Adolesc Psychiatry* 16:16-24.

61. Wöhr M et al. (2008) Effects of genetic background, gender, and early environmental factors on isolation-induced ultrasonic calling in mouse pups: an embryo-transfer study. *Behav. Genet* 38:579-595.

62. Inoki K, Zhu T, Guan K (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115:577-590.

63. Proud CG (2007) Amino acids and mTOR signalling in anabolic function. *Biochem. Soc. Trans* 35:1187-1190.

64. Kelleher RJ, Govindarajan A, Tonegawa S (2004) Translational regulatory mechanisms in persistent forms of synaptic plasticity. *Neuron* 44:59-73.

65. Sutton MA, Taylor AM, Ito HT, Pham A, Schuman EM (2007) Postsynaptic decoding of neural activity: eEF2 as a biochemical sensor coupling miniature synaptic transmission to local protein synthesis. *Neuron* 55:648-661.

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