Supporting Information

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SI Results

Abnormal EEG Activity in Conditional Knockout (cKO) Mutants. cKO mutants displayed abnormal EEG activity similar to Dlk1<−/−> mice. Dlk1<−/−> mice develop epilepsy after p30 (1); however, partial hearing loss can increase susceptibility to audiogenic seizures (2–4). EEG recordings showed that cKO mutants displayed abnormal, seizure-like EEG activity even though their peripheral hearing is normal. The events we observed were recorded in 8-h sessions in 2- to 3-month-old animals, an age when seizures are only starting to develop in Dlk1<−/−> mice. The events we observed were of short duration (~4 s, Fig. S1C), but longer than interictal spikes, which were also observed. These events were isolated to single channels and therefore not artifacts due to movement.


Detail of ANOVA for Testing Bandwidth. Bandwidths were tested in 5-dB steps up to 30 dB above threshold in cortex. Bandwidths were narrower over the range tested (two-way ANOVA, probability of null main effect of the knockout, $P < 0.001$; probability for null interaction with stimulus intensity above threshold, 0.99).

Details of ANOVA for Rate Level Functions (RLFs). To complement the comparison of responses at a single, high intensity, we also compared responses at intensities relative to threshold for each unit. As predicted by the increased threshold in cKO units, control and cKO RLFs are significantly different (two-way ANOVA, probability of null main effect of the knockout, $P < 0.001$; probability for null interaction with stimulus intensity above threshold, $P = 0.41$). There are no significantly different intensity levels with Tukey-Kramer corrected $t$-tests. Because thresholds vary from unit to unit, it is only meaningful to compare the entire RLF directly by aligning the RLFs at the threshold for each unit. In agreement with the effect observed at 80 dB, there is no difference between control and cKO single-unit responses at any intensity once aligned by threshold (two-way ANOVA, probability of null main effect of the knockout, $P = 0.30$; probability for null interaction with stimulus intensity above threshold, $P = 0.99$, Fig. S4A). In contrast, cKO multiunit responses are still different from control responses after aligning by threshold (two-way ANOVA, probability of null main effect of knockout, $P < 0.001$; probability of an interaction with stimulus intensity above threshold, $P = 0.15$, Fig. S4B).

We also examined RLFs from thalamic units. As predicted by the small decrease in threshold of cKO thalamic units, control and cKO RLFs are different (two-way ANOVA, probability of null main effect of the knockout, $P < 0.01$; probability for null interaction with stimulus intensity above threshold, $P = 0.98$, Fig. S4C). The main effect of the knockout is not changed by removing the characteristic frequency (CF) near 21 kHz outlier as in Fig. S3 ($P < 0.01$). This could result from a lack of descending corticothalamic input via the nucleus of the reticular thalamus. However, this again appears to be merely a change in threshold. Neither control nor cKO thalamic single units were different once aligned by threshold (two-way ANOVA, probability of null main effect of the knockout, $P = 0.28$; probability for null interaction with stimulus intensity above threshold, $P = 0.90$, Fig. S4D).

SI Methods

Generation of Mice. The generation and characterization of I12b-Cre transgenic mice and Dlk1 constituent mutant alleles was described previously (1, 2). To generate the conditional (floxed) allele of Dlk1 (Dlk1<fl>/fl>), a targeting vector was generated that inserted a floxP sequence and Flippase Recognition Target (FRT)-flanked neomycin resistance gene cassette into the BamHI site in the first intron of Dlk1 and a loxP sequence into the first XhoI site in the 3′ untranslated region. A TK cassette outside of the homologous arms was used for negative selection. The targeting vector was linearized with PvuI and transfected into JM1 ES cells. Clones were selected with G418 and ganciclovir. Clones were screened for homologous recombination by Southern blot and PCR. Two clones were identified with correct recombination events and expanded for blastocyst injection (~1% recombination efficiency). Chimeric mice were crossed with C57BL/6 wild-type mice, and germline transmission of the floxed Dlk1 allele was confirmed by PCR on genomic DNA extracted from mouse tails. Floxed Dlk1 mice were bred with flippase recombinase (FLP)-deleter mice purchased from Jackson Labs to remove the neomycin cassette, and removal of the neomycin gene was confirmed by PCR. The floxed Dlk1 allele (Dlk1<fl>) without the neomycin cassette was used for subsequent experiments. Floxed Dlk1 mice are genotyped by PCR: Dlk1<fl>−/fl> I12b-Cre mutant and Dlk1<fl>/fl>−I12b-Cre controls were generated by crossing mice with floxed Dlk1 alleles to mice carrying the I12b-Cre transgene in a Dlk1 heterozygous background. Mouse colonies were maintained at the University of California, San Francisco (UCSF), in accordance with National Institutes of Health and UCSF guidelines.


