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Supplemental Information

Trio Haploinsufficiency Causes Neurodevelopmental Disease-Associated Deficits

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Supplemental Information

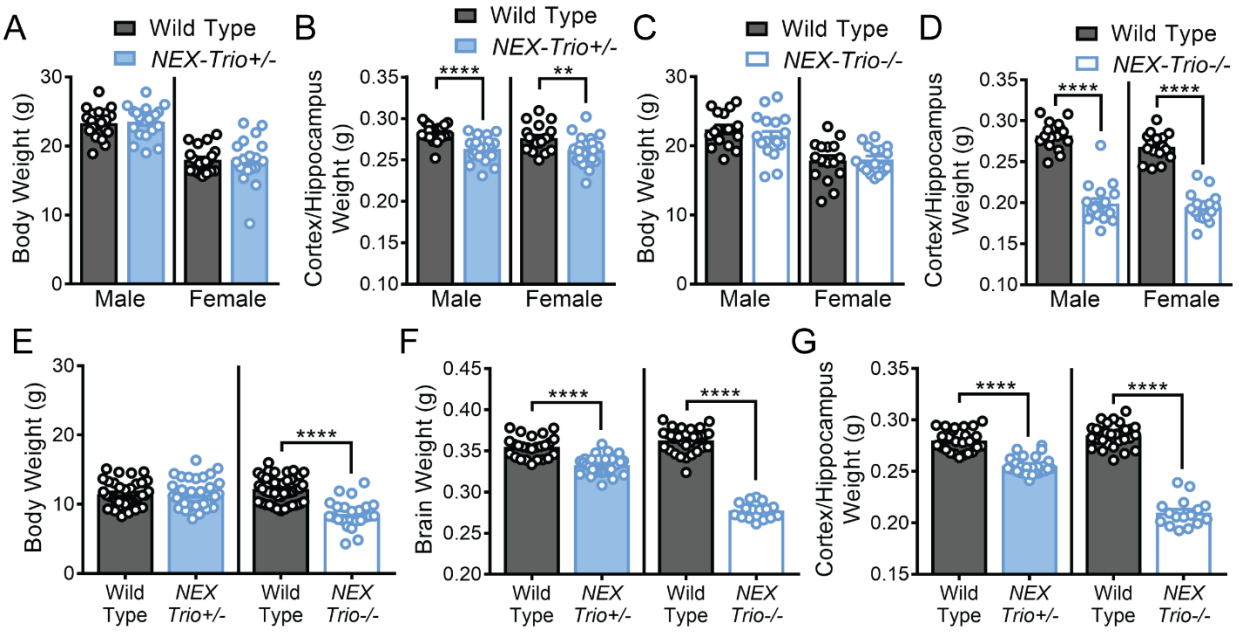

Figure S1. *NEX-Trio*+/- and *NEX-Trio*-/- mice have similar body weights yet smaller brains than sex-matched littermates. *Related to Figure 1.*

(A, C) Body weight does not differ significantly between *NEX-Trio*+/- (A) or *NEX-Trio*-/- (C) mice and WT mice of the same sex at P42.

(B, D) There was a significant reduction in the combined weight of the cortex and hippocampus between *NEX-Trio*+/- (B) or *NEX-Trio*-/- (D) mice and WT mice of the same sex at P42. RM 2-way ANOVA with post-hoc Bonf MC test identified differences (n = 16-21 mice per genotype).

(E) Body weight does not differ significantly between *NEX-Trio*+/- mice and WT mice at P21. Body weight was significantly decreased in *NEX-Trio*-/- mice relative to WT controls at P21.

(F, G) There was a significant reduction in the total brain weight (F) and combined weight of the cortex and hippocampus (G) between *NEX-Trio*+/- or *NEX-Trio*-/- mice and WT mice at P21. Unpaired t-tests identified differences between groups (n = 16-46 mice per genotype).

