SUPPLEMENTAL INFORMATION

Supplemental Figure Legends

Figure S1 *(related to figure 1)*

(A-D) Validation of the specificity of the affinity purified anti-Neurl1 antibody. (A) Immunoblot detection of different forms of Neurl1 transiently expressed in HEK293T cells (i). The antibody detects the full-length Neurl1 protein and a truncated form lacking the last 100 C-terminal aminoacids (Neurl1ΔC). The antibody cannot detect Neurl1 lacking the first 194 N-terminal aminoacids (Neurl1ΔN), which contain the epitope. Neurl1 proteins are not detected when the anti-Neurl1 antibody has been pre-incubated for 1h at room temperature with the peptide (amino acids 27–42) that was used for the production of the anti-serum (ii). (iii) Immunoblot of lysates from HEK293T cells transfected with plasmid expressing the full length Neurl1 (1) and of hippocampal homogenates from adult (3 ½ months) wild type mice (2). Untransfected HEK293T cells were used as control (3). The anti-Neurl1 antibody recognizes a protein band of the same molecular size (~60kD) in both Neurl1-expressing HEK293T and hippocampal homogenates.

(B) Confocal micrographs (stack of series) showing immunostaining of cultured hippocampal neurons using the affinity purified anti-Neurl1 antibody. The neurons were transfected with either plasmid encoding for GFP and the control vector (mock) or GFP and plasmid encoding for shRNA specific for Neurl1 (shRNA-95 and shRNA-97). Neurl1 immunostaining was significantly reduced in the cell body and dendrites compared to the surrounding untransfected neurons only when the Neurl1-specific shRNAs were transfected, indicating the specificity of antibody against Neurl1 protein.

(C) Western blot analysis of protein lysates from HEK293T cells either co-trasnsfected with plasmids encoding for Neurl1 and Neurl1-specific shRNAs or co-transfected with the plasmid encoding for Neurl1 and the empty vector used for the expression of the shRNAs (mock). Neurl1 was detected with the

affinity purified anti-Neurl1 antibody. Plasmid encoding for GFP was also co-transfected for normalization. Alpha tubulin is loading control. Two different shRNAs were used (shRNA-97 and shRNA98). The Neurl1-specific shRNAs significantly reduce the levels of the expressed Neurl1 in a dosage-dependent manner (shown for shRNA-97), indicating the specificity of the anti-Neurl1 antibody. The shRNA-95 (see B) was also used. Despite that this shRNA reduces the levels of Neurl1 in hippocampal neurons, it did not affect the levels of transiently expressed Neurl1 protein in HEK293T cells (not shown). This is because shRNA-95 is directed against the 3'-UTR of Neurl1 that is missing from the cloned cDNA sequence, confirming the specificity of the used shRNAs against Neurl1.

(D) Immunostaining of CA1 pyramidal neurons from adult wild type mice using the anti-Neurl1 antibody without (i) or with (ii) pre-incubation with Neurl1 peptide. (iii) Immunostaining with pre-immune serum.

(E) Immunostaining of CA1 hippocampal region from adult Neurl1-Flag expressing mouse (i) and single *tetO-Neurl1-Flag* control animal (ii) using anti-Flag antibody. The antibody recognizes the recombinant Neurl1-Flag protein, while no immunostaining is detected in the control mouse.

Figure S2 *(related to figure 2)*

(A) Schematic representation of the tetO/tTA system. In the double transgenic mice (DT), tTA binds to the *tetO* promoter and activates the expression of the transgene only in the forebrain where the *CaMKIIα* promoter is active. The expression of the transgenes is temporarily regulated in a reversible manner. This is because the binding of the tTA transactivator to the *tetO* promoter is inhibited by tetracycline or its analogs (doxycycline; dox).

(B) Schematic representation of the diet protocols that the Neurl1DN DT mice and their control littermates underwent prior to behavioral tasks and molecular studies. The expression of *Neurl1DN-Flag* transgene is shown.

(C) Expression of Neurl1DN-Flag in the adult forebrain. (i) *In situ* hybridization analysis of *Neurl1DN-*

Flag mRNA on sagittal brain sections from adult (P95) DT mice tested off dox (gene on) or on dox (gene off) (on/off and on/off/on diet protocols, respectively; see B). (ii) Western blot showing the expression of Neurl1DN-Flag and endogenous Neurl1 proteins in the hippocampus of 95-day-old DT animal off dox (DT). DT mouse on dox and *tetO-Neurl1DN-Flag* single transgenic mouse (st) are negative controls. (iii) Confocal sections of CA1 pyramidal neurons from adult DT mouse off dox. Coimmunostaining with anti-Neurl1 (detection of endogenous Neurl1 and Neurl1DN-Flag proteins) and anti-Flag antibodies (detection of Neurl1DN-Flag) shows that both proteins are expressed in the same CA1 neurons and exhibit similar localization.

(D) Data from the Morris water maze which complement the data in figures 2A and B (DT off dox: Neurl1DN expressing mice). Mean escape latencies ($\pm$ SEM) plotted against the day of the experiment. Control and DT displayed similar performance in the visible version of the task (repeated-measures ANOVA; training day effect: $p<0.0001$; genotype effect: $p=0.9128$). Hidden version of the maze (acquisition and transfer): DT and control reduced their latencies in a similar way (repeated-measures ANOVA; $p>0.12$). All groups learned the tasks by the end of training (repeated-measures ANOVA for day effect; Acquisition: controls: $p < 0.0001$ & DT: $p < 0.0016$; Transfer: controls: $p < 0.0001$; DT: $p < 0.0021$).

(E) Data from Morris water maze from a group of Neurl1DN DT mice and their control littermates kept on dox during the task. (i) DT and control on dox displayed similar performance during the acquisition phase of the hidden platform [repeated-measures ANOVA; significant effect of training day ($p<0.0001$) but not genotype ($p >0.58$)]. Both groups displayed similar performance in probe trial 1 day after the end of training (repeated-measures ANOVA; genotype effect: $p>0.15$; genotype*quadrant effect: $p>0.37$). (ii) DT and control on dox learned equally well the new platform location [repeated-measures ANOVA; significant effect of training day ($p<0.0001$) but not of genotype ($p>0.67$)]. Both groups exhibited similar performance in probe trial 1 day after the end of training (repeated-measures ANOVA; genotype effect: $p>0.14$; genotype*quadrant effect: $p>0.42$). These data confirm that the memory deficits in the

Neurl1DN expressing mice (DT off dox) do not have any developmental aetiology and do not result from position effects of the transgenes.

(F) LTP in Neurl1DN DT mice and control siblings on dox in adulthood. LTP induced by 1TBS was similar in DT and controls (average of the whole recording, p=0.41).

See also Table S1.

Figure S3 *(related to figure 3)*

(A) Neurl1 overexpression in the adult forebrain. (i) RNA *in situ* hybridization analysis of *Neurl1* (right panel) and *Neurl1-Flag* (left panel) on sagittal brain sections from adult (P95) double transgenic (DT) animals tested off dox or on dox (on/off and on/off/on diet protocols, respectively). (ii) Western blot and RNA *in situ* hybridization showing the expression of Neurl1-Flag protein and transgene in the hippocampus of 95-day-old DT animal off dox (DT). DT mouse on dox (*Neurl1-Flag* OFF) and *CaMKIIa-tTA* single transgenic mouse (st) are negative controls. (iii) Western blot of total Neurl1 protein in the adult hippocampus. Averaged fold difference ($\pm$ SEM) of Neurl1 protein level in the adult hippocampus is also shown [comparisons with control (ctl); DT: n=5; DT on dox: n=3; ctl (pooled): wild type: n=2, single tetO: n=3, single tTA: n=2)]. Neurl1 level in DT is significantly increased ($p<0.0001$). There is no difference in control and DT on dox ($p=0.9820$). *$p<0.0001$. (iv) Confocal sections of CA1 pyramidal neurons from adult Neurl1 overexpressing mouse. Coimmunostaining with anti-Neurl1 (detection of endogenous and recombinant Neurl1 proteins) and anti-Flag (detection of recombinant Neurl1) antibodies. Endogenous and recombinant Neurl1 proteins are expressed in the same CA1 neurons and exhibit similar localization.

(B) Data from the Morris water maze which complement those in figure 3A. (i) Mean escape latencies $\pm$ SEM. Acquisition and transfer: DT reduced their escape latencies significantly faster than controls (repeated-measures ANOVA; significant genotype effect; Acquisition: $p=0.001$; Transfer: $p=0.0224$). (ii)

DT and control spent more time in the training quadrant (TQ) [ANOVA for quadrants 1-4; $p<0.0001$ for both groups; post hoc analysis: significant preference for TQ (week1/day5: controls: $p<0.002$ & DT: $p<0.0001$; week2/day5: $p<0.0001$ for both groups]. There was no significant genotype or genotype*quadrant effects (repeated-measures ANOVA; $p>0.34$ and $p>0.083$, respectively). However, DT formed an accurate knowledge of the platform location faster than controls as they crossed it significantly more often in the probe trial at week1/day5 (see figure 3A). (iii) DT spent significantly more time searching in the new training quadrant than controls in the probe trial at week1/day5 (repeated-measures ANOVA; genotype*quadrant effect: $p=0.0002$; t test for training quadrant: $^{**}p<0.0001$), and 8 days after the end of training [repeated-measures ANOVA; genotype*quadrant effect: $p=0.0177$; t test for training quadrant: $p=0.0076$ (*)]. TQ: training quadrant. PQ: TQ in (ii).