Histology. Adult Dlk1<fl>/fl>−I12b-Cre and Dlk1<fl>/fl>−I12b-Cre littermate mice were deeply anesthetized with xetavet (Sigma; 0.2 mL/10 g body weight) and perfused intracardially with 4% (wt/vol) paraformaldehyde (PFA) in PBS solution (PBS 0.1 M, pH 7.4). The brains were removed and postfixed overnight in the same fixative, except brains prepared for vasoactive intestinal peptide (VIP) immunohistochemistry that where fixed for 30 min. Brain sections were prepared at a thickness of 50 μm on a vibratome and used for free-floating immunohistochemistry. The slices were washed in PBS solution, incubated in blocking solution [0.5% (vol/vol) Triton X-100, 10% (vol/vol) normal goat serum, 2% (vol/vol) nonfat milk, 0.2% gelatin in PBS] for 1 h, and incubated overnight at 4°C in the primary antibody diluted in 0.5% Triton X-100, 3% normal goat serum, 0.2% gelatin in PBS. The antibodies used were as follows: calretinin polyclonal antibodies (1:500; Millipore), neuropeptide Y (NPY) polyclonal antibodies (1:250; Immunostar), parvalbumin monoclonal antibodies (1:500; Millipore), somatostatin monoclonal antibodies (1:250; Millipore), and VIP polyclonal antibodies (1:200; Immunostar). Immunoreactivity was detected with appropriate Alexa-488 or Alexa-594 (1:300; Molecular Probes) conjugated secondary antibodies. Quantifica-
tion of calretinin\textsuperscript{+}, NPY\textsuperscript{+}, parvalbumin\textsuperscript{+}, somatostatin\textsuperscript{+}, and VIP\textsuperscript{+} cells in the primary auditory cortex was determined in coronal sections from three mice of each genotype. Cell counts were performed on digitized images obtained with a CoolSNAP EZ Turbo 1394 digital camera (Photometric) on a Nikon ECLIPSE 80i microscope (Nikon Instruments) using a 4× objective. The numbers of positive cells were assessed in a 1 mm\textsuperscript{2} area of primary auditory cortex.

Fig. S1. Auditory brainstem responses are normal and seizure-like activity is observed by EEG in cKO mice. (A) Auditory brainstem responses (ABRs) from control and cKO mice. (B) ABR thresholds from control and cKO mice (P > 0.05, n = 4 and 3). For comparison purposes, data from C57BL/6 mice and previously reported values from a constitutive Dlx1\textsuperscript{−/−} mutant are shown (1). (C) Abnormal EEG activity observed in cKO mice. Upper, three different episodes from two animals. Lower, simultaneous signals on other channels.

