Ulk4 regulates GABAergic signaling and anxiety-related behavior

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Abbreviated title: *Ulk4* regulates GABAergic signaling and anxiety

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Figures and Figure Legends

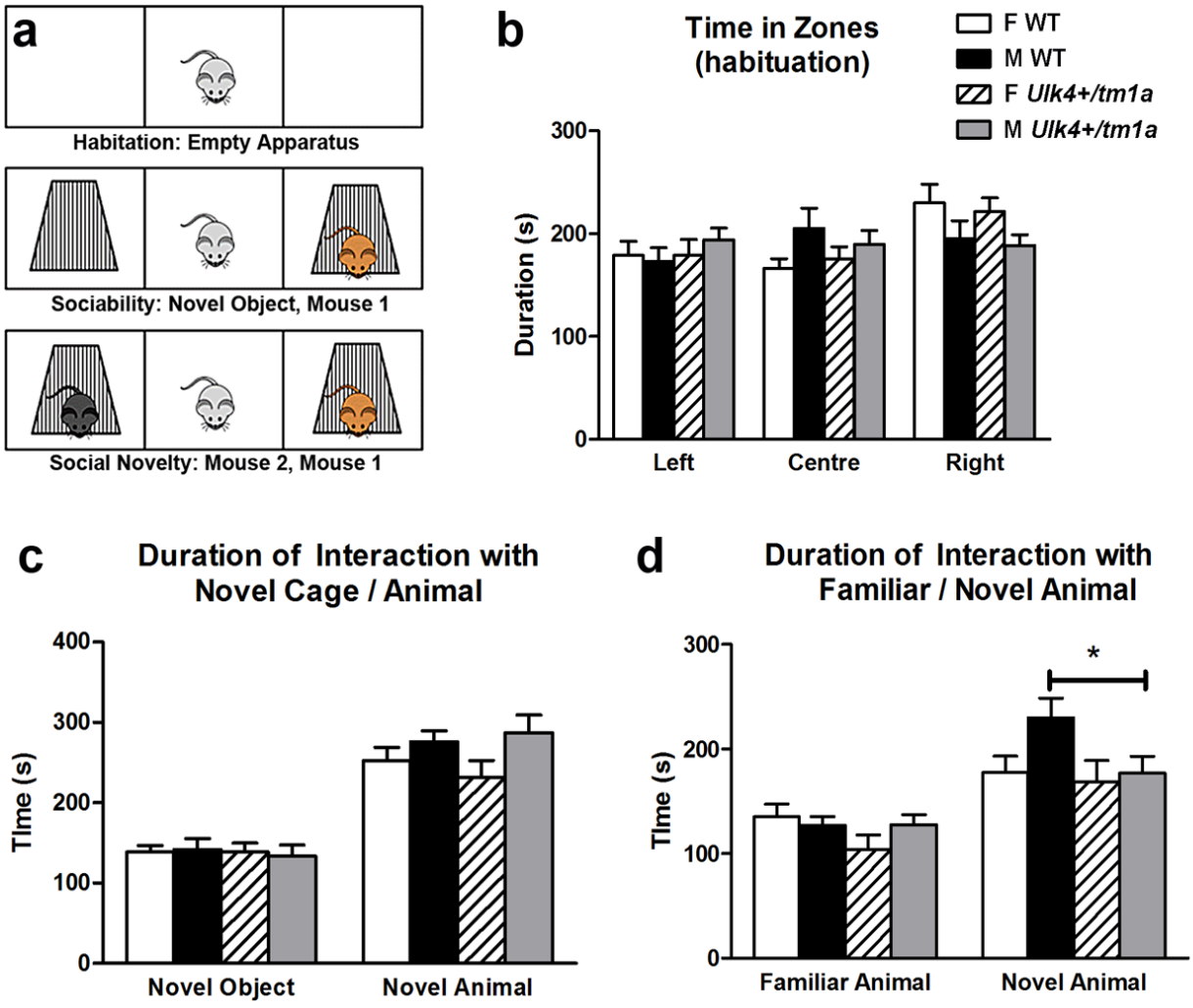


Figure 1 *Ulk4*$^{+/tm1a}$ **male mice display reduced social novelty preference. (a)** WT male (n=18), WT female (n=20), *Ulk4*$^{+/tm1a}$ male (n=20) and *Ulk4*$^{+/tm1a}$ female (n=14) were tested in Three-Chamber apparatus. **(b)** No zone preference during the habitation period (p>0.05). **(c)** No

genotype difference in sociability test. All animal spent more time in the arena with the novel stimulus animal *vs*. time in center or in arena of novel object ($p<0.01$). **(d)** Social novelty tests showing that *Ulk4*$^{+/tm1a}$ male spent significantly less time interacting with the novel animal in comparison to the WT male (* for $p<0.05$).