(C) (i) LTP induced by 1 TBS was still enhanced in the presence of 100 μM of the $GABA_A$ inhibitor picrotoxin (1hr recording; mean % of baseline: unpaired t-test: $p<0.001$). The membrane resting potential (ii), input resistance (iii) and capacitance (iv) in controls and DT were similar ($p> 0.65$). There was no increase in the number of CA3 axons in DT as the input-output relationship for the fiber volley amplitude was similar in DT and control (v) (repeated-measures ANOVA; $p=0.3$).

(D) Data from Morris water maze of Neurl1 DT and control littermates on dox in adulthood. Both groups displayed similar performance during acquisition (i) and probe trials (ii) [repeated-measures ANOVA; Acquisition: significant effect of training day ($p<0.0001$) but not genotype ($p>0.63$); Probe trials: no genotype effect ($p>0.43$)]. They also learned equally well the new platform location (iii) and displayed similar performance in the probe trials (iv) [repeated-measures ANOVA; Learning: significant effect of training day ($p<0.0001$) but not of genotype ($p>0.20$); Probe trials: no genotype effect; ($p>0.1$)]. These results confirm that the enhanced learning and memory in the Neurl1 overexpressing mice (DT off dox) does not have any developmental aetiology and does not result from position effects of the transgenes (tetO & tTA).

(E) LTP in Neurl1 DT and control littermates on dox in adulthood. LTP in both groups was similar (1x100Hz and 4x100Hz: average of the whole recordings' durations: $p>0.5$; 1TBS: average of the 3hr recording: $p=0.33$). No differences were observed for input/output (iv) and PPR (v) (average of the 3hr recording: input/output: $p>0.5$; PPR: $p>0.05$).

See also Table S2.

Figure S4 *(related to figure 4)*

(A) Neurl1 overexpression in the adult hippocampus does not affect the arborization (i & ii) and the total length (iii) of the apical dendrites of CA1 neurons. (i & ii) Sholl analysis. (iii) Mean dendritic length $\pm$ SEM. No differences were observed among all groups of mice ($p>0.5$). ON & OFF: on & off dox in adulthood, respectively. See also figure 4A.

(B) Neurl1 and CPEB3-Ub_{KO} overexpression do not change the relative surface expression of GluA1receptors in the basal state or after glutamate application in cultured hippocampal neurons (16 DIV). Surface GluA1 receptors at the basal state and 15min after glutamate application. Neurl1: neurons infected with Neurl1-Flag lentivirus (Neurl1 overexpression). CPEB3-Ub_{KO}: neurons expressing CPEB3 fused to single ubiquitin that cannot form poly-Ub chains. Mock: neurons infected with control lentivirus.

Surface GluA1 receptors were isolated using Neutravidin beads and detected by western blotting with anti-GluA1 antibody (i). Immunoblot analysis of total GluA1 was used for normalization. Control: No biotinylation prior to pull downs (CPEB3-Ub_{KO} neurons). (ii) Mean percentage $\pm$SEM of surface GluA1 receptors compared to their total amount for each type of neurons described in (i) from 3 independent experiments. No difference was found (comparisons with mock; $p>0.94$). Inset: Averaged fold difference $\pm$ SEM of relative amount of surface GluA1. No difference was observed ($p>0.54$). (iii) Remaining surface GluA1 receptors 15min after glutamate application (comparisons with respective surface GluA1 in the basal state). No differences were observed ($p>0.82$). See also Table S3.

(C) Overexpression of Neurl1 does not affect the internalization of GluA1 receptors either in the basal state or in response to glutamate stimulation. Detection (i) and quantification (ii and iii) of internalized GluA1 in cultured hippocampal neurons (16 DIV) infected with control lentivirus (mock) or lentivirus expressing Neurl1-Flag (Neurl1-overexpression; Neurl1). Surface GluA1 receptors were first biotinylated and then the neurons were kept at 37^0C for 15 minutes with or without stimulation with glutamate. The conjugated biotin to the remaining surface GluA1 receptors was removed using glutathione-stripping buffer. The protected (internalized) GluA1 receptors were isolated using NeutrAvidin beads and detected by immunoblotting with GluA1-specific antibody (i). Normalization was performed by immunoblot analysis of the respective total GluA1 used for NeutrAvidin pull downs. NeutrAvidin-isolated GluA1 receptors at 0min and without biotin stripping represent the initial levels of surface receptors. Biotinylated GluA1 receptors detected in all the other conditions represent the amount of the initial surface receptors that have been internalized. Right panel: independent experiment testing the efficiency of the stripping buffer. (ii) & (iii) Averaged data ($\pm$ SEM) from 3 independent experiments. No differences were found ($p > 0.59$). Comparisons were made with mock (ii) or the respective initial levels of surface GluA1 (iii). See also Table S3.

(D) Overexpression of Neurl1 in the adult hippocampus does not affect the mRNA levels (i) and the degradation rate (ii & iii) of GluA1 and GluA2. (i) RNA *in situ* hybridization (probes: GluA1A and GluA2B; Extended Experimental Procedures) on adult brain slices from Neurl1 DT and control mice (st1 and st2: single tetO and single tTA, respectively). (ii) Western blot analysis of hippocampal GluA1 and GluA2 90min after subcutaneous injection of anisomycin (+) or equal volume of saline (-). Each lane represents different animal. The averaged decrease $\pm$ SEM of GluA1 and GluA2 90 min after injection of anisomycin is shown (see also "Extended Experimental Procedures"). The net change was calculated by comparing the protein levels in the anisomycin-injected versus the vehicle-injected animals of the same genotype. No differences were found ($p>0.5$). See also table S4. (iii) ^{35}S-GluA1 and ^{35}S-GluA2 degradation in hippocampal cultures (16DIV) after a 1hr pulse of ^{35}S-Met/^{35}S-Cys. Mean $\pm$ SEM from 3

independent experiments (see also figure 4F and Table S4). Neurl1: neurons overexpressing Neurl1. Mock: neurons infected with control lentivirus. No differences were found ($p>0.5$). See also Table S4.

(E) Western blot analysis (i) and mean fold difference ± SEM (ii) of hippocampal lysates from Neurl1DN expressing (DT), control, and DT on dox mice. (i) St1 & St2: tetO-*Neurl1DN-Flag* and *CaMKIIα-tTA* single transgenic mice, respectively. Each lane represents different animal. (ii) CPEB3, GluA1 and GluA2 were significantly reduced in DT compared to control and DT on dox (*$p<0.0001$). (iii) Averaged fold difference ± SEM of mRNA levels (real-time qPCRs). No differences were found ($p>0.5$). See also Table S4.

(F) Expression of Neurl1DN reduces the translation of GluA1 and GluA2 mRNAs without affecting the degradation rate of the encoded proteins in cultured hippocampal neurons (16DIV). (i) Time course of ^{35}S-GluA1 and ^{35}S-GluA2 levels after a 1hr pulse of ^{35}S-Met/^{35}S-Cys. GluA1 and GluA2 were immunoprecipitated from total lysates and analyzed by SDS-PAGE and autoradiography (detection of ^{35}S-GluA1 and ^{35}S-GluA2). Silver stains of isolated total GluA1 and GluA2 are shown (loading controls). Neurl1DN: neurons infected with lentivirus expressing Neurl1DN-Flag. Mock: neurons infected with control lentivirus. (ii) Averaged data (±SEM) from 3 independent experiments. Results are presented as ratio of ^{35}S-GluA1 and ^{35}S-GluA2 ± SEM at each time point compared to their levels immediately after the pulse. The rate of decrease was similar (repeated-measures ANOVA; $p>0.2$). (iii) Averaged half-life ± SEM. No differences were observed ($p=0.5$). (iv) ^{35}S-GluA1 and ^{35}S-GluA2 at 0 min after the pulse were significantly reduced in Neurl1DN neurons compared to mock (*$p<0.0001$). Inset: Averaged fold difference (±SEM) of mRNA levels (real time qPCRs). No differences were observed ($p>0.7$).

Figure S5 *(related to figure 5)*

(A) Mouse CPEB3 binds *GluA1* mRNA *in vivo* and *in vitro*. (i) Hippocampal lysates from adult wild type mice (3-4 months) were immunoprecipitated with anti-CPEB3 antibody. RT-PCR was performed

using oligonucleotides specific for *GluA1* mRNA (Extended Experimental Procedures). Lanes 3-5: GluA1 amplicons that were present in the total lysates used for the indicated IPs. GluA1 transcript was detected in the IP with anti-CPEB3 antibody (lane 7; 1/3 of sample) while it was not detected in the control IPs (lanes 6 and 8; beads and IgG, respectively). Lane 9: total input used for IP with anti-CPEB3 antibody. (ii) GluA1 3'-UTR fragment used in EMSA experiments corresponds to the amplicon from (i). Dig-labeled fragments of the *GluA1* 3'-UTR were incubated in the presence of CPEB3 (lanes 2-4). Control reactions were performed in buffer alone (n.p; lane 1) or in 10 fold molar excess of unlabeled probe (lanes 3 and 4; c). The reactions were analyzed on native polyacrylamide gel. Unbound RNA fragments (free probe) and RNA-protein complexes (*) are indicated. The formation of RNA-CPEB3 complex results to a shift of *GluA1* 3'-UTR molecular size (*). This interaction was abolished in the presence of 10 fold molar excess of unlabelled probe (lanes 3 and 4).

