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Supplemental Data

Mutations Affecting the SAND Domain of DEAF1 Cause Intellectual Disability with Severe Speech Impairment and Behavioral Problems

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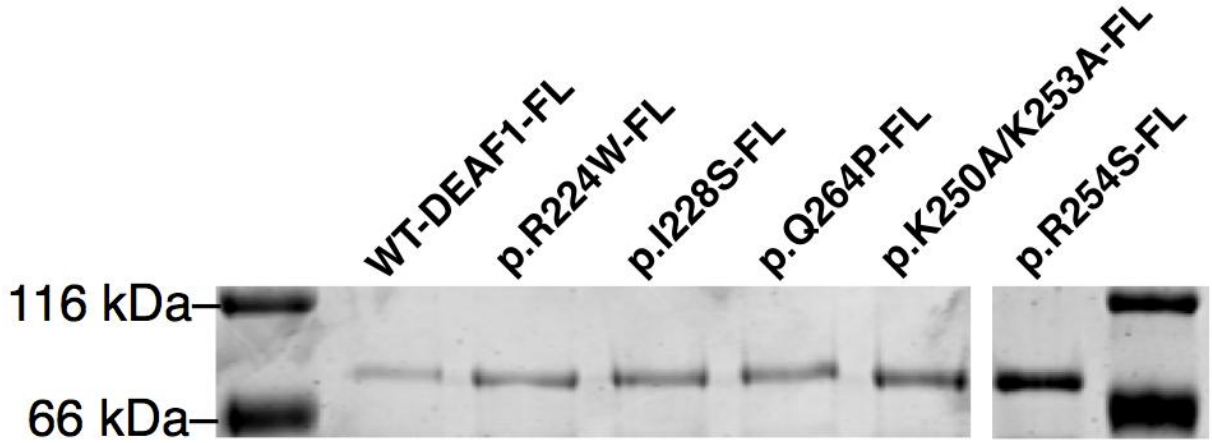
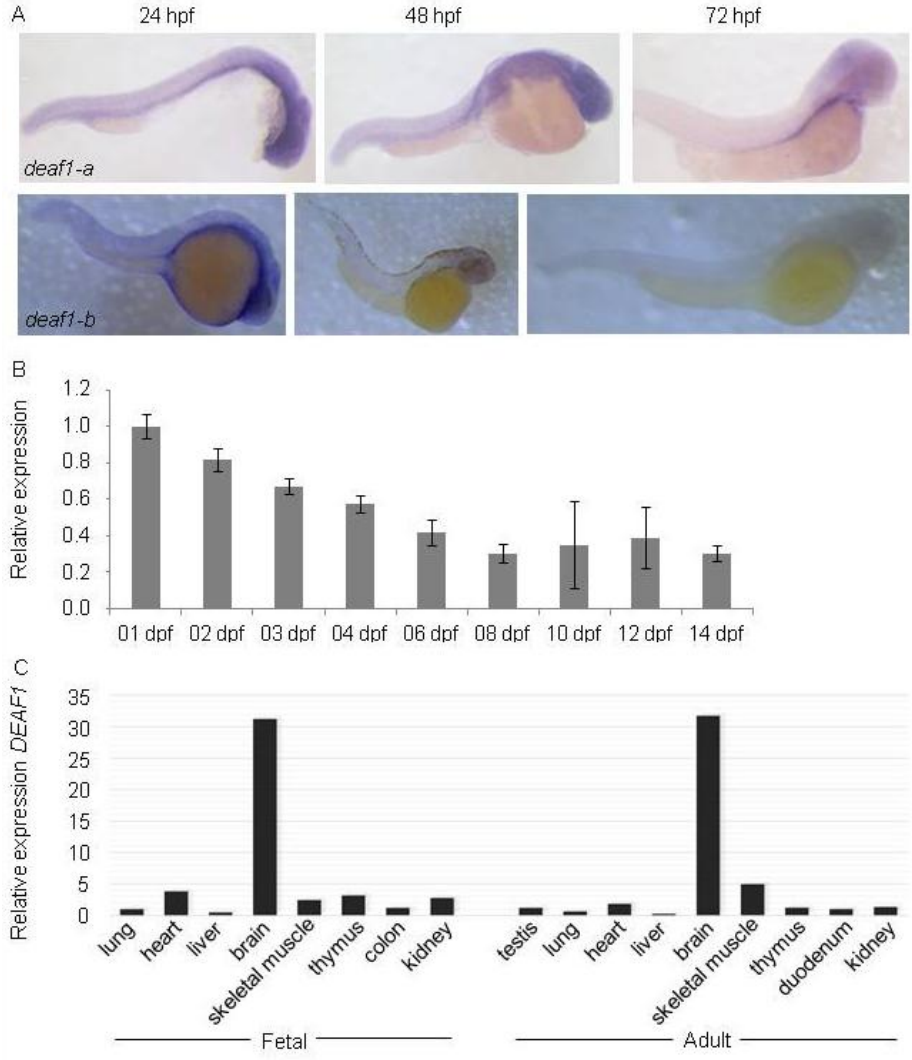
Figure S1 Recombinant proteins used in EMSA



Recombinant WT and mutant DEAF1-FLAG were isolated from HEK 293 cells, separated by SDS-PAGE and stained with Coomassie blue. Left lane contains protein size standards; abbreviations of mutant proteins can be found in Figure 1 and 2 of the manuscript.