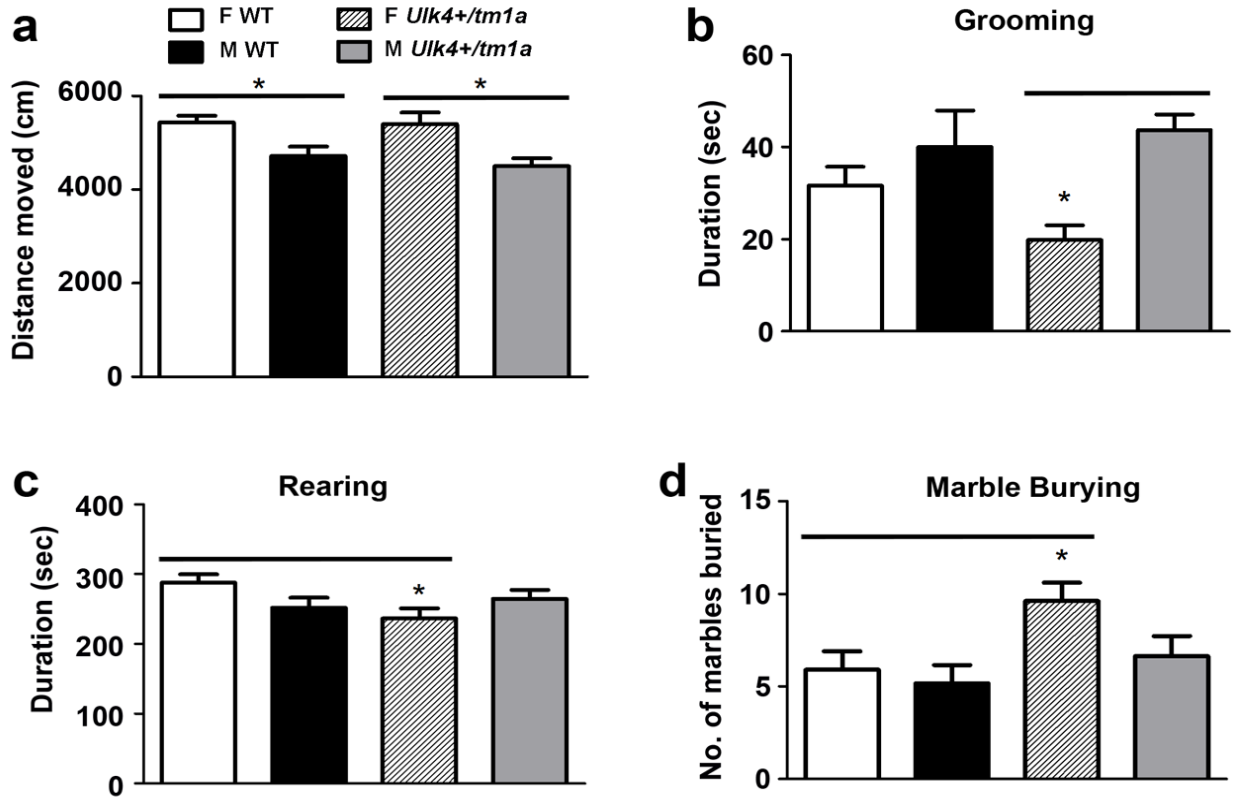


Figure 2 *Ulk4*$^{+/tm1a}$ female mice displayed decreased grooming activity, reduced rearing, but increased marble burying. (a-c) 20 WT female, 18 WT male, 13 *Ulk4*$^{+/tm1a}$ female and 17 *Ulk4*$^{+/tm1a}$ male at 2~3-month-old were placed in an open field arena for 20 minutes. (a) There was no genotype-specific but gender-associated difference in distance travelled. (b) The duration spent on grooming was reduced in *Ulk4*$^{+/tm1a}$ female (39.8+5.1, n=13) compared with *Ulk4*$^{+/tm1a}$ male (77.3+6.3, n=17, $p<0.01$). (c) *Ulk4*$^{+/tm1a}$ female also showed reduced rearing activity (240.2+14.2, n=13, $p=0.02$) than WT Female (287.7+12.1, n=20). (d) *Ulk4*$^{+/tm1a}$ female and total *Ulk4*$^{+/tm1a}$ mice showed significant increase in marble burying compared to WT female and total WT mice.