(B) Neurl1-Flag recombinant protein interacts with endogenous CPEB3 in the adult hippocampus of Neurl1-Flag expressing mice.

(C) Averaged fold difference $\pm$ SEM of normalized levels of monoubiquitinated CPEB3 in the adult hippocampus (complements figure 5Eiv). *$p<0.0003$. Monoubiquitinated CPEB3 returned to control levels when the DT mice were kept on dox in adulthood [DT vs DT on dox: significant difference (Neurl1: $p<0.0001$; Neurl1DN: $p<0.0003$); DT on dox vs controls: no significant difference ($p>0.89$)].

(D & E) Synaptic stimulation by glutamate induces Neurl1-dependent monoubiquitination of CPEB3 in hippocampal neurons.

(D) Western blot analysis of endogenous CPEB3 and Neurl1 levels 30min after glutamate application in dissociated hippocampal cultures (16DIV). Mock: Neurons expressing control lentivirus. Neurl1DN: neurons expressing Neurl1DN. (ii) and (iii) Mean fold difference $\pm$ SEM from 3 independent experiments. Application of glutamate resulted in parallel and significant increase of Neurl1 and CPEB3 in control neurons, consistent with the upregulation of CPEB3 protein levels in the Neurl1

overexpressing mice (mock/Glut(-) vs mock/Glut(+): $p<0.0001$). Similar to what we observed in Neurl1DN mice, the basal protein levels of CPEB3 were significantly reduced by Neurl1DN (mock/Glut(-) vs Neurl1DN/Glut(-): $p=0.0323$). Neurl1DN also blocked the activity-dependent upregulation of CPEB3 (Neurl1DN/Glut(-) vs Neurl1DN/Glut(+): $p=0.9925$; mock/Glut(-) vs Neurl1DN/Glut(+): $p=0.0487$). Neurl1DN did not affect the basal levels of Neurl1 or its activity-dependent increase (mock/Glut(-) vs Neurl1DN/Glut(-): $p=0.9930$; Neurl1/Glut(-) vs Neurl1DN/Glut(+): $p<0.0001$; mock/Glut(+) vs Neurl1DN/Glut(+): $p=0.4472$). *$p<0.05$, ***$p<0.0002$.

(E) Isolation of ubiquitinated CPEB3 from lysates of Neurl1DN and control neurons (mock) from (D) using sequential IP with anti-CPEB3 and anti-Ub antibodies. (i) Normalized input of CPEB3 used in IPs. (ii) Detection of ubiquitinated CPEB3. CPEB3 *(in vitro ub):* Sample from *in vitro* ubiquitination of CPEB3 by Neurl1. CPEB3: non-ubiquitinated CPEB3 (from *in vitro* assay without Neurl1). (iii) Averaged data from 3 independent experiments. Monoubiquitinated CPEB3 was significantly increased 30min after the application of glutamate in control neurons (Glut (+): 2.77%$\pm$0.31 of total CPEB3 vs Glut (-): 1.31%$\pm$0.12 of total CPEB3, $p=0.0001$). Monoubiquitinated CPEB3 was significantly reduced in the basal state in Neurl1DN neurons compared to mock (Mock/Glut(-): 1.31%$\pm$0.12 of total CPEB3 vs Neurl1DN/Glut(-): 0.56%$\pm$0.05 of total CPEB3, $p=0.0148$). Neurl1DN also blocked the increase of monoubiquitinated CPEB3 after glutamate application [the relative level of monoubiquitinated CPEB3 was similar between Neurl1DN/Glut(+) and Neurl1DN/Glut(-) neurons (Neurl1DN/Glut(+): 0.63$\pm$0.06 of total CPEB3 vs Neurl1DN/Glut(-): 0.56%$\pm$0.05, $p=0.9935$); the relative level of monoubiquitinated CPEB3 in Neurl1DN/Glut(+) neurons was significantly lower compared to control/Glut(-) neurons ($p=0.0421$)]. *$p<0.05$, **$p<0.002$, ***$p<0.0002$.

(F) Western blot analysis (i) and quantification of CPEB3 protein (ii) and RNA (iii) levels in hippocampal lysates from Neurl1 overexpressing (DT) and control mice. (i) st1 and st2: tetO-*Neurl1-Flag* and *CaMKIIα*-tTA single transgenic mice, respectively). (ii) Averaged fold difference $\pm$ SEM of

CPEB3 levels. CPEB3 was increased in DT (n=6) (**p<0.0001). Controls: single tetO-*Neurl1-Flag* (n=6), single *CaMKIIa-tTA* (n=4) and wild type mice (n=6). Control genotypes displayed similar levels of CPEB3 and were pooled (p=0.4880). (iii) Averaged fold difference ± SEM of himppocampal CPEB3 mRNA levels (real time qPCRs). No differences were observed (p=0.34). DT: n=6. Controls (pooled; p=0.5890): n=10 (single tTA, n=4; single tetO, n=4; wild type, n=2).

(G) The N-terminal domain of CPEB3 is critical for its interaction with Neurl1 and its ubiquitination. (i) IP from total protein lysates of cultured hippocampal neurons (14DIV) overexpressing the indicated proteins by lentiviral gene transfer. CPEB3 lacking its N-terminal domain was fused to the HA epitope tag (CPEB3ΔNter-HA) so that it could be discriminated from the endogenous CPEB3. Anti-HA antibody was used for IP of CPEB3-ΔNter-HA. Red asterisk: Neurl1 coimmunoprecipitates endogenous CPEB3 but not CPEB3ΔNter-HA. (ii) CPEB3 and CPEB3ΔNter-HA produced by coupled *in vitro* transcription/translation were incubated with 2µg or 4 µg of Neurl1-Flag and the necessary components for *in vitro* ubiquitination. Reaction products were detected by immunoblotting with anti-CPEB3 antibody. Anti-Ub antibody was used to test Neurl1-Flag ubiquin ligase activity (Neurl1 forms poly-Ub chains). Neurl1-Flag failed to ubiquitinate CPEB3ΔNter-HA. c: reactions without Neurl1-Flag (controls).

Figure S6 *(related to figure 6)*

(A) Quantitative real-time PCRs of reverse transcription products of total mRNAs isolated from neurons expressing the indicated proteins. The averaged fold difference ± SEM of GluA1 and GluA2 mRNA levels compared to mock (neurons infected with control lentivirus) from 3 independent experiments is shown. The experiments were performed in parallel with the experiments in Figure 6B. No differences were observed among all types of expression (p>0.95).

(B) ^{35}S-beta-actin immediately after a 1hr ^{35}S-Met/^{35}S-Cys pulse in cultured hippocampal neurons (16DIV) expressing the indicated proteins. Silver stain of isolated beta-actin is shown (loading control).

(ii) Averaged data from 3 independent experiments. No differences were observed among all types of expression ($p>0.42$)

(C) Western blot analysis (i & iii) and averaged fold difference $\pm$ SEM (ii & iv) of DsRed reporter protein levels in HEK293T cells transiently trasnfected with DsRed-carrying vectors alone (controls) or DsRed-carrying vectors and the indicated constructs. The coding sequence of DsRed was fused to either the SV40 polyA signal or the 3'-UTRs of GluA1 and GluA2. EGFP was co-transfected for normalization. Alpha-tubulin: loading control. (ii) & (iv) Averaged fold difference $\pm$ SEM of DsRed reporter protein levels in HEK293T cells transiently transfected with the constructs indicated in (i) and (iii), respectively. Each experiment was independent and repeated 3 times. Comparisons were made between samples of the same experiment. DsRed protein levels were compared to the respective control of each experiment. Black asterisk: significant difference compared to control (<0.0004). Red asterisk: significant difference compared to CPEB3 (<0.003). See also Table S3.