Figure S2 *DEAF1* expression in zebrafish and human tissues



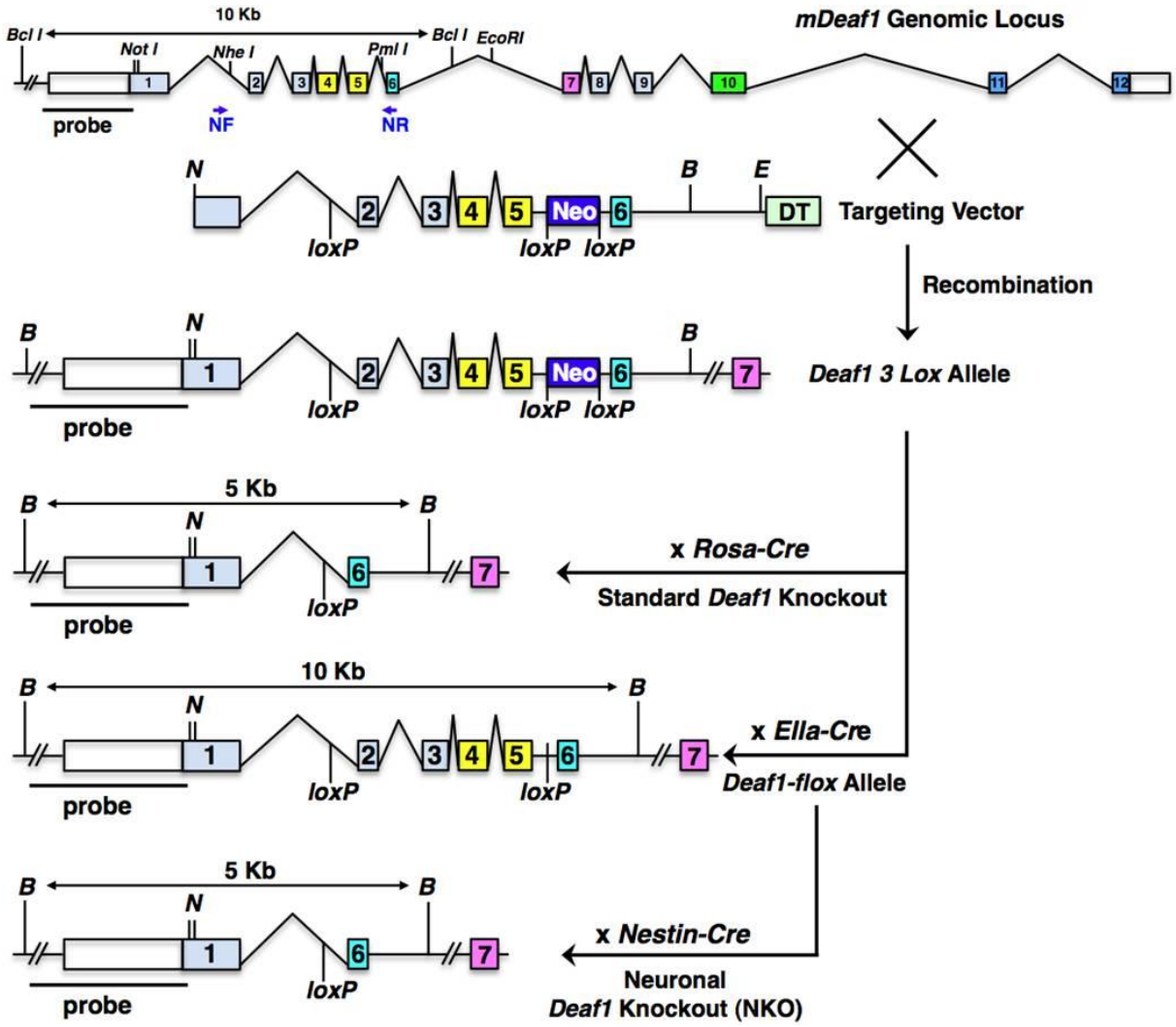
A. *Deaf1 in-situ* hybridization at various stages post fertilization in zebrafish embryos as previously described[2] with minor modifications. The primers used to design the hybridization probes are available in Table S1. *Deaf1* shows highest expression in brain and spinal cord. hpf = hours post fertilization.

B. Quantitation by qPCR of d*eaf1-a* expression at various post fertilization stages (8 and 16 hpf; 1-2-3-4-6-8-10-12-14 days post fertilization (dpf)) in zebrafish. Ten to twenty zebrafish embryos were dechorionated and collected per time point. The samples were homogenized in 200 µl trizol (QiaZol) and mixed with 40 µl chloroform. After centrifugation (4°C, 15 min, 12 rcf) the water phase was isolated, mixed with 100 µl isopropanol and incubated at room temperature (15 min). The precipitated RNA was

pelleted (4°C, 10 min, 12 rcf) and washed with 200 µl 75% ethanol (4°C, 5 min, 7.45 rcf). The pellet was air dried and subsequently resolved in 30 µl RNAse free water (60°C, 10 min). cDNA synthesis of these RNA samples was performed on 0.5 µg total RNA with the iScript kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. qPCR was performed with the Sso Advanced SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA) in a 5 µl reaction volume with 5 ng cDNA and 5 µM primers. Analysis was performed using the qbase^{+} software (http://www.biogazelle.com) and in-house validated reference genes (Vanhauwaert *et al.*, in preparation). Primers used for *deaf1-a* (zgc:194895) and *deaf1-b* (zgc:171506) are available in Table S1. *Deaf1-a* has highest expression at early developmental stages.