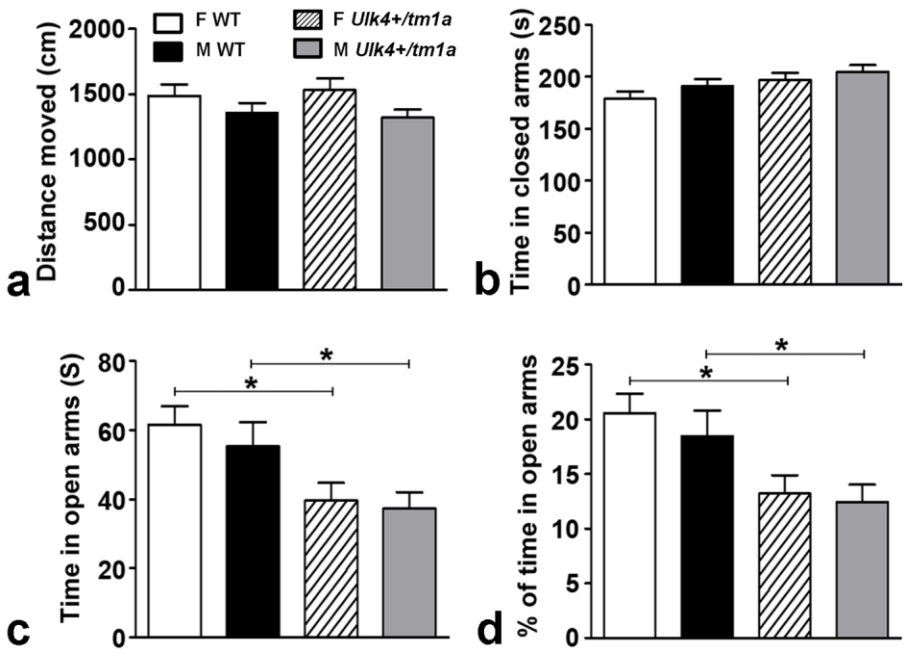


Figure 3 Both *Ulk4*$^{+/tm1a}$ male and female mice exhibit an increased anxiety on EPM test. (a) Distance moved; (b) time spent in the closed arms; (c) time spent in open arms and (d) percentage of time spent in the open arms. Male and female *Ulk4*$^{+/tm1a}$ mice and their WT littermates were subjected to the EPM. Data are expressed as mean ± SEM; n=12-19 per group. *p<0.05.