(D) Nucleotide sequences of the 3'UTRs of GluA1 and GluA2 mRNAs. (i) GluA1-specific PCR product (grey highlight) was amplified using the primers in red. For poly(A) assays, the GluA1-specific forward primer was used in combination with a poly(A)-specific primer (USB-Affymetrix). Highlighted with green and blue: polyadenylation signal and polyadenylation site, respectively (see also "Extended Experimental Procedures"). Underlined sequence: PCR product in the RNA-IP experiments that were performed for the identification of GluA1 mRNA as specific target of CPEB3. Blue sequences: primers used in the RNA-IP experiments. (ii) Sequence highlighted with grey: GluA2-specific PCR amplicon using the oligonucleotides in green. The amplified PCR product is part of the PCR product detected in the RNA-IP experiments performed by Huang et al. (2006) for the identification of GluA2 mRNA as specific target of CPEB3 in rat hippocampal neurons (underlined sequence). Sequence in red: forward primer used by Huang et al. The reverse GluA2-specific primer (green and italics) was common in both experiments (ours and Huang et al.). The polyadenylation signal is highlighted with green. The starting size of the detected poly(A)-tail-specific amplicons for both GluA1 and GluA2 conform with the existing

literature and prediction softwares (Extended Experimental Procedures). Note: The GluA2 3'-UTR deposited in GenBank includes an additional sequence downstream of the polyadenylation site. PCRs using various conditions and primer sets did not produce products from reverse transcribed RNAs isolated from adult hippocampus and cultured hippocampal neurons, indicating either mistakenly deposited sequence or absence of this sequence in neurons. This is consistent with the work from Huang et al. (2006) who studied the 3'-UTR of rat GluA2 mRNA. The isolated 3'-UTR of rat GluA2 that Huang et al. used in their experiments did not contain the abovementioned sequence either.

(E) Polyadenylation assays of actin mRNA (same samples as in figure 6C; control experiments). (i) Poly(A) assay of actin mRNA isolated from adult hippocampus of double transgenic (DT) mice either overexpressing Neurl1 or Neurl1DN and their single tetO control animals (st). The mice were kept either off or on dox in adulthood. No differences were observed among all groups. (ii) The poly(A) length of actin mRNA was also not altered in cultured hippocampal neurons expressing the indicated proteins.

Figure S7 *(related to figure 7)*

Immunostainings showing the dendritic localization of the indicated proteins in cultured hippocampal neurons. CPEB3ΔNter, CPEB3-Ub_{KO} and CPEB3-SUMO recombinant proteins were detected with antibody specific to the HA epitope that was fused to these proteins. Neurl1-F: Neurl1-Flag. $Neurl1^{Rm}$-F: $Neurl1^{Rm}$-Flag. Scale bars: 5μm.

Extended Experimental Procedures

Generation of Transgenic Mice

The mouse *Neuralized1 and Neuralized1DN* open reading frames (encoded amino acids: 1-574 and 1-520, respectively; Genebank accession numbers for Neurl1 nucleotide sequence: Y15160 and BC099702) were fused to the Flag tag epitope and subsequently cloned into the pMM400 plasmid (Mayford et al., 1996). pMM400 was modified to carry the human growth hormone polyadenylation sequence instead of the described SV40 poly A (Kellendonk et al., 2006). The Flag tag was not fused at the N-terminal end of Neurl1 proteins as it has been shown to severely affect its subcellular localization (Koutelou et al., 2008; and E.P., unpublished data). The NotI fragment containing the *tetO-Neurl1-Flag* and *tetO-Neurl1DN-Flag* transgene was isolated and used to generate transgenic mice by injection into pronuclei of one-cell C57Bl/6-CBA(J) F2 oocytes, which were transferred via the oviduct into pseudopregnant foster females the next day. Transgenic founder animals were identified by Southern blots using a probe specific for the tetO promoter sequence. Progeny of founder animals were crossed with mice expressing the tTA transgene under the control of the *CamKIIα* promoter (Mayford et al., 1996). Offspring were genotyped by independent Southern blots for tTA and tetO. For regulating tetO-driven gene expression, mice were fed chow supplemented with 40 mg/kg doxycycline (Mutual Pharmaceutical).

Maintenance of mice and Genetic Background

Mice were maintained and bred under standard conditions, consistent with NIH guidelines and approved by the Institutional Animal Care and Use Committee (IACUC). In order to control genetic background, we followed the recommendations made by the Banbury conference on genetic background in mutant mice (Silva, 1997). The *tetO-Neurl1* mice were backcrossed at least six times to C57Bl/6J background and bred with *CaMKII-tTA* mice that were backcrossed 16 to 18 times to the 129SveVTac background.

Doxycycline diet protocols

On/off dox diet protocol: The transcription of the transgenes in the double transgenic mice was inhibited during development by maintaining the pregnant females and their litters on food containing 40 mg/kg doxycycline until day P40, at which point we switched the animals to food without doxycycline. The expression of the transgenes was detectable seven days later. The mice were tested when they were 3 ½ months (day P105).

On/off/on dox diet protocol: This protocol allowed us to examine whether the memory deficit in the DT mice results from acute action of the recombinant proteins, and not from either an early effect of their expression between the day of their activation (day P40) and adulthood or position effects of the transgenes. The double transgenic animals and their control littermates remained on doxycycline-containing food until day P40, at which point they were switched to doxycycline-free food. At day P80, the mice shifted back to doxycycline-containing food. Complete inhibition of the transgenes was achieved 15 days later. The mice were examined when they became 3 ½ months old.

Injections

Anisomycin (Sigma) was diluted in PBS (50 mg/ml adjusted to pH 7.4 with HCl). Mice received subcutaneous injections of 150 mg of anisomycin/kg of body weight or an equivalent volume of saline.

RNA *In Situ* Hybridization

RNA *in situ* hybridizations were performed on fresh frozen adult brain slices using a 42-45 base anti-sense oligonucleotide specific for the mRNAs of interest. Mice were sacrificed by cervical dislocation, and the brains were dissected and rapidly frozen in mounting medium (Tissue Tek, O.C.T. Compound 4583, Sakura). Cryostat sections (14 μm) were postfixed for 10 min in 4% paraformaldehyde in PBS (pH 7.4), dehydrated and stored in 100 % ethanol at 4°C until use. 50 ng of oligonucleotide were

labeled with 50 microCi of [α33P]dATP (Perkin Elmer) using recombinant terminal transferase (Roche). 10^7 cpm of oligonucletide per 1ml of hybridization buffer were used (50 % formamide, 4 X SSC, 10% (w/v) dextran sulfate). After hybridization, the slides were washed with 1X SSC for 10 minutes at room temperature, 1X SSC for 30 minutes at 60°C and finally with 1 X and 0.1X SSC for 1 minute each at room temperature. Slides were then dehydrated and exposed on X-ray film for 1-3 weeks. Oligonucleotides used: ***Neurl1:*** Neurl1-A: 5'- CCC TCA CTG CTT GTG CCC TCA TCT CCC TGC CTG AAA CTC TTT C -3', Neurl1-B: 5'- GTC CAC TGC GTG TTC ATA GCA AAT GGT GCA TTC ATC ACT CCA CTG -3' *and* Neurl1-C: 5'- CTC GCT GCA GAG AGG AGA CAC TGG AGA AGT TGT TAC CCA TGG C -3'; ***GluA1***: GluA1A: 5'- TTA CAA TCC TGT GGC TCC CAA GGG CAT CCC TGA ACT GTG GCT -3', GluA1B: 5'- GAT GAA GCA CAC ATG GAG GGC CCC ACA GAA GGA GGT CAG CAT GTT -3', GluA1C: 5'- TCC CCC TAT CTG GAT ATT GTT GGG GAA ATT GGC ACC CAC AAC CGC -3'; ***GluA2:*** GluA2A: 5'- TCA GTC CCC ATA AAA CAG GAG AAA GGA GGA CAG AAA TAT GCA-3', GluA2B: 5'- GTG TCA GTC TGA ACT CCG AAG TGG AAA ACT GAA CCA TCC CTA CCC 3', GluA2C: 5'- ATG TTC CCC ACA TTG ATA GCA GTC ACC TGC CAC TTC TTC TCC GCA -3'; ***Neurl1-Flag and Neurl1DN-Flag:*** 5'- CGG GAT CCG GCT ACT TAT CGT CGT CAT CCT TGT AAT CGG AGC TG -3'.

Synaptoneurosome Preparation and PSD Isolation

Hippocampi from 3 ½ month old mice were homogenized in synaptoneurosome buffer (10mM HEPES, 1mM EDTA, 2mM EGTA, pH 7.0 and protease inhibitors from Roche), mixed by end-over-end rotation for 10 minutes at 4 ^{0}C and centrifuged (1000rpm) for 5 minutes. The supernatant was collected and its volume was increased to 1.5ml by adding synaptoneurosome buffer. Filtration followed, first through 2 layers of pre-wetted 10μm filter (twice) and then through 2 layers of 5μm pore filters (Mitex^{TM}; Millipore). The filtrated sample was centrifuged for 10 minutes at 4 ^{0}C (1000xg) and the synaptoneurosomal pellet was resuspended in 300 ul of buffer containing 50mM HEPES pH 7.4, 2mM

EDTA, 0.5% Triton X-100 and protease inhibitors, mixed for 15 minutes at 4 ^{0}C and centrifuged for 20 minutes (32.000xg). The pellet was resuspended in 50mM HEPES pH 7.4, 2mM EDTA, Triton X-100 to 0.5% and protease inhibitors, incubated again at 4 for 15 min and then centrifuged at 200,000 x g for 20 minutes. The PSD pellet was resuspended in 50mM HEPES pH 7.4, 2mM EDTA and protease inhibitors, and analyzed by SDS-PAGE and immunoblotting.