C. Expression of *DEAF1* by mRNA expression analysis in different human fetal and adult tissues. Relative expression levels are given as the fold change in comparison to the tissue with the lowest expression level (duodenum). Five microgram of total RNA from different human fetal (20-21 weeks) and adult tissues (Stratagene Europe, Amsterdam, the Netherlands) was reverse transcribed to cDNA using the iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. SYBR green-based real-time quantitative PCR expression analysis was performed on a 7500 *Fast* Real-Time PCR System with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer´s instructions. Primers were designed by the primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and encompassed at least one exon-exon boundary. *GUSB* was used as a reference.[1] Primer sequences are provided in Table S1. Differences in *DEAF1* expression in the various tissues were calculated by the comparative Ct or $2^{\Delta\Delta Ct}$ method,[2] with the relative expression being determined by normalization of the *DEAF1* expressing tissues to the tissue with the lowest detectable expression of *DEAF1* set at 1.0.

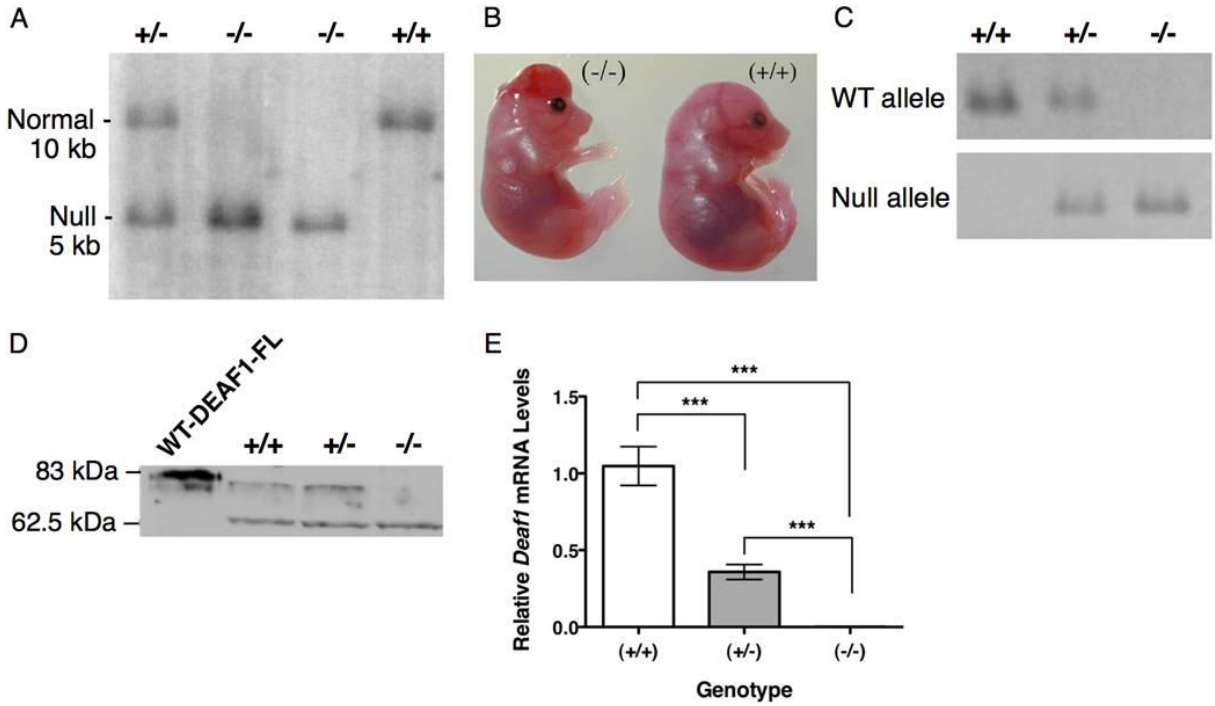
Figure S3 Genomic structure of *Deaf1* targeted alleles in mice



Schematic representations of the gene structures for the normal mouse *Deaf1* locus, the targeting vector, the *3loxP* allele, the standard *null* allele and the *Deaf1-floxed* allele and breeding schemes to produce them. *Deaf1* genomic clones were isolated from a mouse genomic 129/SvJ DNA lambda library (Stratagene #946313). The targeting vector contained a 10.1 Kb *Not*I/*EcoR*I fragment of *Deaf1* with a loxP sequence inserted into the *Nhe*I site, a *neomycin* gene cassette (Neo) flanked with loxP sites inserted into the *Pml*I site, and a *diptheria toxin* (DT) gene cassette inserted at the 3' end of the construct for positive selection of recombination in ES cells. The targeting construct was linearized with *Not*I and electroporated into 129J x 129J ES cells. Two independent ES cell clones were injected into C57BL/6 blastocysts to produce three chimeric mice that gave germ line transmission of the