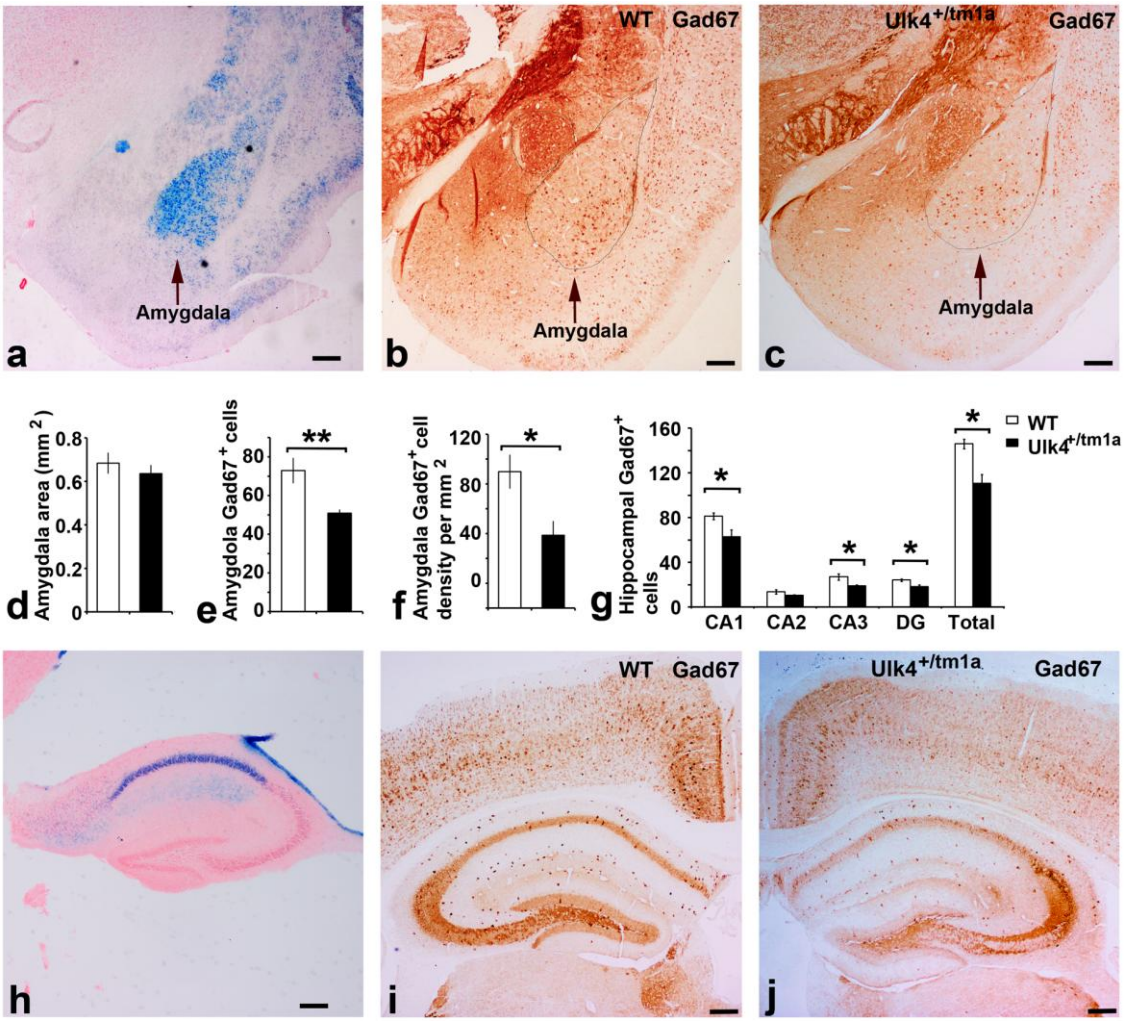


Figure 4 Reduced Gad67 positive cells in the amygdala and hippocampus of *Ulk4*$^{+/tm1a}$ mice. X-gal staining showed Ulk4 expression in the basolateral amygdala (A) and hippocampus (B). Anti-Gad67 immunohistochemistry was carried out on brain section of 5 female WT and 5 *Ulk4*$^{+/tm1a}$ female mice at 2-month. The Gad67^{+} cells were quantified from the equivalent regions as outlined in WT (0.68 ± 0.05 mm^2, n=5) and *Ulk4*$^{+/tm1a}$ (0.64±0.04 mm^2, n=5) mice. The Gad67^{+} cells were quantified from comparable sections of the CA1 (81.2±3.0 in WT vs. 62.9±6.1 in *Ulk4*$^{+/tm1a}$, p=0.03), CA2 (13.7±1.9 in WT *vs.* 10.3±0.7 in *Ulk4*$^{+/tm1a}$, p=0.13), CA3 (27.0 ±2.8in WT *vs.* 19.0±0.9 in *Ulk4*$^{+/tm1a}$, p<0.02), DG (24.1±1.2 *vs.* 18.4±1.4, p<0.02), WT hippocampus (146.0±4.3) and *Ulk4*$^{+/tm1a}$ hippocampus (110.6±8.2, p<0.01) and statistically analyzed (G), showing significant reduction of Gad67 cells (E) and cell density in *Ulk4*$^{+/tm1a}$ Amygdala (F) and a significant reduction (p<0.05) of Gad67 cells in the hippocampus (G) of *Ulk4*$^{+/tm1a}$ mice. Bar=200 µm in a,b,c,h,i,j. *p<0.05. **p<0.01.

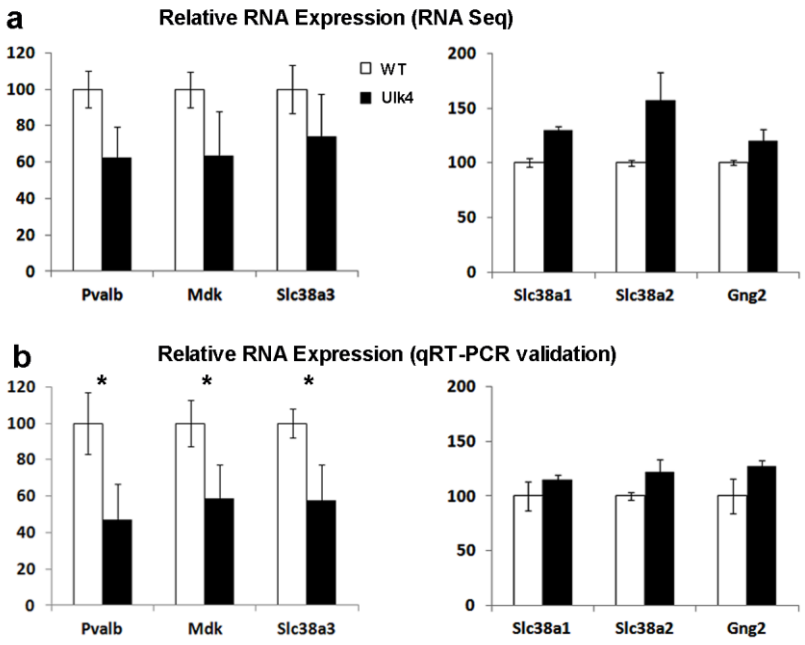


Figure 5. Altered gene expression identified by whole genome RNA sequencing (A), and validated by quantitative RT-PCR (B). RNA sequencing was carried out in 3 pairs of P12 WT and *Ulk4* $^{tm1a\ /tm1a}$ cortex, and quantitative RT-PCR in 4 pairs of cortical RNA (B). Whereas *Slc38a1*, *Slc38a2* and *Gng2* genes showed increased mRNA transcripts by both methods, the *Pvalb*, *Mdk* and *Slc38a3* gene transcription were significantly reduced (* for $p<0.05$).

Table 1. Ulk4 regulates GABAergic signaling, hyper-anxious and hypo-anxious genes