Data are represented as mean ± SEM. (**p<0.01, ****p<0.0001)

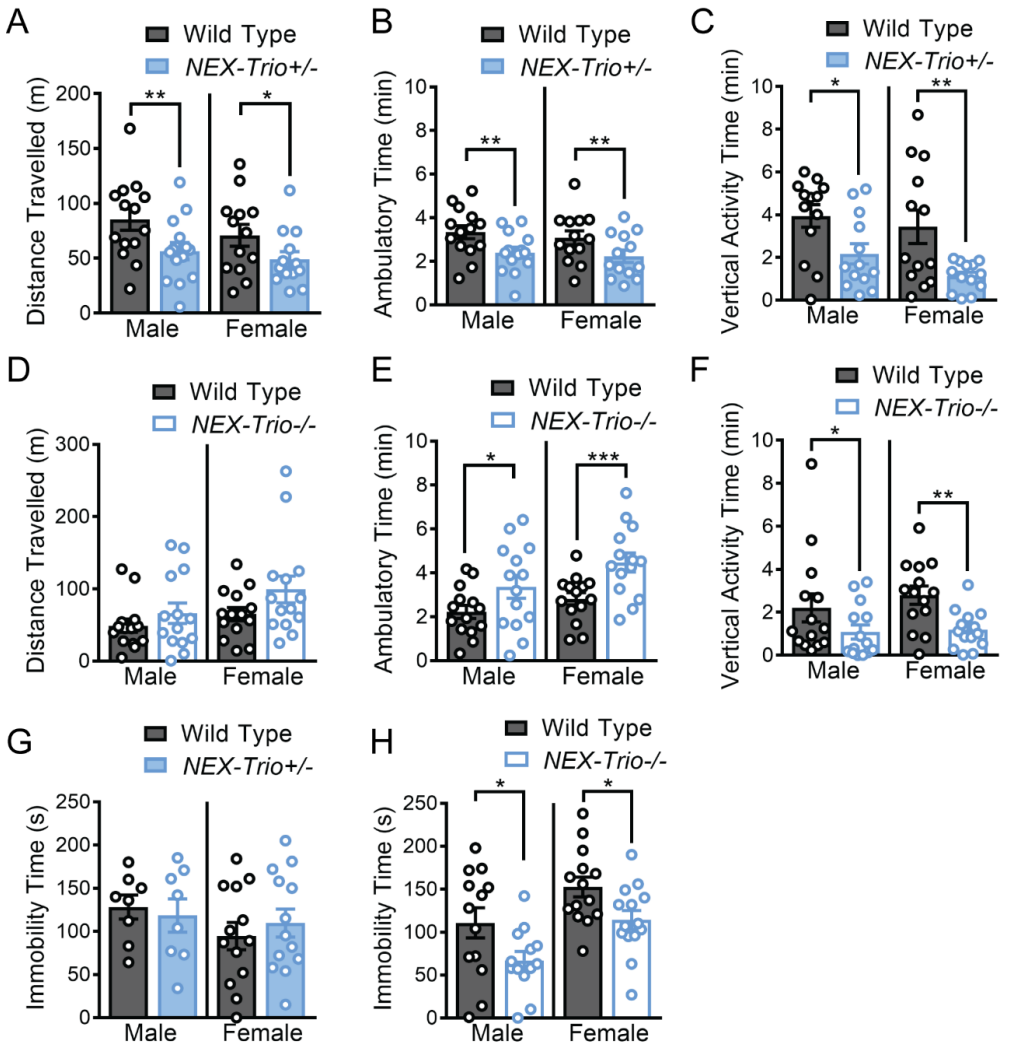

Figure S2. *NEX-Trio*+/- mice show decreased locomotor activity in the open field test (OFT) and no behavioral despair. *Related to Figures 2 and 3.*

(A, B, C) During the open field test (OFT), *NEX-Trio*+/- mice of both sexes traveled less distance overall (A), spent less time ambulating (B), and spent less time rearing (C) than WT littermates.

(D, E, F) Male and female *NEX-Trio*-/- mice showed no difference in distance travelled (D), spent more time ambulating (E), and spent less time rearing (F) relative to WT littermates in the OFT. RM 2-way ANOVA with post-hoc Bonf MC test identified differences (n = 13-14 littermate pairs).

(G) Male and female *NEX-Trio*+/- mice showed no change in immobility time during the forced swim test (FST) relative to WT littermates.

(H) Male and female *NEX-Trio*+/- mice spent significantly less time immobile during the FST relative to WT littermates. RM 2-way ANOVA with post-hoc Bonf MC test identified differences (n = 8-16 littermate pairs).