"*3lox*" allele (transgenic mouse core at University of Washington, Seattle). For production of the null allele, mice with the *Deaf1 3lox* allele were bred to *Rosa-Cre* transgenic mice and offspriing with germline transmission of the null allele were outcrossed to C57BL/6 for greater than seven generations before genotype distribution of F2 mice and embryos were initially assessed (Table S2 and S4). Mice with a conditional *Deaf1* allele were produced using the "3loxP" approach of Xu et al.[3] Mice heterozygous for the *3lox* allele were backcrossed for nine generations onto a C57BL/6 strain background and were then bred to mice transgenic for *EIIa-Cre* gene (also on a C57BL/6 background). Partial recombination of loxP sites and removal of the neomycin cassette produced a *Deaf1* “2lox” or "floxed" allele with the genotype $Deaf1^{+/fl}$. The offspring were backcrossed again to C57BL/6 to assure congenic status, and subsequent $Deaf1^{+/fl}$ mice were maintained by breeding of $Deaf1^{+/fl}$ to each other. $Deaf1^{fl/fl}$ genomic structure was confirmed by genomic DNA sequencing at both loxP sites. In the upper diagram of the genomic locus functional domains are colored: the SAND domain is represented in yellow exons 4 and 5, a zinc-finger homology domain is shown in aqua exon 6, nuclear localization signal is in pink exon 7, nuclear export signal is in green exon 10, and the MYND domain is in blue exons 11 and 12. Restriction enzyme sites: *Bcl*I (B), *Not*I (N), *EcoR*I (E). When *loxP* is inserted into the *Nhe*I and *Pml*I sites of genomic DNA, the restriction sites are destroyed. NF and NR represent primers mDeaf1NP-F and mDeaf1NP-R (Table S1) in introns 1 and 5 that assay for the *null* allele following deletion of exons 2-5. "Probe" indicates a 1.2-Kb *Xho*I-*Apa*I DNA fragment used as a hybridization probe for Southern blot analysis of genomic DNA from F1 offspring (Figure S4A).

Figure S4 Confirmation of the *Deaf1* standard knockout mouse model



A. Southern blot analysis of *Bcl*I digested genomic DNA from F1 mouse embryo cells and hybridized to the radiolabeled DNA probe depicted in Figure S3.

B. Exencephaly phenotype observed in *Deaf1*$^{-/-}$ embryos.

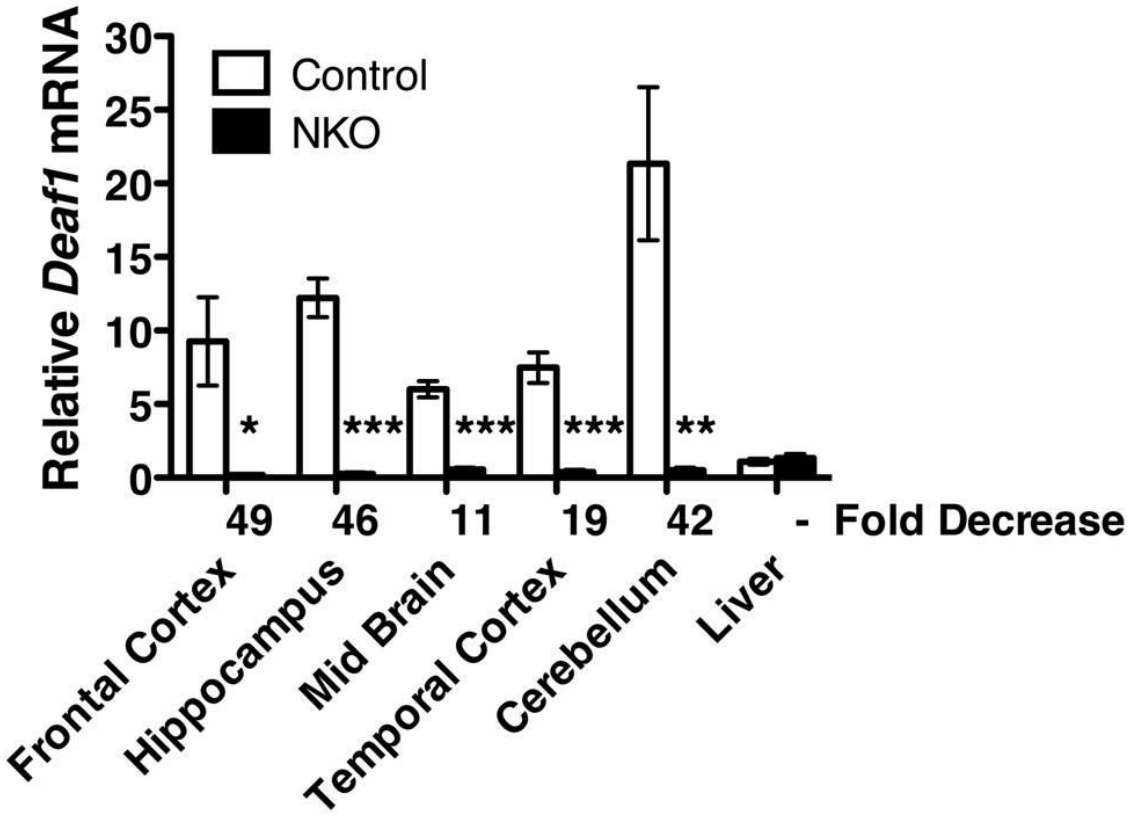
C. PCR-based genotyping of genomic DNA from tail using primers mDlox5695F and mDlox5869R for assay of the WT allele, and primers mDeaf1NP-F and mDeaf1NP-R to assay the null allele (Table S1).