Quantitative mRNA Analysis by Real-Time PCR

Hippocampi were homogenized in 1 ml of TRIzol reagent (Invitrogen) and total RNA was extracted and quantified. cDNA was synthesized from 800 ng total RNA by reverse transcriptase reaction using Superscript III (Invitrogen). Real-time PCR was performed using iQ SYBR Green Supermix according to the manufacturer's instructions (Bio-Rad). PCR parameters were 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C, and 1 minute at 60°C. Finally, a melting curve from 70° to 90°C was performed to assure product purity. The primer pairs used for specific transcript amplification were: GluA1: Qiagen Mm_Gria1_1_SG QT01062544), GluA2: MmGria2_1_SG QT00140000), and CPEB3: Qiagen Mm_Cpeb3_1_SG QT00177345. Ct values for each reaction were normalized to GAPDH (Qiagen Mm_Gapdh_3_SG QT01658692). Each reaction was performed six times and the results were pooled. Mean fold difference over the control group was determined by the $2^{\Delta Ct}$ method of quantification.

Plasmids

The Neurl1-F, Neurl1^{Rm}-F (two highly conserved RING domain cysteine residues, Cys^{521} and Cys^{524} were changed to serine) and 6xHis-Ubiquitin expression plasmids were kind gifts of Joan Conaway and Nicholas Moschonas (Stowers Institute for Medical Research, USA and University of Crete, Greece, respectively). The Neurl1 shRNA plasmids (shRNA-95, shRNA-97 and shRNA98) were from Sigma-Aldrich (TRCN0000106295, TRCN0000106297, and TRCN0000106298, respectively). CPEB3 was

isolated from adult hippocampus of C57BL6/J mice (3 months) using RT-PCR, and subcloned into pCR2.1-TOPO vector. CPEB3 was subsequently subcloned into pEGFP-N1 and pDsRed-monomer-N1 vectors from Clontech (EcoRI/XhoI). CPEB3ΔNter was generated by PCR-cloning. For the generation of CPEB3-Ub_{KO} and CPEB3-SUMO, the coding sequences of CPEB3, $Ubiquitin_{KO}$ and SUMO were sequentially subcloned to pCMV-SPORT6 vector (Stratagene) using PCR and primers with linkers containing the appropriate restriction sites (EcoRI/SalI for CPEB3 and SalI/HindIII for $Ubiquitin_{KO}$ and SUMO). To avoid the recognition of CPEB3-Ub_{KO} as ubiquitin and its conjugation to target proteins, the two C-terminal glycines in the ubiquitin portion of CPEB3-Ub_{KO} were deleted. This deletion prevents the isopeptide bonds between ubiquitin and target lysines and thus prevents use of CPEB3-Ub_{KO} fusion protein in this way (Carter and Vousden, 2008). To distinguish CPEBΔNter, CPEB3-Ub_{KO} and CPEB3-SUMO from wild type CPEB3, the HA epitope was fused to the N-terminus of the proteins. **Neurl1-FOR:** 5'- ATGGGTAACAACTTCTCCAG -3' (5'end of Neurl1 coding sequence-common primer). **Neurl1-Flag-REV:** 5'-CTACTTATCGTCGTCATCCTTGTAAT CGGAGCTGCGGTAGGT CTTG -3' (3'end of Neurl1 open reading frame; used for the generation of Neurl1-Flag transgene). **NeurlDN-REV:** 5'- TCATTACTTATCGTCGTCATCCTTGTAATCTTCA TCACTCCACTGGCCCA-3' (3'end of Neurl1 open reading frame up to aminoacid 520; used for the generation of Neurl1DN-Flag transgene). **Neurl1-REV:** 5'- CTAGGAGCTGCGGTAGGTCTTG -3' (3'end of Neurl1 coding sequence; used for cloning of Neurl1 in various expression vectors). **Neurl1-ΔC:** 5'- GCAGGGGGTCAGAGAGG CGG -3' (used with Neurl1-FOR for the generation of Neurl1 lacking the last 100 C-terminal aminoacids). **Neurl1ΔN:** 5'- TGCTTTTCTTCAGTGGGGT -3' (used with Neurl1-REV for the generation of Neurl1 lacking the first 194 C-terminal aminoacids). **CPEB3-A-FOR:** 5'- ATGCAGGATGATTTACTGATGG -3' (5'end of coding sequence; used for the isolation and cloning of CPEB3 cDNA and for the generation of CPEB3-Ub). **CPEB3-A-REV:** 5'- TCAGCTCCAGC GGAACGGGACG -3' (3'end of coding sequence; common primer for the isolation and cloning of CPEB3 cDNA). **CPEB3-B-REV:** 5'-CCGGTCGACGCTCCAGCGGAACGGGACGTG -3' (3'end of coding sequence with SalI restriction

site (linker); used for the generation of CPEB3-ub). **CPEB3-ΔN:** 5'- ATGACCAGCAAGCCGTCCTC -3' [used with CPEB3-A-REV for the generation of CPEB3ΔN (aa 223-716)]. **CPEB3-ΔN-HA:** 5' CCACCATGTACCCATACGATGTTCCAGATTACGCTACCAGCA AGCCGTCCTC -3' (used for the generation of CPEB3ΔN-HA*;* underlined sequence: HA epitope*).* **CPEB3-HA:** 5'-CCACCATGTACCCATACGATGTTCCAGATTACGCTATGCAGGATGATTT ACTGATGG -3' (used for the generation of CPEB3-Ub_{KO} and CPEB3-SUMO HA tagged proteins*;* HA epitope is underlined). **Ub-FOR:** 5'- CCGGTCGACATGCAGATCTTCGTGAAGACC -3' (5'end of coding sequence with SalI restriction site in frame with CPEB3 ORF). **Ub-REV:** 5'- CTATCTCAGGCG AAGGACCAG GTG -3' (3'end of coding sequence). **SUMO-FOR:** 5'-CCGGTCGACATGTCTGACC AGGAGGCAAAAC-3 *(*5'end of coding sequence with SalI restriction site in frame with CPEB3 ORF). **SUMO-REV:** 5'- CTGTACACAAGCT TCTACTAAACTGTTGAATGACCC -3' (3'end of coding sequence).

Antibodies and Reagents

Affinity-purified rabbit polyclonal sera directed against an N-terminal peptide sequence of mouse Neurl1 (amino acids 27–42; peptide generated by Global Peptides), were produced by Covance. Anti-CPEB3 (ab10833), anti-MAP2 (ab11267) and anti-GFAP (ab7260) and anti-beta actin (ab6276) antibodies were from Abcam. Anti-GluA1 and anti-GluA2 antibodies were from Millipore (AB1504 and AB1768, respectively). Anti-Flag (M2), anti-alpha-tubulin and anti-histone3 antibodies were purchased from Sigma (F3165, T6199 and H0164, respectively). Anti-HA mouse monoclonal antibody was from Covance. Anti-Shank was from UC Davis/NINDS/ NIMH NeuroMab facility (panShank, 73-089). Anti-PSD-95, anti-Synaptophysin and anti-Ubiquitin antibodies were from Santa Cruz Biotechnology (sc-71933, sc-25786, sc-7568 and sc-9133, respectively). Normal IgG and all horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch. Alexa 488, 568 and 647-

conjugated secondary antibodies were purchased from Invitrogen. Anisomycin was purchased from Sigma.

Western Blotting and Immunoprecipitation

Dissected hippocampi were homogenized in 50mM Tris-HCl pH 7.4 and 2% SDS buffer with protease inhibitors (Roche). 10-20 μg of homogenates were separated by SDS-PAGE and transferred to PVDF membranes (BioRad). Membranes were blocked in milk solution (5% milk in TBS and 0.1% tween 20). Each sample was analyzed by immunoblotting at least two times.

For coimmunoprecipitations, hippocampi were homogenized in RIPA buffer. The homogenates (300μg) were incubated overnight at 4^0C with 5μg of affinity purified anti-Neurl1 or anti-CPEB3 antibodies. Protein A sepharose beads equilibrated in the same buffer (plus 0.5%BSA) were incubated with the lysate/antibody mix for one hour at 4 ^{0}C. The beads were washed five times with lysis buffer (5 minutes each at 4 ^{0}C). Bound proteins were eluted with Laemmli sample buffer and analyzed by western blotting. Silver stains were performed using the SilverQuest kit (Invitrogen).

For the detection of Neurl1 after LTP induction, we used western blotting to measure the ratio of Neurl1 protein levels in the CA1 neurons of the adult hippocampus in pairs of acute hippocampal slices recorded at the same time; one member of the pair served as a control and the other one was used to induce LTP. Frozen samples were homogenized by the addition of boiled solution containing 1% SDS, 10mM Tris-HCl pH 7.4 and 0.1M NaCl), they were sonicated and incubated at 100°C for 5 min. Protein samples (10-20μg) were separated by standard SDS-PAGE and immunoblotted. Five independent experiments were performed. For the quantification of the protein bands, we performed a methodical approach. Specifically, we analyzed the five independent experiments four times by western blotting; in other words, we performed 4 independent western blots for each individual experiment. This approach is commonly used for such type of analyses, and its purpose is to eliminate errors related to differences of the transfer efficiency that could result in either over- or under-estimated differences. The X-ray films

were pre-flashed and this was followed by image analysis of the scanned X-ray films. Western blots that gave saturated protein bands were excluded from the analysis. The rest of the western blots were used for quantification. The analysis included at least two western blots for each one of the five independent experiments. The values of the graph are: 35% increase ± 10% (SEM). The observed variability is not uncommon in experiments examining protein levels in response to LTP induction (the levels of several synaptic plasticity-related proteins highly depend on the delay after the preparation of the slices; Taubenfeld et al., 2002). For that reason, we always recorded pairs of slices that had undergone the exact same preparation (one member of the pair served as a control and the other one was used to induce LTP).