Data are represented as mean ± SEM. (*p<0.05, **p<0.01, ***p<0.001)

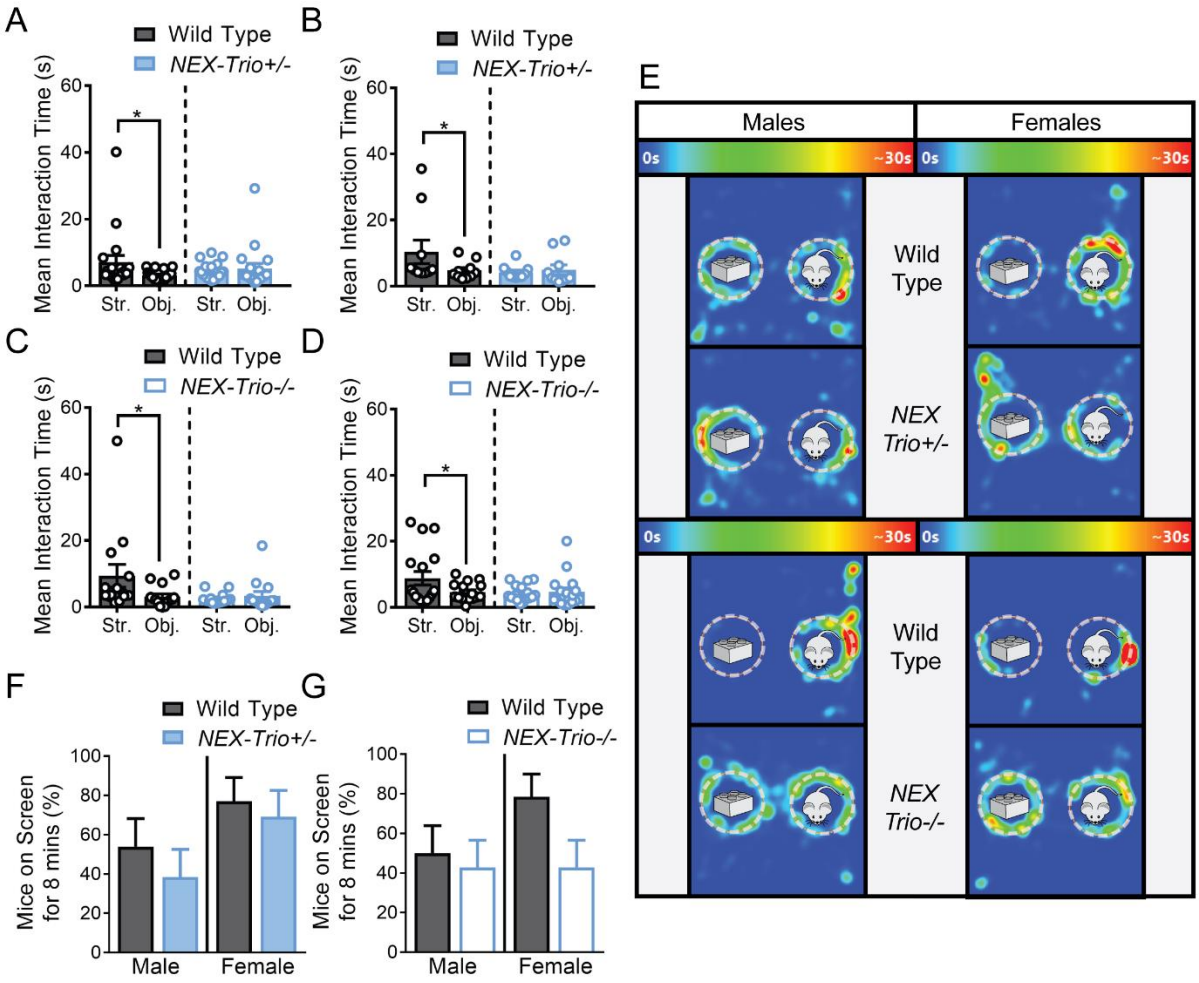

Figure S3. *NEX-Trio+/-* and *NEX-Trio-/-* mice do not spend more time with the stranger mouse, relative to the object, per visit and show no change in muscle strength. *Related to Figures 2 and 3.*

(A, B) Unlike WT mice, male (A) and female (B) *NEX-Trio+/-* mice spent equal time per visit interacting with the stranger mouse (Str.) and the object (Obj.) (mean interaction time).

(C, D) Unlike WT mice, male (C) and female (D) *NEX-Trio-/-* mice spent equal time per visit interacting with the stranger mouse (Str.) and the object (Obj.) (mean interaction time). RM 2-way ANOVA with post-hoc Bonf MC test identified differences (n = 10-18 littermate pairs).