D. Loss of DEAF1 in *Deaf1*$^{-/-}$ embryos. Immunoblot blot of proteins in lysates from freshly isolated embryos and analyzed using a rabbit anti-DEAF1 antibody. Cell lysate from HEK 293T cells transfected with DEAF1-FLAG (DEAF1-FL) expression vector is used as a positive control in the far left lane. The 80 kDa DEAF1 protein is absent in *Deaf1*$^{-/-}$ embryos. The blot is representative of two independent experiments.

E. Loss of WT *Deaf1* mRNA in *Deaf1*$^{-/-}$ embryos. RNA was isolated from e18.5 F1 embryos: *Deaf1*$^{+/+}$ n=7, *Deaf1*$^{+/-}$ n=14, and *Deaf1*$^{-/-}$ n=9. Relative *Deaf1* mRNA levels were measured by qPCR using primers mDEAF696F and mDEAF769R that are specific for exon 5 and normalized to β-Actin (Table S1). Levels relative to *Deaf1*$^{+/+}$ were calculated by the $2^{-\Delta\Delta Ct}$

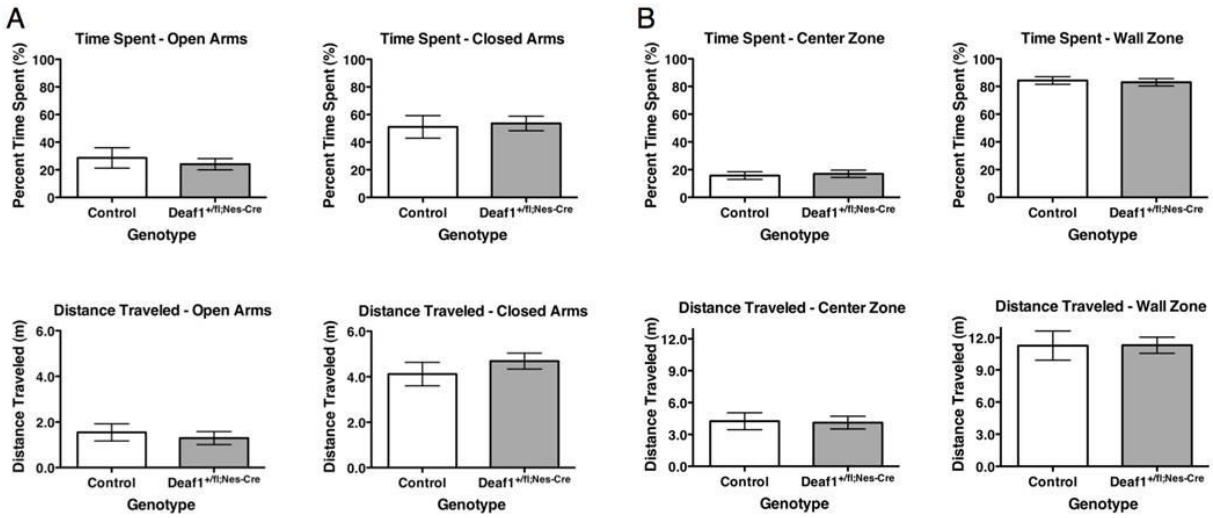
method[2] and reported as the mean ± SEM of three experiments, one-way ANOVA, Bonferroni post test, *** $p<0.001$

Figure S5 *Deaf1* mRNA expression in brain regions and liver of adult male control and NKO mice



Relative *Deaf1* mRNA levels were measured by qPCR using primers mDEAF696F and mDEAF769R to exon 5 (Table S1) and normalized to β-Actin. RNA (3.5 µg) was primed with oligo(dT) and cDNA was generated using GoScript (Promega). *Quantitative PCR (qPCR)* was performed using a Bio-Rad CFX90 Real Time C1000 Thermal Cycler and Bio-Rad iQ SYBR Green Supermix. with the exon 5 specific primers. The Ct values obtained were normalized to mouse *β-actin* mRNA levels in each sample and relative gene expression in the samples were determined using the $2^{-\Delta\Delta Ct}$ method.[2] Levels relative to control liver were calculated by the $2^{-\Delta\Delta Ct}$ method[2] and are reported as the mean ± SEM. Control n=5, NKO n=5. Significant decreases were observed for NKO brain regions with no change in liver expression, two-way ANOVA with Bonferroni post test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

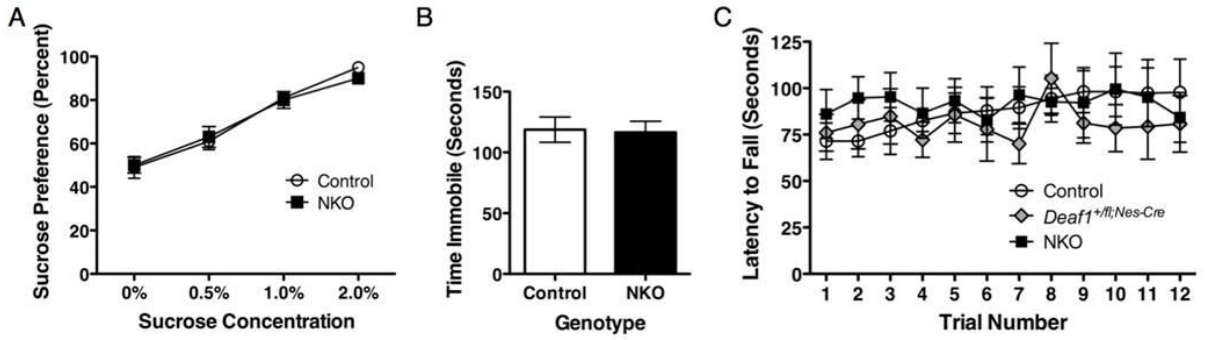
Figure S6 Anxiety testing of mice with conditional heterozygous knockout of *Deaf1* in brain