GABA subtypes	WT (FPKM)	*Ulk4* mutants (FPKM)	Average Mutant /WT	Up-Down (Mutant /WT)	P-value	FDR
Pvalb (39%)	28.7 ±2.9	18.0 ±3.0	0.63	Down	4.33E-07	9.01E-06
Sst (23%)	382.4 ±22.8	324.9 ±11.9	0.85	Down	7.97E-10	2.61E-08
Cck (5%)	437.7 ±3.8	358.0 ±1.9	0.82	Down	2.52E-17	1.80E-15
Npy (8%)	257.1 ±9.9	196.8 ±21.4	0.77	Down	1.19E-14	6.84E-13
Nos3 (<1%)	4.6 ±0.4	3.1 ±0.6	0.67	Down	1.09E-04	1.35E-03
Calb2 (24%)	11.2 ±0.3	16.3 ±1.8	1.45	Up	5.29E-05	7.07E-04
Vip (11%)	18.9 ±1.3	23.2 ±3.4	1.23	Up	4.04E-03	3.05E-02
GABAergic Synapse Pathway						
Gng4	47 ±3.1	40.1 ±4.3	0.85	Down	5.26E-05	7.04E-04
Gng7	142.6 ±15.9	107.8 ±33.4	0.76	Down	5.00E-53	1.82E-50
Slc38a3	42.5 ±5.6	31.5 ±7.4	0.74	Down	2.37E-10	8.49E-09
Slc38a5	7.1 ±1.4	3.7 ±1.4	0.53	Down	8.81E-06	1.43E-04
Adcy9	9.7 ±0.8	13.4 ±2.2	1.39	Up	1.91E-13	9.77E-12
Cacna1c	7.2 ±0.4	8.6 ±0.5	1.19	Up	3.57E-05	4.98E-04
Gabra1	78.4 ±8.5	95 ±8.7	1.21	Up	2.55E-22	2.60E-20
Gabra3	36.5 ±2.5	44.4 ±3	1.21	Up	1.24E-08	3.39E-07
Gabra4	32.7 ±1.6	38.8 ±1.5	1.19	Up	3.91E-07	8.21E-06
Gabra5	35.5 ±0.3	43.3 ±4.9	1.22	Up	2.03E-06	3.76E-05
Gabrb3	74.8 ±5.8	95.5 ±8.9	1.28	Up	6.62E-35	1.29E-32
Gng2	85.6 ±2	103.2 ±10.7	1.20	Up	1.35E-16	8.93E-15
Hap1	15.7 ±2.2	26.6 ±5	1.70	Up	4.26E-24	4.92E-22
Nsf	174.4 ±15.1	214.9 ±9.8	1.23	Up	3.11E-38	7.03E-36
Slc38a1	21.7 ±0.8	28.1 ±1	1.30	Up	5.89E-18	4.41E-16
Slc38a2	21.3 ±0.6	33.6 ±8.4	1.58	Up	5.01E-33	9.04E-31
Hypo-anxious Genes						
Atp1a2	228.0±18.7	175.47±19.7	0.77	Down	1.28E-117	1.40E-114
Ptn	178.6±26.3	142.9±33.5	0.80	Down	9.28E-19	7.57E-17
Mdk	22.8±3.9	14.5±6.1	0.63	Down	1.47E-04	1.75E-03
Plcb4	11.4±2.4	14.9±2.5	1.31	Up	1.50E-05	2.28E-04
Apoe	1085.4±162.0	1127.2±46.3	1.04	Up	1.56E-05	2.36E-04
Cacna1e	15.1±3.5	17.0±0.3	1.13	Up	2.75E-04	3.06E-03
Adra2a	8.4±1.1	10.7±1.7	1.28	Up	3.72E-04	3.97E-03
Hyper-anxious Genes						
Ncam1	96.0±4.7	82.8±8.5	0.86	Down	4.78E-12	2.09E-10
Gria1	48.4±6.7	59.4±8.3	1.23	Up	4.14E-16	2.65E-14
Syngap1	106.8±10.0	121.9±27.1	1.14	Up	3.03E-10	1.06E-08
Npy2r	0.9±0.3	1.9±0.6	2.13	Up	1.15E-04	1.41E-03
Ptpra	56.6±2.2	62.9±2.4	1.11	Up	1.98E-04	2.29E-03