(E) Representative heat maps show the amount of time (0 s in purple; >30 s in red) that the test mouse spent in each location (nonsocial zone, Duplo block; social zone, gray mouse).

(F, G) Male and female *NEX-Trio+/-* mice (F) and *NEX-Trio-/-* mice (G) successfully completed the inverted screen test at the same rate as WT littermates. N-1 Chi Square tests identified differences (n = 12-14 littermate pairs).

Data are represented as mean ± SEM. (*p<0.05)

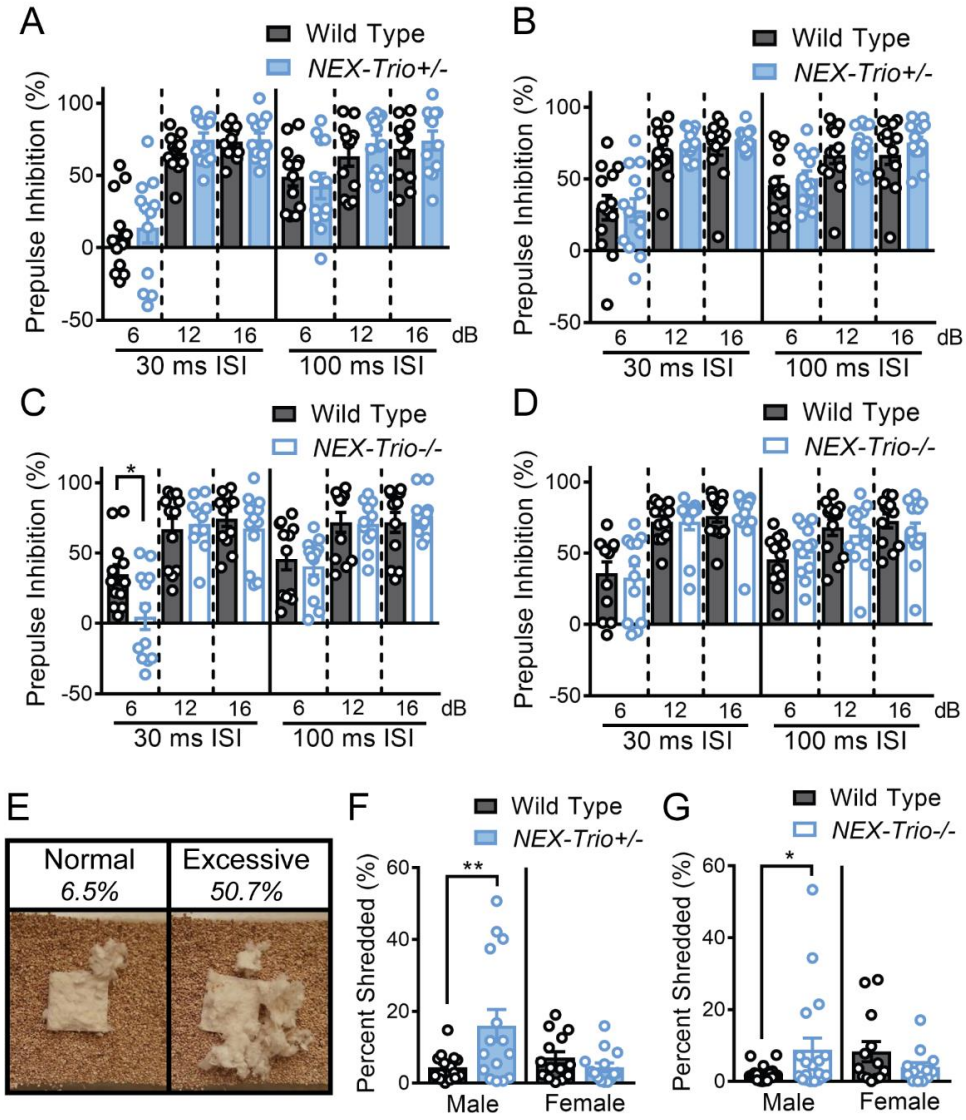

Figure S4. Some male *NEX-Trio+/-* and *NEX-Trio-/-* mice have deficits in sensorimotor gating and nestlet shredding. *Related to Figures 2 and 3.*

(A, B, C, D) Male *NEX-Trio+/-* (A), female *NEX-Trio+/-* (B), male *NEX-Trio-/-* (C), and female *NEX-Trio-/-* (D) mice displayed normal prepulse inhibition of acoustic startle with 30 or 100 ms interstimulus intervals (ISI) for all prepulse intensities (6, 12, and 16 dB), except for male *NEX-Trio-/-* mice (C) who showed significantly decreased inhibition at a 6 dB prepulse intensity with 30 ms ISI. RM 2-way ANOVA with post-hoc Bonf MC test identified differences (n = 12-13 littermate pairs).