A. Anxiety testing using the elevated plus maze. Bars represent the mean ± SEM of time spent or distance traveled in open and closed arms. Control n=12, *Deaf1*$^{+/fl;Nes\text{-}Cre}$ n=13. No significant difference was observed between the two genotypes, unpaired two-tailed t-test.

B. Anxiety testing using the open field exploration test. Bars represent the mean ± SEM of time spent or distance traveled in center or wall zones. Control n=12, *Deaf1*$^{+/fl;Nes\text{-}Cre}$ n=15. No significant difference was observed between the two genotypes, unpaired two-tailed t-test.

Figure S7 Tests for depression-like behavior and mobility of mice with conditional knockout of *Deaf1* in brain



A. Sucrose preference, Control n=11, NKO n=11, mean ± SEM, No significant difference was observed between genotypes. The sucrose preference test was conducted similarly to that previously described.[4] Mice were individually housed during the testing period (48 hours) followed by group housing in between testing of different sucrose concentrations (24 hours). Food was provided *ad libitum*. During the testing period, the individually housed mice were given two bottles from which to drink, one containing plain tap water and the other containing sucrose with varying sucrose concentrations (0%, 0.5%, 1% and 2%) in tap water. The bottles were weighed at the beginning of the testing period (0 hour) and at the end (48 hours). The position of the bottles was switched at 24 hours to avoid possible side bias. Following the 48 hour testing period, mice were group housed along with their cage mates and provided with two bottles both containing plain tap water. After 24 hours of group housing, mice were again separated into individual cages and tested with a different concentration of sucrose. The process was repeated and after the final concentration, mice were permanently returned to group housing. The difference in weight in grams at 0 hour and 48 hours was calculated for the bottle containing plain tap water (ΔW) and the bottle containing tap water with sucrose (ΔS). Sucrose preference was calculated using the formula

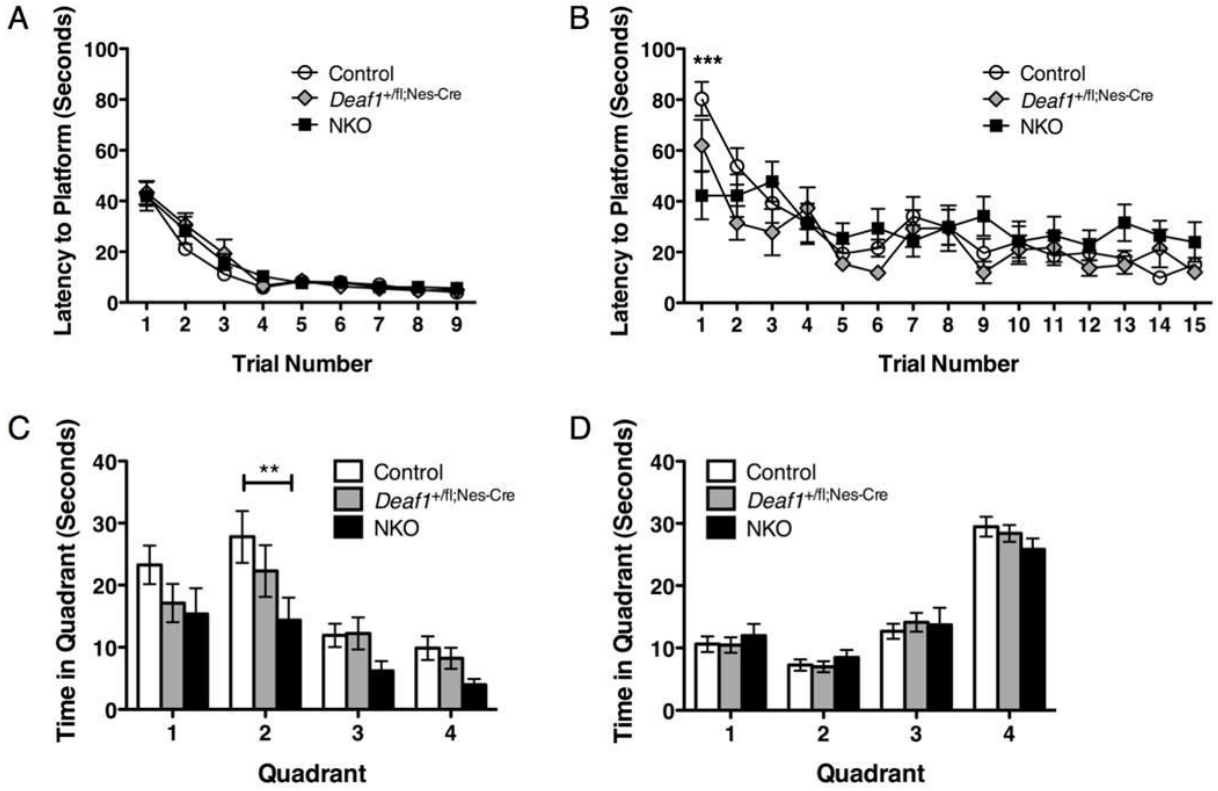
$$\text{Sucrose preference } \% = \frac{\Delta S}{\Delta W + \Delta S} * 100$$