Anti-α-tubulin (Sigma) was used as control for loading. All the HRP-conjugated secondary antibodies were used at 1:5000 dilutions. Immunoblots were visualized with either Supersignal West Pico (Pierce) or ECL plus chemiluminescent reagents (Amersham).

For the detection of ubiquitinated CPEB3 from adult hippocampus, our approach was based on sequential immunoprecipitations (IP on IP approach) as most of the existing methods for the isolation of ubiquitinated species of individual proteins in the basal state are not competent for the isolation of mono- and polyubiquitinated proteins, especially those species directed for degradation. Adult hippocampal lysates from different genotypes were first normalized against CPEB3 by western blot analysis and quantification. The starting hippocampal lysates/inputs from control mice, Neurl1DN-expressing mice and DT animals on dox were 1-1.2mg (total protein) while the input from Neurl1 overexpressing mice was 400-500μg (depending on the increase of total CPEB3 compared to the respective controls). CPEB3 was immunoprecipitated with anti-CPEB3 antibody (Abcam; ab10883; 6μg/ml). Elution of CPEB3 was performed by competition with 100X of the respective peptide (Abcam; ab24478). For the subsequent immunoprecipitation of ubiquitinated CPEB3, anti-Ub antibody from Santa Cruz Biotechnology was used (sc-9133; 10μg/ml). The final immunoprecipitant was eluted with Laemmli sample buffer after eight washes with RIPA buffer (15 minutes each).

Image quantification analysis (Imagequant software) was used for the calculation of protein level differences. The StatView software (SAS institute) was used for statistical analysis.

Immunohistochemistry

Three-month-old mice were anesthetized and perfused intracardiacally with 50 ml of ice-cold 4% paraformaldehyde in 0.1M Na_2HPO_4/NaH_2PO_4 pH 7.4 buffer. The brains were post-fixed overnight at 4^0C. Coronal sections (30 μm of thickness) were taken using a vibratome (Vibratome). The collected floating sections were rinsed four times in Tris-buffered saline (TBS; 0.1M Tris pH.7.4 and 0.9% (w/v) NaCl), and incubated with blocking solution (10% fetal bovine serum and 0.2% Triton X-100) for 2 hours at room temperature. The slices were incubated with the primary antibodies overnight at 4 ^{0}C. After six rinses (30 minutes each) in TBS (0.3% Triton X-100), sections were incubated for 1 h at room temperature with the appropriate secondary antibodies conjugated with Alexa dyes (Invitrogen), then washed 6 more times (30 minutes each) with TBS (0.3% Triton X-100) and mounted on slides with Fluorsave (Roche) for observation. Confocal optical sections were taken with step size 0.2μm.

RNA-Immunoprecipitation and Gel-Shift Experiments

Protein purification. Bacterially expressed CPEB3 fusion protein was generated as described by Si et al. (2003), with modifications. Briefly, the CPEB3 open reading frame was cloned into the hexahistidine vector pRSET and the expression of the recombinant protein was induced in *E. coli* BL21 by 0.5 mM IPTG. The his-tagged proteins were purified in Ni^{+2} NTA resin using the manufacturer's protocol (ProBond; Invitrogen). The following buffers were used for purification. Lysis and binding buffer: 6 M guanidine hydrochloride, 100 mM $NaPO_4$ (pH 8.0), and 10 mM Tris base. Wash buffer: 8 M urea, 100 mM $NaPO_4$ (pH 8.0), 10 mM Tris base, 10 mM imidazole. Elution buffer: 8M urea, 100 mM $NaPO_4$ pH 8.0, 10 mM Tris base, and 250 mM imidazole. The purified proteins were dialyzed against buffer

containing 2 M urea, 100 mM Kcl, 10 mM Na-HEPES (pH 7.6), 1 mM DTT, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5% glycerol at 4C for 24–32 hours. Solutions were stored at -80°C until used.

RNA labeling. RNA was prepared by *in vitro* transcription using Dig-labeling kit from Roche following the manufacturer instructions.

RNA gel shift assay. Shift experiments were performed as described by Hake et al. (1998) and Si et al. (2003), with modifications. Purified protein was incubated with 100ng of Dig-labelled purified RNA in a 20µl reaction containing 100mM KCl, 10 mM Na-HEPES (pH7.6), 5mM DTT, 0.1mM $CaCl_2$, 1mM $MgCl_2$, 100µM ZnCl2, 5% glycerol, 0.1mg/ml BSA and 50µg/ml *E. coli* t-RNA. The reaction mixture was incubated at 4 ^{0}C for 20 min and was then analyzed on 4% polyacrylamide. Following electrophoresis, the RNA was transferred to a positively charged nylon membrane by electroblotting at 30 V in 0.5x TBE buffer. DIG-labeled RNA-protein complexes were detected with alkaline phosphatase-conjugated anti-DIG antibody and CSPD chemiluminescent alkaline phosphatase substrate.

Preparation of whole-hippocampus lysate. Hippocampi from two mice (3-4 months old) were homogenized in cold immunoprecipitation buffer (10mM HEPES pH 7.4, 200mM NaCl, 30mM EDTA pH 8.0 and 0.5% Triton X-100) in the presence of protease inhibitor cocktail and 0.4 U/ul RNasin. The homogenate was centrifuged at 1,000xg for 10 min at 4 °C to remove nuclei and non-lysed cells. The cleared lysate (supernatant) was stored at -80 ^{0}C.

CPEB3 IPs and Reverse-Transcription PCR analysis. Hippocampal lysates were pre-cleared with protein A sepharose beads and immunoprecipitated with 10 ul RNasin, 10 µM CPEB3 antibody (or no antibody controls), and 100 ul packed fresh protein A beads for 3 h at 4 °C. Immunoprecipitants were washed with IP buffer (10 mM HEPES pH 7.4, 200 mM NaCl, 30 mM EDTA pH 8 and 0.5% Triton X-100) and resuspended in 1 ml TRI-Reagent. Total RNA was isolated and precipitated in the presence of 2 µM tRNA. The final pellet was resuspended in DEPC water, solubilised at 60 ^{0}C for 10 min, and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) using random hexamer oligonucleotides, following the manufacturer's instructions. 1 ul of the cDNA was analyzed for GluA1 by PCR. GluA1

primers: sense: 5'-GGAGCAGTGGGAAAACTC-3' (nucleotides 5364-5381) and anti-sense: 5'-TATCACAAGAATTAAATCAC-3' (nucleotides 5633-5652). The cycle conditions were as follows: 2 min at 95 ^{0}C and (35 cycles: 30 s at 95 ^{0}C, 1 min at 60 ^{0}C, 30 s at 72 ^{0}C), followed by a final extension of 5 min at 72 ^{0}C. Control RT reactions on templates in the absence of reverse transcriptase were analyzed by PCR to ensure absence of DNA contamination. PCR products were gel purified and sequenced to confirm their identity.

Polyadenylation Assays

The Poly(A) tail-length assay kit from USB-Affymetrix was used following the manufacturer's instructions (product #: 76450). The detection of the poly(A) tails of GluA1 and GluA2 mRNAs was performed using a gene-specific sense oligonucleotide located upstream of the polyadenylation signal sequence and a universal primer provided by the manufacturer. PCRs using two gene-specific primers located upstream of the polyadenylation signal sequence (same sense oligonucleotide as in polyadenylation assays) were used to test the initial input for each tested sample (reverse transcription product) and the expected starting molecular size of the poly(A)-specific amplicons (for schematic representation see figure 9B). The one step PCR protocol was followed. Annealing temperatures: 69 ^{0}C for GluA1 and 64 ^{0}C for GluA2. GluA1 sense: 5'-GAAATGAGGGTCTTGCTGGGAGGG-3'. GluA1 anti-sense: 5'-CAGAGTTTTCCCACTGCTCCATT-3'. GluA2 sense: 5'-GGAAAGACCAAAATAATT ATGAACT-3'. GluA2 anti-sense: 5'-GAGAGTTATTTTTTTCCCTCACACATCTTCCA-3'. PCR products were analyzed on 2.5% agarose gels. The GluA1 oligonucleotides were selected based on the polyadenylation signal and polyadenylation site (highlighted with green and blue, respectively) indicated by the NCBI reference sequences for both mouse and human GluA1 transcripts (NM_001113325.1 and NM_000827, respectively). The GluA2 polyadenylation signal was selected based on the work by Kohler et al., (1994) and the polyadq software (Cold Spring Harbor Laboratory; http://rulai.cshl.org/tools/ polyadq/polyadq_form.html). NCBI sequence: NM_001083806.