(E) Representative images of nestlets showing both normal shredding (6.5%) and excessive shredding (50.7%).

(F, G) Male *NEX-Trio+/-* (F) and *NEX-Trio-/-* (G) mice showed a significant increase in the percentage of nestlet shredded over 30 minutes. Female *NEX-Trio+/-* (F) and *NEX-Trio-/-* (G) mice shredded the same percentage of nestlet as WT. RM 2-way ANOVA with post-hoc Bonf MC test identified differences (n = 13-19 littermate pairs).

Data are represented as mean ± SEM. (*p<0.05, **p<0.01)

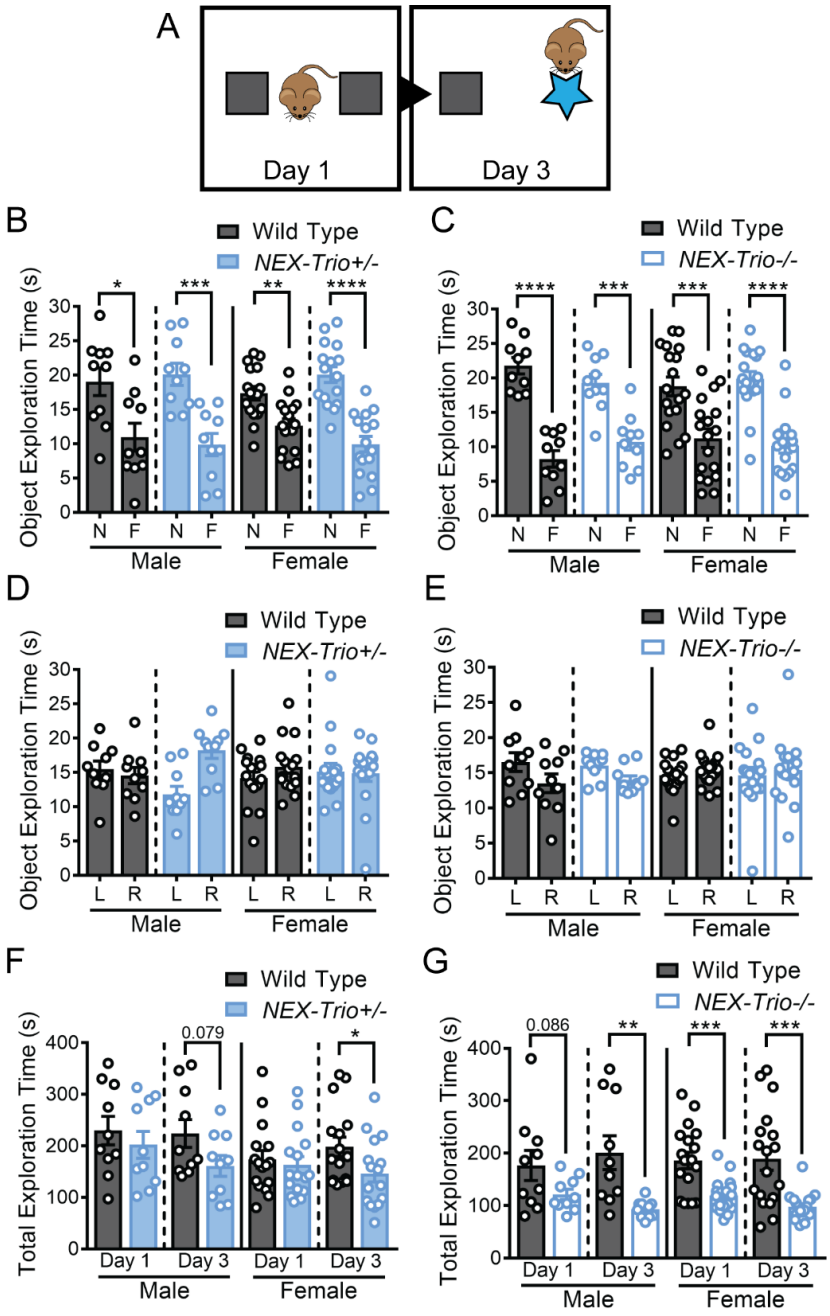

Figure S5. *NEX-Trio*+/- and *NEX-Trio-/-* mice showed normal object recognition. *Related to Figures 2 and 3.*
(A) The hippocampus-dependent novel object recognition task involved familiarizing the mice with an object on Day 1 and allowing the mouse to discriminate between the familiar object and a novel object on Day 3. WT mice preferred the novel object on Day 3.