B. Forced swim, Control n=12, NKO n=12, mean ± SEM, No significant difference was observed between genotypes. Mice were individually placed in a clear plastic cylinder

(diameter 17 cm, height 24.5 cm) filled with water at room temperature (22-25°C) to a depth of 10 cm. Fresh water was used for each animal. The animal was placed in the cylinder for a total of six min, and behavior was recorded and analyzed using ANY-maze software for the last four min. Behavior was categorized as “swimming” or “immobility” (defined as the absence of active, escape-oriented behaviors such as swimming, jumping, rearing, sniffing or diving). A mouse was judged to be immobile when it stopped struggling and floated in an upright position, making only small movements to keep its head above water.

C. Rotarod, Control n=9, *Deaf1*$^{+/fl;Nes\text{-}Cre}$ n=9, NKO n=9, mean ± SEM, No significant difference was observed among genotypes. Mice were placed on a rotating-rod apparatus that accelerated linearly from 4 to 40 rpm over five minutes of the run. The mice were given three trials on each of four consecutive days with a one hour resting period between trials. The amount of time a mouse stayed on the rotating rod was plotted to a maximum time of five minutes, the duration of each trial. Two episodes of holding on instead of walking, as the rod rotated through 360 degrees was scored as a fall. The average for three trials for each day is plotted.

Figure S8 Morris water maze for learning and memory of mice with conditional knockout of *Deaf1* in brain



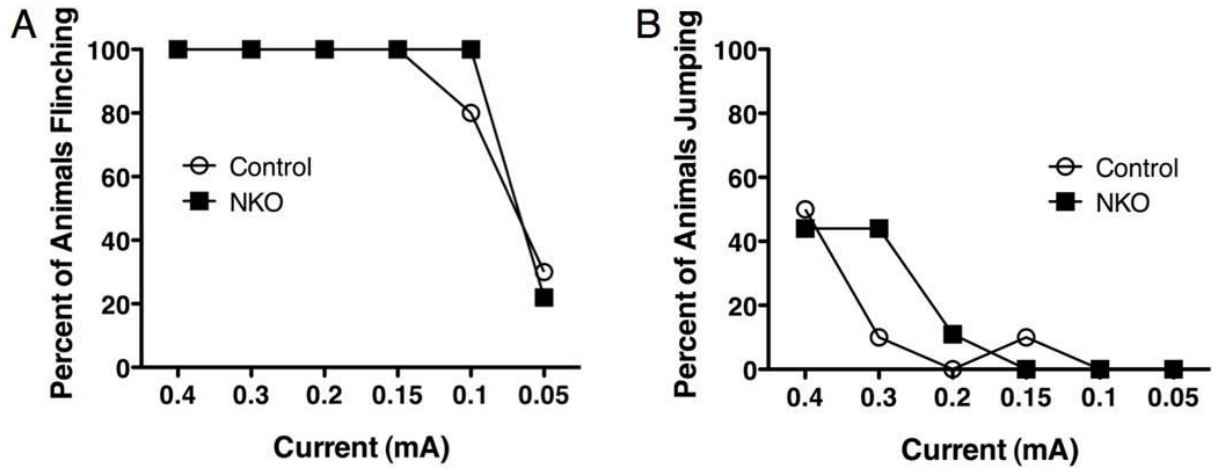
A. Morris Water Maze-Visible Platform, Control n=16, *Deaf1*$^{+/fl;Nes\text{-}Cre}$ n=12, NKO n=14, mean ± SEM, No significant difference was observed among genotypes.

B. Morris Water Maze-Hidden Platform, Control n=16, *Deaf1*$^{+/fl;Nes\text{-}Cre}$ n=12, NKO n=14, mean ± SEM, Two-way ANOVA, Bonferroni post test *** $p<0.001$ for Trial 1 between NKO and Control.

C. Morris Water Maze, Trial 1 of Quadrant Analysis of quadrant 4 with hidden platform (quadrant 2 with previously visible platform), Control n=16, *Deaf1*$^{+/fl;Nes\text{-}Cre}$ n=12, NKO n=14, mean ±SEM, Two-way ANOVA, Bonferroni post test ** $p<0.01$ between NKO and Control in quadrant 2.

D. Morris Water Maze, Quadrant Analysis, Probe Trial after 1 week, Control n=16, *Deaf1*$^{+/fl;Nes\text{-}Cre}$ n=12, NKO n=14, mean ± SEM, No significant difference was observed among genotypes.