Metabolic Labelling of AMPA Receptors

This approach does not affect translation or any other cellular processes and therefore allows 1) the accurate examination of the degradation rate and half-life of proteins and 2) the rate of translation of the proteins of interest. High density cultured hippocampal neurons (16DIV) were incubated for 1 hour in 600 uCi/ml of ^{35}S Met/^{35}S-Cys express labelling mix (Perkin Elmer) in serum and Met/Cys-free DMEM after an initial incubation for 1 1/2 hours at 37^{0}C. After three subsequent washes with PBS (Pierce), neurons were either immediately lysed in RIPA buffer (50mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors; 0min time point) or returned back to their growth medium and lysed at various time points depending on the experiment. Immunoprecipitations of GluA1 and GluA2 were conducted at 4^{0}C for 4 hours followed by 4 washing steps (10 minutes each) at 4^{0}C. Immunoprecipitated GluA1and GluA2 were eluted with Laemmli sample buffer and analyzed on SDS-PAGE. Gels were then dried and exposed to phosphoimager screens (Cyclon, Perkin Elmer). Three independent experiments were performed for the investigation of either the translation of GluA1 and GluA2 or their degradation rate. Quantification was performed using the Imagequant software. The neurons were infected with lentiviruses expressing the described proteins at 11 DIV.

Biotinylation Assay of AMPA Receptor-Surface Expression

High density cultured hippocampal neurons (16 DIV) were incubated with aCSF (1.25 mM CaCl, 0.5 mM MgCl, 0.4 mM MgSO4, 5 mM KCl, 134 mM NaCl, 4mM $NaHCO_3$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4 and 5.5 mM glucose) at 37 ^{0}C for 1 hour and then stimulated with 100μM glutamate for 15 minutes at 37^{0}C or kept in aCSF. Neurons were then incubated at 4^{0}C with 1 mg/ml sulfo-NHS-LC-biotin (Pierce) in aCSF for 30 min and lysed with RIPA buffer (150 mMNaCl, 50 mM Tris HCl pH 7.4, 0.1% SDS, 1%NP-40, 0.5% sodium deoxycholate, 1% Triton TX-100) in the presence of protease inhibitors (Roche) after four washes with ice-cold TBS (Pierce). Lysates were centrifuged at 16.000 g for 10 min at

4 ^{0}C. Biotinylated surface receptors were precipitated with immobilized NeutrAvidin-coated beads (Pierce) for 4 hours at 4 ^{0}C followed by 6 washes (10 minutes each) with RIPA buffer and eluted in Laemmli sample buffer. Biotinylated GluA1 receptors were analyzed by SDS-PAGE and western blotting using anti-GluA1 antibody (Millipore). For the analysis of the relative levels of surface GluA1 compared to the receptors present in the lysates used for the NeutrAvidin pull downs, western blot analysis of these lysates were performed in parallel using identical conditions. Imagequant software was used for quantification. For normalization, the total GluA1 levels in the respective inputs were used. Three independent experiments were performed (each experiment was duplicated).

Biotinylation Assay of AMPA Receptor Internalization

16 DIV cultured hippocampal neurons were surface-biotinylated with 1mg/ml sulfo-NHS-SS-Biotin (Pierce) as described above, in the presense of 100μM/ml leupeptin. Neurons were first washed 3 times in aCSF to remove unbound biotin and then returned to 37^{0}C for 15 minutes to allow endocytosis, in the presence or absence of 100 μM glutamate, followed by 3 washes with ice-cold aCSF. The remaining surface-bound biotin was removed with glutathione-stripping buffer (50 mM glutathione in 75 mM NaCl, 10 mM EDTA, 1% BSA, and 0.075 M NaOH; 2x15min at 4^{0}C). Cells were then rinsed 3 times with TBS (Pierce) and the remaining biotinylated receptors (internalized) were isolated with NeutrAvidin beads and analyzed as described above. 100μM/ml leupeptin was continuously present prior to lysis of the neurons. Total GluA1 receptors of each lysate were used for normalization. Three independent experiments were performed (each experiment was duplicated). To test the efficiency of the glutathione-stripping buffer, cells were incubated with this buffer immediately after biotinylation. Subsequent NeutrAvidin pull down and western blot analysis did not detect biotinylated AMPA receptors.

Injections of Anisomycin

We injected 180mg of anisomycin per kilogram body weight. High dosage of anisomycin (50-150mg/Kg of body weight) yield >90% inhibition of protein synthesis in the brain during the first 2 hours (Lattal and Abel, 2004). Injection with saline was used as control.

Mass Spectrometry

Using Neurl1-specific antibody, we immunoprecipitated Neurl1 from adult hippocampal protein lysates from wild type mice. We first separated the samples on 8% SDS-PAGE and isolated the migrating bands representing potential Neurl1-interacting proteins. The protein bands were transferred to micro centrifuge tubes and cut into small pieces with tweezers. The preparation of the samples for mass spectrometric sequencing has been described previously (Puthanveettil et al. 2008).

***In Vitro* Ubiquitination Assays**

CPEB3 was produced by coupled in vitro transcription/translation in reticulocyte lysate (Promega). 5 ul of reticulocyte sample were incubated with wild type Neurl1-Flag (1 or 2μg) or mutant Neurl1^{Rm}-Flag (1μg) that lacks its ubiquitin ligase activity (two highly conserved RING domain cysteine residues, Cys^{521} and Cys^{524} were changed to serine) for 1 h at 37 °C with 50nM Ube1, 0.2μM UbcH5a, 3μm Ubiquitin-aldehyde and 0.25 mg/ml ubiquitin in 50mM Tris-HCl, pH 7.6, 50mM NaCl, 5mM $MgCl_2$, 0.5mM EDTA (pH 7.9), 5% glycerol, 0.5mM dithiothreitol and an energy regenerating mix (Boston Biochem). Ube1, UbcH5a, Ubiquitin and Ubiquitin-aldehyde were purchased from Boston Biochem.

***In Vivo* Ubiquitination Assays**

HEK293T cells (Invitrogen) were transiently transfected in 150-mm dishes with plasmids encoding 6xHis-Ub, CPEB3 and Neurl1-Flag or Neurl1^{Rm}-Flag. The cells were lysed twenty-four hours

later. A small number of cells was lysed in 50mM Tris-Cl pH 7.4 and 2% SDS and used for detection of CPEB3 and Neurl1 levels. The rest of the cells were lysed in 800 µl/dish of 6M guanidium-HCl, 100mM Na_2HPO_4, 30mM imidazole, and protease inhibitors (Roche). Lysates were sonicated and cleared by ultracentrifugation (100,000 x *g* for 1 h at 4 °C). His-Ub-conjugated proteins were purified by nickel chromatography using Ni-NTA-agarose (Qiagen). Nickel-binding proteins were separated by SDS-PAGE and CPEB3 was detected by immunoblotting using CPEB3-specific antibody.

Lentivirus Infections of Cultured Hippocampal Neurons

The coding regions of CPEB3, Neurl1-F, Neurl1-F^{Rm}, CPEB3-Ub_{KO}, CPEB3-EGFP, CPEB3-SUMO and CPEB3ΔNter were amplified by PCR from their original vectors and subcloned into the FUGW vector (kind gift of Pavel Osten, Dittgen et al., 2004; 5'linker: AgeI or BamHI; 3'linker: EcoRI or BsrGI). For the production of lentiviruses, the FUGW constructs were co-transfected with the two helper plasmids pMD2.G and psPax2 plasmids (Addgene) in HEK293FT cells (Invitrogen) following the manufacturer's instructions.

Hippocampal Cultures

Hippocampi were dissected from C57BL6/J mice at postnatal day 1-2, sliced into 1 mm pieces, and incubated in Hanks' balance salt solution (Invitrogen) containing 10 units/ml papain (Sigma) at 34 °C for 30 min. Trypsin inhibitor (Sigma) was added to a final concentration of 0.5 mg/ml, and the tissue was mechanically dissociated by passing through a flame-polished Pasteur pipette. Cells were plated at 1 x 10^5 cells/cm^2 on poly-L-lysine-coated (0.1 mg/ml) plates and maintained in Neurobasal basal medium (Invitrogen) supplemented with B27 (Invitrogen), penicillin/streptomycin, and glutamine at 2 mM. To reduce the number of non-neuronal cells, ARA-C (3.3 µg/ml, Sigma) was added to the medium 48 h after plating.

Neurons were transfected at 10 DIV with the desired plasmids and fixed one day later with 4% formaldehyde, 4% sucrose for 20min at room temperature. Primary and secondary antibodies were applied in PBS with 10% fetal bovine serum (blocking buffer) overnight at 4^0C and 1 hr at room temperature, respectively. All washing steps were performed at room temperature using PBS buffer. The coverslips with neurons were mounted on slides with Fluorsave (Roche).

For lentivirus infections and detection of AMPAR protein levels, 7 DIV hippocampal neurons were infected with the virus and cultured for another 4 days before protein extraction, unless otherwise indicated. Neurons were lysed in buffer containing 50mM Tris-pH7.4 and 2% SDS in the presence of protease inhibitors (Roche), sonicated and boiled for 5 minutes at 100 ^{0}C. 20-30µg of homogenates were analyzed by SDS-PAGE and immunoblotting.