(B, C) *NEX-Trio*+/- (B) and *NEX-Trio-/-* (C) mice of both sexes displayed normal discrimination between novel (N) and familiar (F) objects on Day 3.
(D, E) *NEX-Trio*+/- (D) and *NEX-Trio-/-* (E) mice of both sexes displayed normal object familiarization between objects on the left (L) and right (R) sides of the cage on Day 1.
(F, G) *NEX-Trio*+/- (F) and *NEX-Trio-/-* (G) mice accumulated 30 seconds of exploration time in significantly less overall time than WT littermates on day 3 and days 1/3, respectively. A linear regression with post-hoc Bonf MC test identified differences (n = 10-18 littermate pairs).
Data are represented as mean ± SEM. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

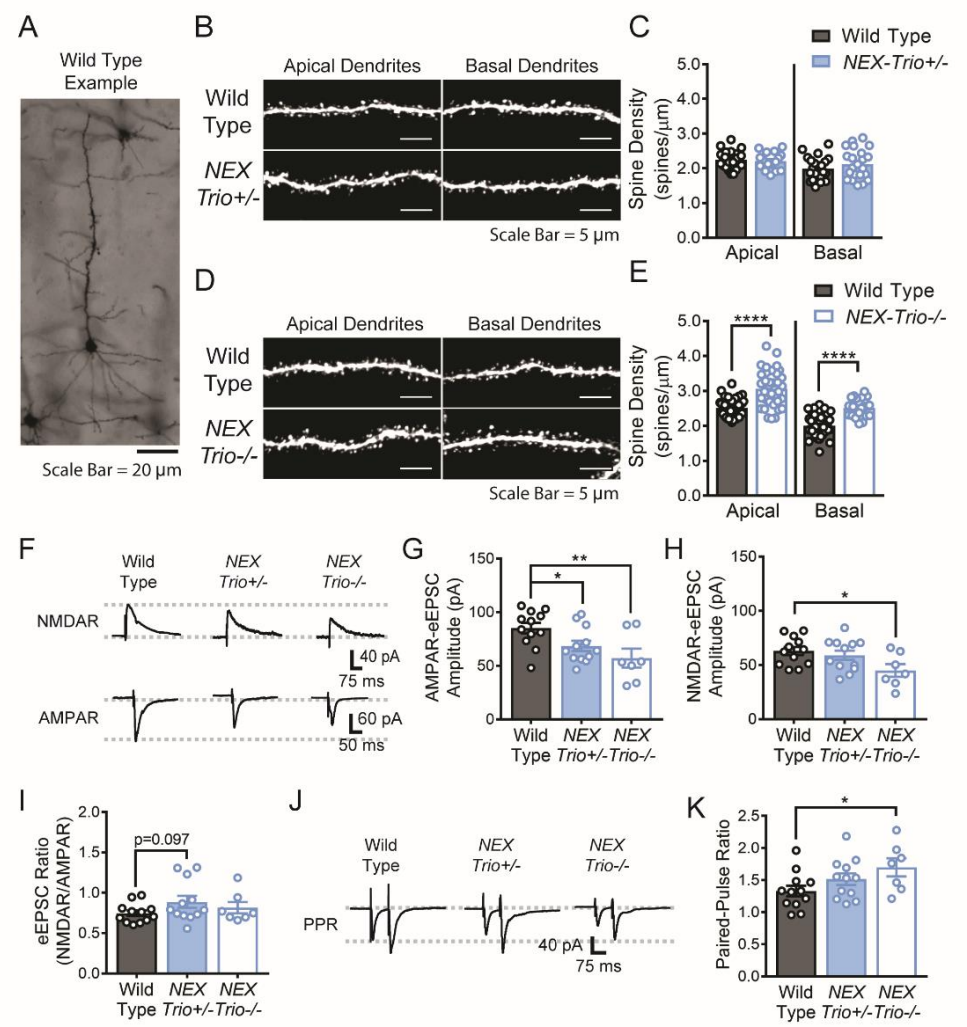

Figure S6. *NEX-Trio-/-* mice have increased dendritic spine density and paired-pulse ratio in the hippocampus.
Related to Figures 4, 5, and 6.