Figure S9 Sensitivity to foot shock of NKO vs control



Foot shock sensitivity in mice was examined following fear conditioning testing. A jump is recorded if either all four limbs or the hind limbs alone leave the floor while the footshock is given. A flinch would be a noticeable response to the shock without a jump. Footshocks were given for one second each and mice were allowed to recover for 1 minute between shocks. Shock was applied in a descending order of intensity. The chamber was wiped with 70% ethanol and dried completely between mice. Mice of the indicated genotype were exposed to the indicated level of foot shock, starting from the highest to lowest mA value. Percent of animals flinching (A) and jumping (B) was recorded. Control n=10, NKO n=9. NKO mice have a similar response relative to control mice at the current used.

Table S1 Primers used in this study

Primer name	Sequence 5'->3'	Product size	Allele or Purpose
mDlox5695F	TAGAATTCGATCTATGGTGGGTGTG	174 bp (WT)	Wild Type
mDlox5869R	CATACATGGGGCACACCTAATTTAG	226 bp (+LoxP)	and LoxP
mDeaf1NP-F	TTGGGATTTGGGGTCAAGG	359 bp	Null
mDeaf1NP-R	AGTGGGTGTAGTGGTTAAGG		
C001	ACCAGCCACTATCAACTCG	199 bp	Cre
C002	TTACATTGGTCCAGCCACC		
mβactin1756	CCCTTTTTTTTGTCCCCCCAA	99 bp	qPCR of β-
mβactin1855R	AAGTCAGTGTACAGGCCAGC		actin mRNA
mDEAF696F	GTGTATCAAGCAGGGAGAAAAC	73 bp	qPCR of WT
mDEAF769R	CGGATGCTTCTCTTCCAGTC		*Deaf1* mRNA
Eif4g3-609F	aaagctagcCTGAATCCGCTCCATCCCTTCC	667 bp	Reporter
Eif4g3+58R	aaaaagcttCTCAACGAGCAGAGCATCCAAC		
N52-69F	AATTCTTCGGCTTCCCACTTCGGAATT	27 bp	dsDNA ligand
N52-69R	AATTCCGAAGTGGGAAGCCGAAGAATT		11 bp sp CG
S6conF	TTGAGTTCGGGTGTTCGGGCTCAA	24 bp	dsDNA ligand
S6conR	TTGAGCCCGAACACCCGAACTCAA		6 bp sp CG
Deaf1-aF	GGGTAAAGGTCGCTGTATC		ISH
Deaf1-aR	CTGCGTGTCCTCTTGAATG		*deaf1-a*
Deaf1-bF	TGGAAGGTGGACGAAGGAAA		ISH
Deaf1-bR	GGCAAACCTACCAGACTCCA		*deaf1-b*
Deaf1-aF	AATCTAAACCTCCTCCGGCC	154 bp	qPCR *deaf1-a*
Deaf1-aR	CTCTGACTGGCTTGTTTGGC		
Deaf1-bF	AGCATGGCATCTGACAGTGA	308 bp	qPCR *deaf1-b*
Deaf1-bR	CCCCACAAGTGACAGGAAGT		
DEAF1_ex4/5_F	TACGGTGCCGGAACATCAG	108 bp	qPCR of
DEAF1_ex4/5_R	CAAACTCGGTGGGACTGTACC		human tissues

Table S2 Distribution of *Deaf1* Genotypes of 133 F2 embryos from *Deaf1*$^{+/-}$ intercross after ≥7 generations of backcross on C57BL/6 strain

Genotype	Number of embryos (percent)
(+/+)	32 (24%)
(+/-)	68 (51%)
(-/-)	33 (25%)

Table S3 Distribution of exencephaly in 33 *Deaf1*$^{-/-}$ F2 embryos (e14.5 to e18.5) from *Deaf1*$^{+/-}$ intercross after ≥7 generations of backcross on C57BL/6 strain

Gender	Number analyzed	Number with exencephaly (percent)
Female	14	9 (64%)
Male	19	11 (58%)

Table S4 Survival to weaning of 131 F2 offspring from *Deaf1*$^{+/-}$ intercross after ≥8 generations of backcross on C57BL/6 strain

Genotype	Number of mice surviving to weaning (percent)
(+/+)	43 (31%)
(+/-)	96 (69%)
(-/-)	0 (0%)

Table S5 Birth statistics of 126 F2 offspring from *Deaf1*$^{+/-}$ intercross after ≥6 generations of backcross on BALB/c strain

Genotype	Number of dead neonates (with exencephaly)	Number of mice surviving to weaning (percent of 92 mice)
(+/+)	6 (0)	39 (43%)
(+/-)	14 (3)	50 (54%)
(-/-)	14 (4)	3 (3%)

Supplemental References

1. de Brouwer, A.P., van Bokhoven, H., and Kremer, H. (2006). Comparison of 12 reference genes for normalization of gene expression levels in Epstein-Barr virus-transformed lymphoblastoid cell lines and fibroblasts. Molecular diagnosis & therapy 10, 197-204.
2. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.
3. Xu, X., Li, C., Garrett-Beal, L., Larson, D., Wynshaw-Boris, A., and Deng, C.X. (2001). Direct removal in the mouse of a floxed neo gene from a three-loxP conditional knockout allele by two novel approaches. Genesis 30, 1-6.
4. Cai, X., Kallarackal, A.J., Kvarta, M.D., Goluskin, S., Gaylor, K., Bailey, A.M., Lee, H.K., Huganir, R.L., and Thompson, S.M. (2013). Local potentiation of excitatory synapses by serotonin and its alteration in rodent models of depression. Nature neuroscience 16, 464-472.