Fig. S2. Cortical responses are significantly different across similar frequencies. (A) Distribution of CFs recorded in control and cKO mutant mice. Vertical dashed line at 15 kHz used as the cutoff for similar frequency distributions. (B) Threshold as a function of CF. Horizontal dashed lines indicate population means. Vertical dashed line as in A. (C) Bandwidth at 20 dB above threshold as a function of CF. Dashed lines as in B. (D) Response areas from units with CFs less than 15 kHz are significantly smaller in cKO units (medians: control (CT), 179 dB*octaves and cKO, 169 dB*octaves; $P < 0.05$, $n = 53$ and 43). (E) Thresholds from units with CFs less than 15 kHz are significantly higher in cKO mutants (medians: CT, 20 dB and cKO, 25 dB; $P < 0.01$, $n = 53$ and 43). (F) Bandwidths 20 dB above threshold from units with CFs less than 15 kHz are significantly narrower in cKO units (medians: CT, 1.1 octaves and cKO, 1.0 octaves; $P < 0.05$, $n = 53$ and 43).
Thalamic responses show little difference due to the loss of cortical interneurons. (A) Response areas are not significantly different in thalamus (medians: CT, 109 dB*octaves and cKO, 142 dB*octaves; $P = 0.06$, $n = 31$ and 65). Plot is identical to Fig. 2H. (B) Response thresholds are lower in cKO thalamus (medians: CT, 40 dB and cKO, 35 dB; $P = 0.049$, $n = 31$ and 65). Note: this is the opposite of the change observed in cortex and therefore cannot directly give rise to the results observed in cortex. Furthermore, this result is dependent on an outlier of the CF distribution (see E and H). If true, one possible explanation for lower thalamic thresholds is that inhibitory feedback from the nucleus of the reticular thalamus will decrease due to a decrease in low-threshold, excitatory, cortical inputs to that nucleus (1). (C) Bandwidths 20 dB above threshold are not significantly different in thalamus (medians: CT, 1.3 octaves and cKO, 1.3 octaves; $P = 0.91$, $n = 31$ and 65). (D) Distribution of CFs recorded in the thalamus of control and cKO mutant mice. Vertical dashed line at 15 kHz used as a cutoff for similar frequency distributions. Only one cKO unit (at 21 kHz) is beyond this cutoff. (E) Threshold as a function of CF. Dashed lines as in Fig. S2. (F) Bandwidth at 20 dB above threshold as a function of CF. Dashed lines as in E. (G) Response areas from thalamic units with CFs less than 15 kHz are not significantly different (medians: CT, 109 dB*octaves and cKO, 142 dB*octaves; $P = 0.07$, $n = 31$ and 64). (H) Thresholds from units with CFs less than 15 kHz are not significantly different (medians: CT, 40 dB and cKO, 35 dB; $P = 0.051$, $n = 31$ and 64). (I) Bandwidths 20 dB above threshold are not different (medians: CT, 1.3 octaves and cKO, 1.3 octaves; $P = 0.83$, $n = 31$ and 64).

Fig. S4. Cortical responses of cKO mice are less sparse even when aligned by threshold. (A) Mean RLFs for single cortical units aligned by threshold for each unit (dotted lines ± SEM). (B) Mean RLFs for cortical multiunits aligned by threshold for each unit (dotted lines ± SEM). (C) Mean RLFs for single thalamic units (dotted lines ± SEM). (D) Mean RLFs for single thalamic units aligned by threshold for each unit (dotted lines ± SEM).
Gene targeting to generate floxed Dlx1. (A) Schematic representation, from top to bottom, of the targeting vector, the wild-type Dlx1 allele (Dlx1\(^{+}\)), the conditional Dlx1 allele before (Dlx1\(^{f:neo}\)), and after (Dlx1\(^{f}\)) removal of the neoselection cassette by Flp recombinase. A, AseI; B, BamHI; X, XhoI; Neo, neomycin resistance cassette; TK, herpes simplex virus thymidine kinase gene. Black triangle, loxP site; open arrowhead, FRT site. Dlx1 exons (1–3) are shown as boxes. Small arrowheads represent PCR primers. The loxP sites were placed to flank exons 2 and 3, which together encode for the homeobox domain. (B, Left) Southern blot of genomic DNA extracted from transfected ES cells, digested with AseI, and visualized with the 5′ probe depicted in A. (Right) Long-range PCR of the 3′ arms between the loxP site in Dlx1 to outside the targeting vector. +/+, wild-type DNA; f/+, recombinant clone; con, negative control. (C) PCR of tail genomic DNA for the 3′ loxP site shows wild-type, heterozygous, and homozygous floxed Dlx1 mice.