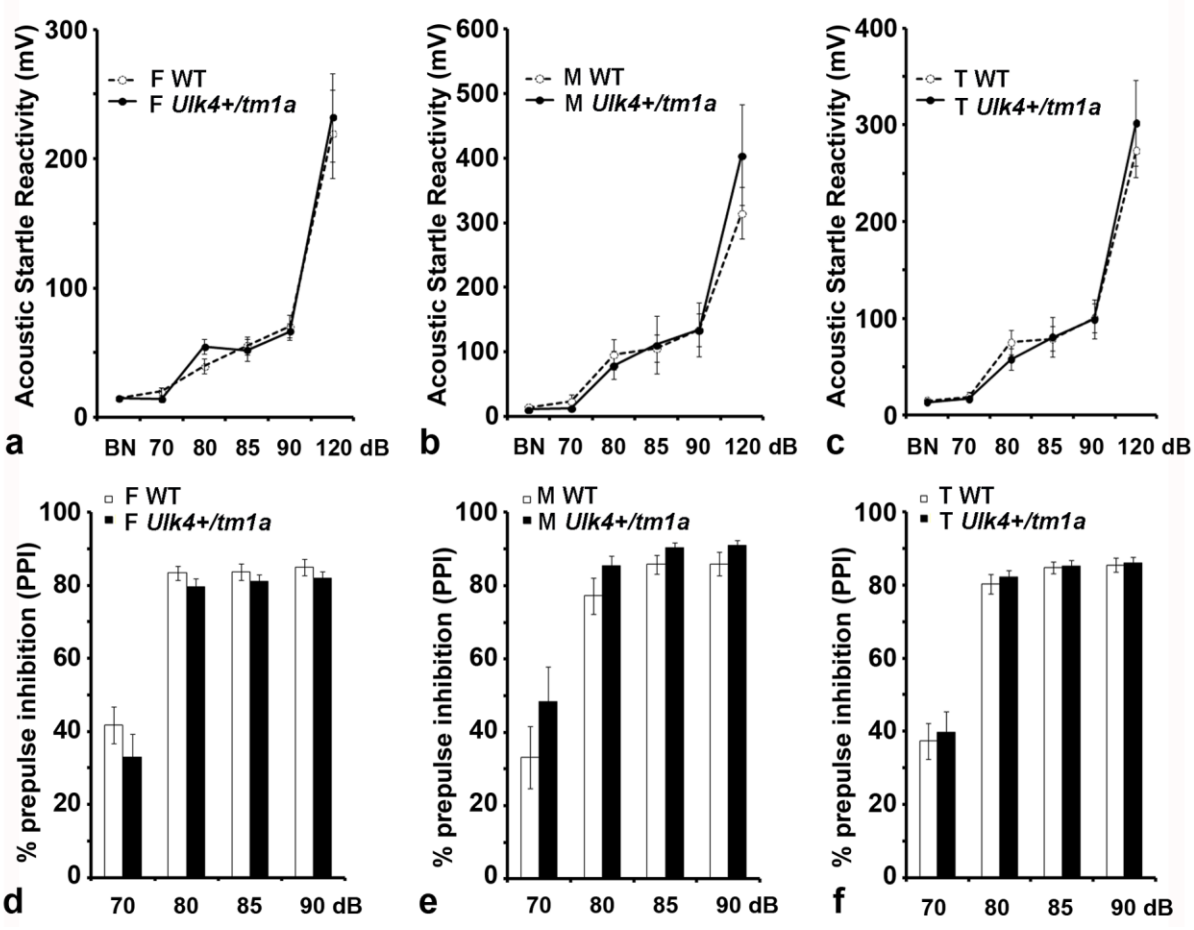
Prepulse Inhibition (PPI)

A separate group of mice, 10 WT male, 10 WT female, 8 *Ulk4*$^{+/tm1a}$ male and 10 *Ulk4*$^{+/tm1a}$ female at the age of ~10 month old, which were not undergoing any other behavioral test, were examined for acoustic startle reactivity (ASR) and PPI using the SR-LAB startle response system (San Diego Instruments)[60, 61]. The system consisted of a sound attenuating box housing an animal enclosure platform, a fan and a speaker, all of which were lit from above by LEDs. Acoustic stimuli were delivered through a speaker at 70 dB, 80 dB, 85 dB, 90 dB and 120 dB, which was calibrated using a decibel counter (A weighting; Radio Shack). The animal enclosure platform that contained an accelerometer was calibrated before testing commenced using the San Diego Instruments Standardization Unit. Initial pilot data (not shown) suggested that a standardized value of 1000 mV from the platform was necessary to have sufficient measurable startle responses at low dB to measure ASR[60,61].

ASR and PPI were measured within the same trial, as outlined in the EMPReSS standard operating procedure (http://empress.har.mrc.ac.uk/viewempress/pdf/ESLIM_011_001.pdf). Startle responses were detected by an accelerometer on the platform and digitized in waveform format. ASR was measured at 'no sound' (65 dB; BN; background noise), 70 dB, 80 dB, 85 dB, 90 dB and 120 dB using a single 40 ms pulse ('no sound' was a trial with only the background noise audible). PPI was measured by the delivery of a tone at either 70 dB, 80 dB, 85 dB, 90 dB (Prepulse) for 10 ms followed by a 100 ms gap at background noise and then a 120 dB 'startle' tone for 40 ms. Intra-trial intervals were averaged at 25 seconds.

A trial consisted of a 5 minute habituation period, during which the 65 dB background noise was present. PPI and ASR were then tested in 10 blocks, of which each block contained 10 trials (ASR: 'no sound', 70 dB, 80 dB, 85 dB, 90 dB and 120 dB; PPI: 70 dB, 80 dB, 85 dB, 90 dB). The delivery of the tones was pseudorandom and the pattern was never repeated across blocks. Startle responses were detected by an accelerometer on the platform, digitized and saved to computer in waveform format, with a post-stimulus time window of 300 ms and resolution of 1 kHz. ASR was analyzed by measuring the Vmax of the mV waveform from 0 to 100 ms. ASR was then averaged for all 10 trials at each dB intensity. PPI trials were analyzed using the same method, except that the waveform was measured in response to the second, 'startle' pulse from 120 to 220 ms. mV values were again averaged for each dB intensity. PPI was calculated as