(A) Representative Golgi-stained neuron used for dendritic arbor reconstructions. Scale bar = 20 µm.

(B, D) Representative dendrite segments from the hippocampus (apical and basal dendrites) of *NEX-Trio+/-* (B) and *NEX-Trio-/-* (D) mice with WT controls. Scale bar = 5 µm.

(C) Dendritic spine density was not significantly changed on the apical or basal dendrites of hippocampal CA1 neurons in *NEX-Trio+/-* mice relative to WT controls.

(E) Dendritic spine density was significantly increased on the apical and basal dendrites of hippocampal CA1 neurons in *NEX-Trio-/-* mice relative to WT controls. A linear regression with post-hoc Bonf MC test identified differences (n = 18-44 dendrite segments from ≥3 mice per group).

(F) Representative traces of NMDAR- and AMPAR-eEPSCs from *NEX-Trio+/-*, *NEX-Trio-/-*, and WT hippocampal CA1 neurons.

(G) The amplitudes of AMPAR-eEPSCs were decreased in the hippocampus of *NEX-Trio+/-* and *NEX-Trio-/-* mice.

(H) The amplitudes of NMDAR-eEPSCs were only decreased in the hippocampus of *NEX-Trio-/-* mice.

(I) There is a trend toward increased NMDAR/AMPAR eEPSC ratio in the hippocampus of *NEX-Trio+/-* mice (p = 0.097) but no change in *NEX-Trio-/-* mice.

(J) Representative traces of eEPSCs are shown from *NEX-Trio+/-*, *NEX-Trio-/-*, and WT hippocampal CA1 neurons evoked by paired-pulse stimulation of hippocampal CA3 Schaffer collateral axons at 75 ms interstimulus intervals.

(K) An increase in the paired-pulse ratio was observed in *NEX-Trio-/-* mice, but no change was observed in *NEX-Trio+/-* mice. To consistently analyze *NEX-Trio+/-* and *NEX-Trio-/-* mice independently, unpaired t-tests identified differences between groups (n = 7-12 neurons from ≥3 mice per group).

Data are represented as mean ± SEM. (*p<0.05, **p<0.01, ****p<0.0001)