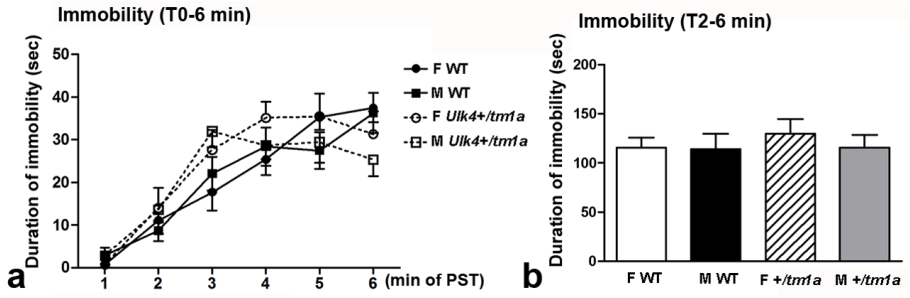
(mean 120 startle alone – mean PPI db startle) / mean 120 startle alone x 100, where mean PPI dB startle represents a separate calculation at each of the 70 dB, 80 dB, 85 dB, 90 dB PPI trials.



Supplemental Figure 1. *Ulk4*$^{+/tm1a}$ **mice show no alteration in Acoustic Startle Reactivity (ASR) or Prepulse Inhibition (PPI).** The PPI test was carried out in 10 WT male, 10 WT female, 8 *Ulk4*$^{+/tm1a}$ male and 10 *Ulk4*$^{+/tm1a}$ female at 7~10 month. ASR was measured at 'no sound' (65 dB; BN; background noise), 70 dB, 80 dB, 85 dB, 90 dB and 120 dB of sound using a single 40 ms pulse. PPI was measured by the delivery of a tone at either 70 dB, 80 dB, 85 dB, 90 dB (prepulse) for 10 ms followed by a 100 ms gap at background noise and then a 120 dB 'startle' tone for 40 ms, with an intra-trial intervals of 25 seconds. There was no significant gender or genotype difference in ASR (A-C) and PPI (D-F).

Porsolt Swim Test (PST)

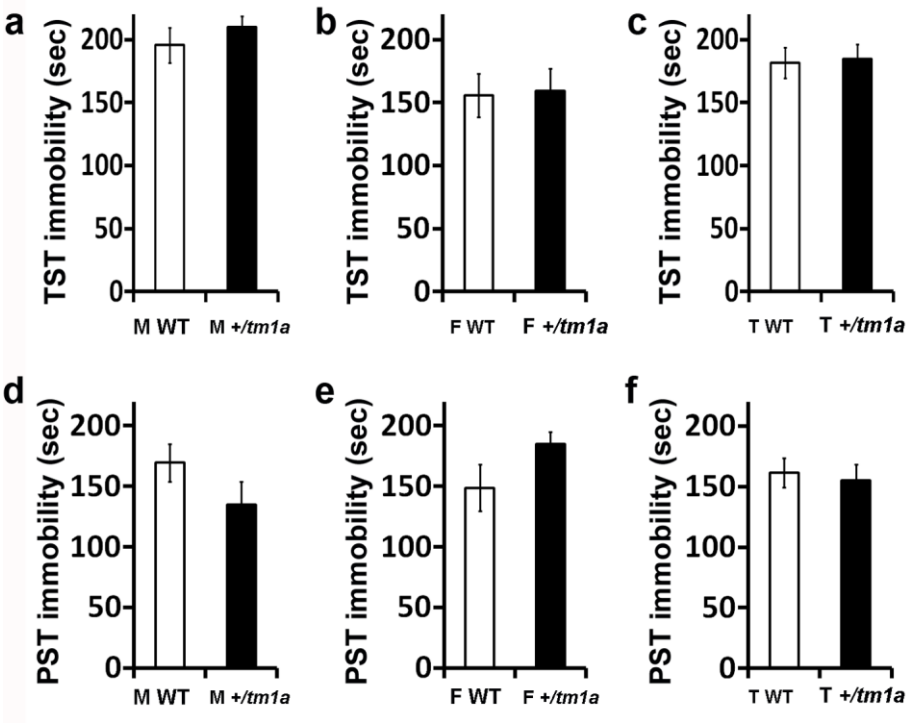
The PST is a reliable test commonly to identify depressive-like behavior in mice and rats[62-64]. In brief, mice were placed in a vertical glass cylinder filled (dimensions) with 25cm deep water at 25 ± 2 ℃ for six minutes and behavior was recorded. Mice were deemed immobile when they floated in an upright position or when only small movements were made to keep the head above water. Immobility was assessed over the six minute trial period, of which the last four minutes were used in analysis as previously described. After the trial, mouse was dried with a towel and placed in a warm cage placed on a heating pad until dry.