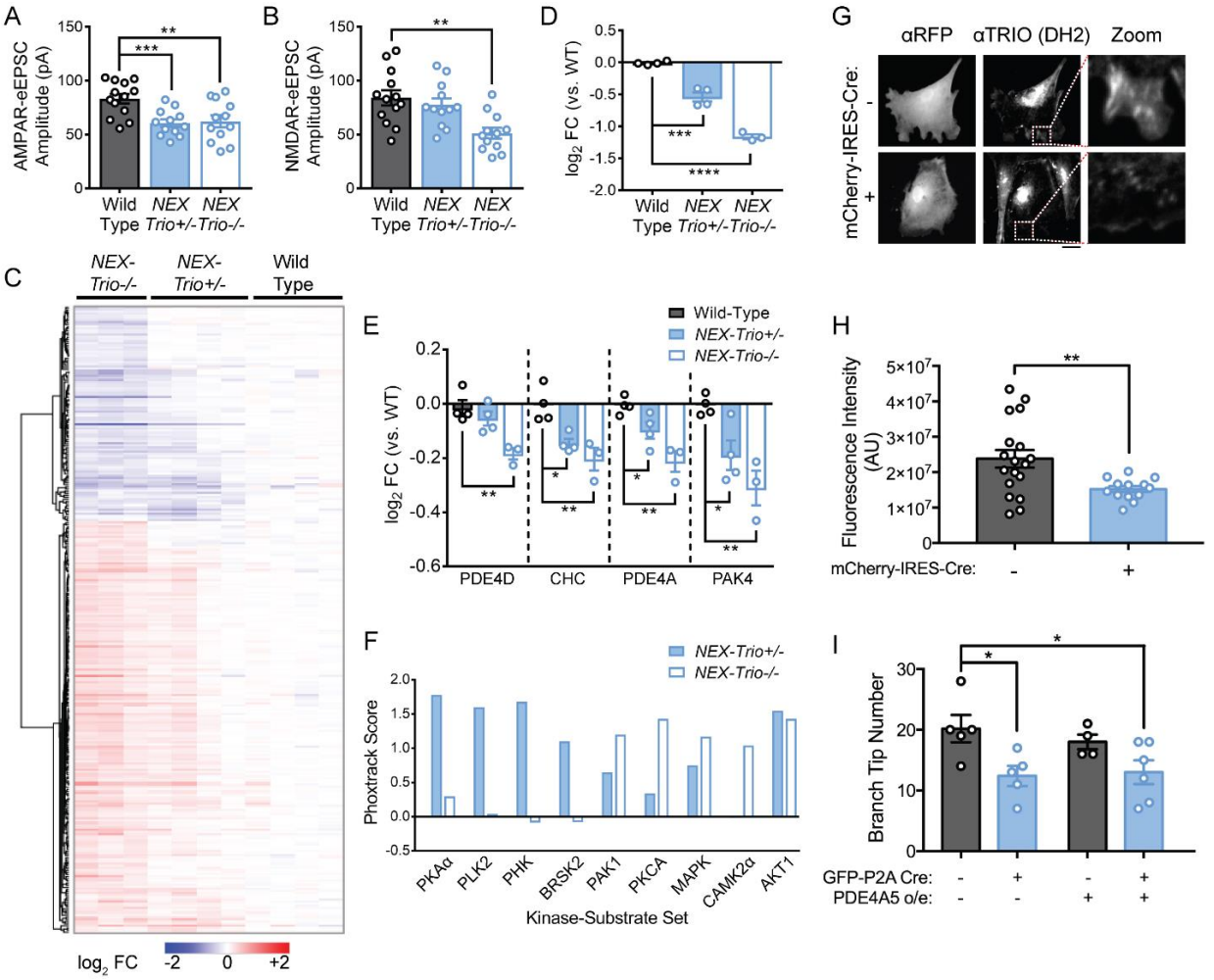

Figure S7. Proteomic and phosphoproteomic analyses reveal that loss of *Trio* alters protein levels and phosphorylation levels in multiple molecular pathways. *Related to Figures 6 and 7.*

(A) The amplitudes of AMPAR-eEPSCs were decreased in motor cortex of *NEX-Trio+/-* and *NEX-Trio-/-* mice.

(B) The amplitudes of NMDAR-eEPSCs were only decreased in motor cortex of *NEX-Trio-/-* mice. To consistently analyze *NEX-Trio+/-* and *NEX-Trio-/-* mice independently, unpaired t-tests identified differences between groups (n = 12-13 neurons from ≥3 mice per group).

(C) Hierarchically clustering of the 294 proteins identified to be differentially abundant (adj. p-value < 0.05) in the motor cortex between WT, *NEX-Trio+/-*, and *NEX-Trio-/-* mice. Individual replicates are shown.

(D) TRIO $\log_2$ fold change (FC) levels were reduced in *NEX-Trio+/-* and *NEX-Trio-/-* mice by mass spectrometry.

(E) The $\log_2$ fold change (FC) levels of PDE4D, CHC, PDE4A, and PAK4 were reduced in *NEX-Trio+/-* and/or *NEX-Trio-/-* mice compared to WT by mass spectrometry. To consistently analyze *NEX-Trio+/-* and *NEX-Trio-/-* mice independently, unpaired t-tests identified differences between groups (n = 3-4 mice per group).

(F) Phoxtracks enrichment score for all kinase-substrate pair sets that were enriched in *NEX-Trio+/-* and/or *NEX-Trio-/-* mice by quantitative phosphoproteomics (p-value < 0.05). Positive values indicate increased kinase activity.

(G) Representative immunofluorescence images of *Trio$^{flox/flox}$* fibroblast cells with or without Cre vector. Cells were immunostained 6 days after transfection for RFP to visualize the outline of individually transfected cells and for TRIO using an anti-DH2 antibody to visualize changes in TRIO levels. Scale bar = 10 μm.

(H) Cre expression in *Trio$^{flox/flox}$* fibroblast cells resulted in a 36% reduction in TRIO immmunopositive signal 6 days after transfection; some of the remaining signal is likely cross-reactivity with Kalirin. Unpaired t-tests identified differences (n = 13-18 cells per group).

(I) *Cre-Trio+/-* cortical neurons showed a reduction in branch tip number 6 days after transfection; this deficit was not rescued by PDE4A5 overexpression. Unpaired t-tests identified differences (n = 5-6 cells per group).

Data are represented as mean ± SEM. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)