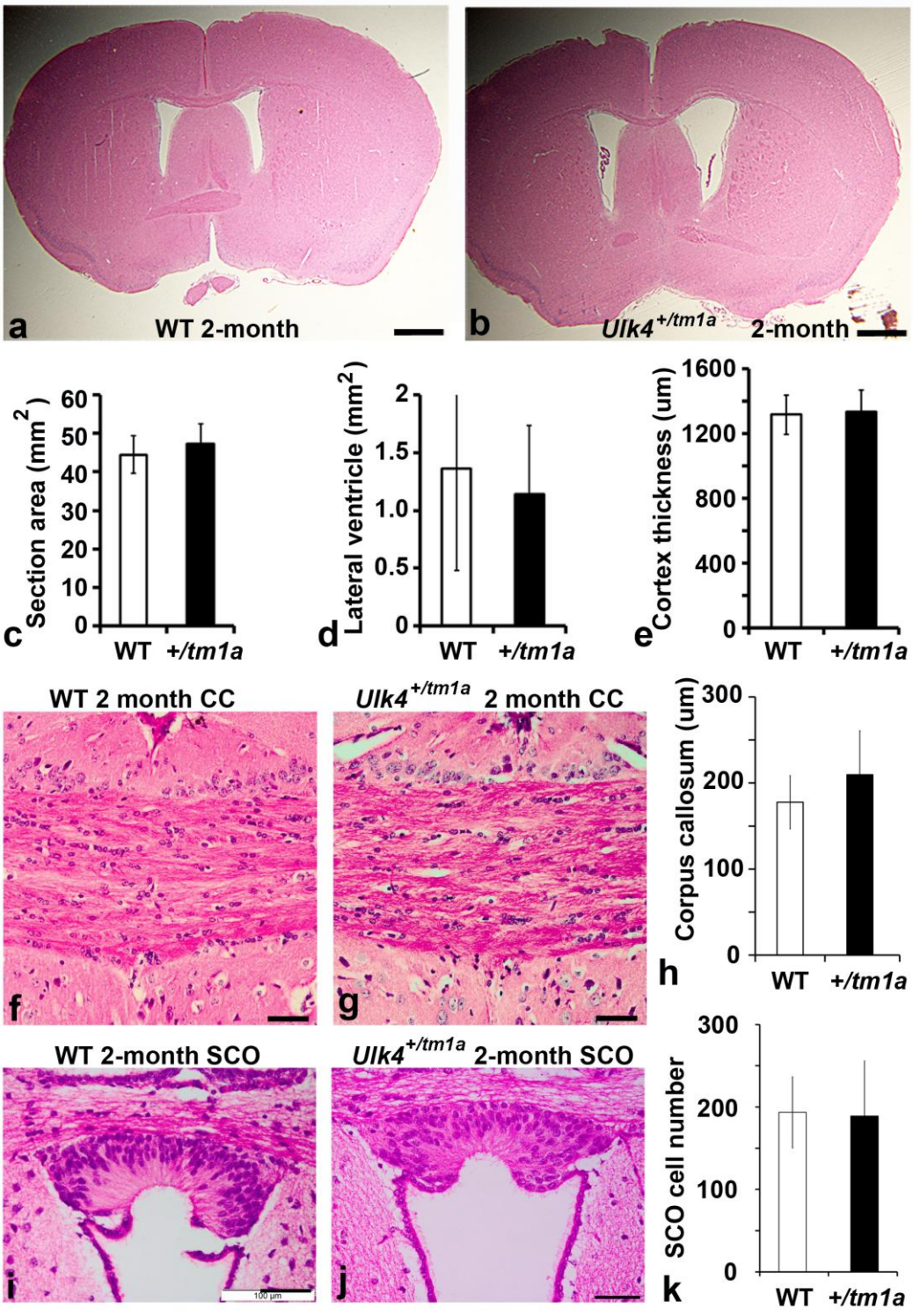
Supplemental Figure S2. PST at 2-3 month did not show significant changes in immobility time. PST was carried out in WT female, WT male, *Ulk4*$^{+/tm1a}$ male and *Ulk4*$^{+/tm1a}$ female mice, n= 7-14 per group. The duration of immobility over test time (A) and total immobility in the last 4min of the PST (B) were statistically analyzed. Data are expressed as mean ± SEM. No significant difference is detected (p>0.05).

Tail Suspension Test (TST)

The TST is a widely used test of depressive-like behavior in mice. Mice were suspended by electrical tape attached to the tail, near the tip, which was affixed to a structure that allowed the mouse to be suspended 20 cm from a catching cage. The trial lasted for 6 minutes. Immobility was defined as the lack of all movement except that required for respiration. At the completion of the trial, the mouse was returned to the home cage [64].



Supplemental Figure S3. *Ulk4*$^{+/tm1a}$ **mice show no depressive phenotype a 7-10 month.** Tail suspension test (TST, A-C) and Porsolt Swim Test (PST, D-F) were carried out on 16 WT male, 9 WT female, 13 *Ulk4*$^{+/tm1a}$ male and 13 *Ulk4*$^{+/tm1a}$ female mice. No genotype- or sex-specific effect was found in accumulated immobility duration in TST (A-C, $p>0.05$) or PST (D-F, $p>0.05$). F for male, M for male and T for total mice tested.



Supplemental Figure 4. No significant neuroanatomical differences between 2-month WT (A,F, I) and *Ulk4*$^{+/tm1a}$ (A, F, I) mouse brains. Morphomertric analyses were carried out to quantify the brain section sizes (A-C), the LV size (D), the cortex thickness (E), the thickness of corpus callosum (F-H), and SCO cell number (I-K). No statistical difference was found (p>0.05 for all).