Neuronal Tracing and Spine Counting

Brains from adult mice (3 ½ months) were removed and processed for Golgi staining according to the manufacturer's protocol (FD Rapid GolgiStain kit; FD Neurotechnologies). Throughout the hippocampus, brains were cut coronally in 120µm sections using a vibratome and mounted on 3% gelatin-coated slides before staining. Neurons chosen for tracing met the following criteria: 1) they were completely impregnated with Golgi stain, 2) they were not obscured by other impregnated neurons or precipitant, 3) they did not have truncated branches, and 4) the dendrites were easily discriminated by focusing through the depth of the tissue (Savonenko et al., 2008; Wellman et al., 2007). Pyramidal cells (from three sections per animal) in the CA1 region were traced, and spines were counted by using Neurolucida software (MicroBrightField). The hemispheric location and the traced neurons were chosen randomly. Using the accompanying software (NeuroExplorer; MicroBrightField), dendritic complexity (via Sholl analysis), dendritic length and spine density were calculated. All protrusions were counted as spines if they were in direct continuity with the dendritic shaft (Sala et al., 2001). Starting from the cell soma, spines were counted along a 130-µm stretch of the main apical shaft and the dendrites originating

from it. The mean spine density was calculated and compared with the examined groups of animals. All samples were number-coded, and the experimenter was "blind" to the genotype and treatment.

Electrophysiology

Preparation of hippocampal slices. Transverse hippocampal slices were prepared from Neurl1 overexpressing mice or control littermates (3.5–4.5 months old). Animals were sacrificed by decapitation in accordance with institutional regulations. Hippocampi were dissected and slices (400 µm thickness) were cut with a "chopper" for field EPSP (fEPSP) recordings. For whole-cell recordings, slices were cut with a vibratome (Vibratome) in ice-cold extracellular solution containing 238 mM Sucrose, 2.5 mM KCl, 10 mM glucose, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1 mM $CaCl_2$ and 2 mM $MgCl_2$. The slices were kept at room temperature with oxygenated artificial CSF (aCSF; 119 mM NaCl, 3 mM KCI, 1.5 mM $MgSO_4$, 2.5 mM $CaCI_2$, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 11 mM glucose) for at least 1.5 hours before transfer to the recording chamber. Cutting and recording solutions were both saturated with 95% O_2 and 5% CO_2, pH 7.4.

Electrophysiological recordings. For field EPSPs recordings, slices were placed in a submerged chamber with ACSF at 28 ± 1°C (flow rate 1.5-2 ml/min) for at least 30 min before recording. Field EPSPs were evoked from CA3–CA1 synapses via stimulation of the Schaffer collateral axons with a bipolar electrode CBAPD75 (FHC) and recorded with a 4–5 MΩ glass pipette (A-M Systems). The stimulation intensity (square pulse, 50 µs duration) was adjusted to give fEPSP slopes of ~40% of the ones obtained with 15-20V stimulation. Baseline and post-stimulation responses were sampled once per minute at this intensity. The LTP protocol for the 100Hz protocol consisted in a single 1 sec burst at 100Hz. For the 4x100 Hz protocol, each burst was given at 5 min interval. Theta-burst stimulation (TBS) was a single theta-burst episode, which consisted of nine bursts of four pulses at 100 Hz with 200 ms interburst intervals. The LTD protocol consisted of a 15 min train at 1Hz.

For the experiment using picrotoxin, in order to avoid epileptic-like activity due to the absence of the inhibitory constrains, the stimulation intensity was adjusted to give fEPSP slope of ~20-25% of the one obtained with 15-20V stimulation.

For whole cell recordings of miniEPSCs, CA1 pyramidal neurons were blind-patched and voltage clamped at -75 mV with a pipette (3-5 MΩ) containing 135mM $KMeS0_3$, 5mM NaCl, 1mM $CaCl_2$, 0.5mM EGTA-Na, 10mM Hepes, 10mM glucose, 5mM ATP and 0.4mM GTP (pH 7.2; 280-290 mOsm). For LTP experiments in whole cells, the internal solution contained 135mM $CsMeSO_3$, 2mM NaCl, 5mM KCl, 1mM $CaCl_2$, 0.1mM EGTA, 10mM HEPES, 5mM ATP, 0.4mM GTP, 10mM Phosphocreatin. Series resistance (typically 10-25 MW) was monitored throughout each experiment with a -4 mV, 80 ms pulse and cells with more than 15% change in series resistance were excluded from analysis. Miniature EPSPs were recorded in ACSF containing 500 nM TTX, 100 mM $CdCl_2$, 4 mM Ca^{2+}, 100mM Picrotoxin, 2 mM CGP 55845A.

For the investigation of the regulation of endogenous Neurl1 by LTP, the stimulating and recording electrodes were positioned at the beginning (CA2/CA1 border) and the ending (CA1/subiculum border) of the CA1 area, respectively, in order to ensure that a large portion of hippocampal slice CA1 neurons was subjected to TBS treatment. For all experiments, two slices from approximately the same hippocampal area were placed on a coverslip in the chamber, and recorded in parallel, one control (only baseline responses) and one stimulated (4 TBS at 5 min intervals). At the end of the recordings (30 min after the last TBS), slices were collected by removing the coverslip, and immediately frozen on dry ice. The CA1 area (comprising the stratum radiatum, stratum oriens and stratum pyramidale) was dissected under a microscope and the samples were stored at –80°C.

Behavior

For all behavioral tasks, 3 ½ month old double transgenic and control littermate males were used. The *CaMKIIα-tTA, tetO* single transgenic and wild type mice (no transgene in the genome) were pooled

together in the control group, as no position effects due to the transgenes' insertion in the genome were observed in any of the behavioral experiments that we performed. The experimenter was "blind" to the genotype in all studies. The StatView software (SAS institute) was used for the statistical analysis of data.

Elevated Plus-Maze. The elevated plus maze is a widely used behavioral task for rodents to measure anxiety. The basic measure of this task is the animals' preference for dark and not exposed places (Walf and Frye, 2007). The plus maze was made of four black wooden arms (67cm long × 7cm wide). Two arms were open (with 1cm rim), and two enclosed (15cm high). The arms formed a cross shape with the two similar arms opposite to each other. The maze was 50 cm above the floor and dimly illuminated. At the beginning of each trial, naïve animals were placed at the center of the maze, facing an enclosed arm. The behavior of the animals was recorded for 5 min with a camera located above the maze. Time spent and entries in the different compartments (closed and open arms) were assessed. Anxiety was assessed by comparing the activity in the open versus enclosed arms, using an index taking into account the time spent in each category of arm as the ratio open arms/enclosed arms. The relationship between index value and anxiety level is inversely analogous, meaning that the higher the index value the lower the level of anxiety. The data were statistically evaluated using ANOVA, with the genotype as the between factor and the arm (open and enclosed) as the within factor.

Open field. In the open field, mice with anxiety-like phenotypes tend to stay along the walls, avoiding the center of the arena. Exploration and reactivity to a novel open field was assessed in Plexiglas activity chambers (model ENV-520; Med Associates, St. Albans, Vermont) (43.2 cm long × 43.2 cm wide × 30.5 cm high). Mice were placed in the front left corner of the open field, and activity was automatically recorded for 60 min (12 x 5 min). ANOVAs with the genotype as the between factor and the zone (center or periphery of the arena) as the within factor were used for the statistical analysis. This task followed the elevated plus-maze task (48 hours difference).

Water maze. The water maze task was performed as previously described (Malleret et al., 1999). It consisted of three phases: 1) 2 days with a visible platform (4 trials/day), 2) 8 days with a hidden platform in quadrant number 3 (1 trial/day; 2 weeks; 4 days/week; 2 days of rest in home cage between the two weeks of training) and 3) 10 days with the hidden platform in opposite quadrant number 1 (2 weeks; 5 days/week; 2 days of rest in home cage). We chose the 1trial/day protocol based on previous studies in *Drosophila* showing that single trial training is adequate for Neuralized overexpressing flies to acquire memory performance that normally requires multiple trials of spaced training (Pavlopoulos et al., 2008). This demanding task involves long-term memory processes that operate cumulatively over several days. Probe trials, during which the platform was removed from the maze, lasted 1 minute and were performed to assess retention of the previously acquired information. The exploration time and the number of platform crossings were examined. The trajectories of mice in the maze were recorded with a video tracking system (HVS Image Analysis System VP-118). Statistical analyses used ANOVAs with genotype as the between-subject factor and day and area (quadrant or platform during the probe trials) as within-subject factors. Scheffe's test was used for post-hoc analysis.

Novel object recognition task. This task was performed as previously described (Bevins and Besheer 2006; Bourtchouladze et al., 2003). In summary, the mice were first habituated in the arena for 15 minutes for three consecutive days. Mice were first allowed to explore two identical novel objects for 15 minutes. In the retention test performed 24 hours later, a novel object was presented in conjunction with the familiar one. The testing phase lasted 5 minutes. The mice were recorded using a videocamera and the exploration time was counted using the ODlog software (www.macropodsoftware.com). The discrimination index was determined by the difference in exploration time expressed as a ratio of the total time spent exploring the two objects.

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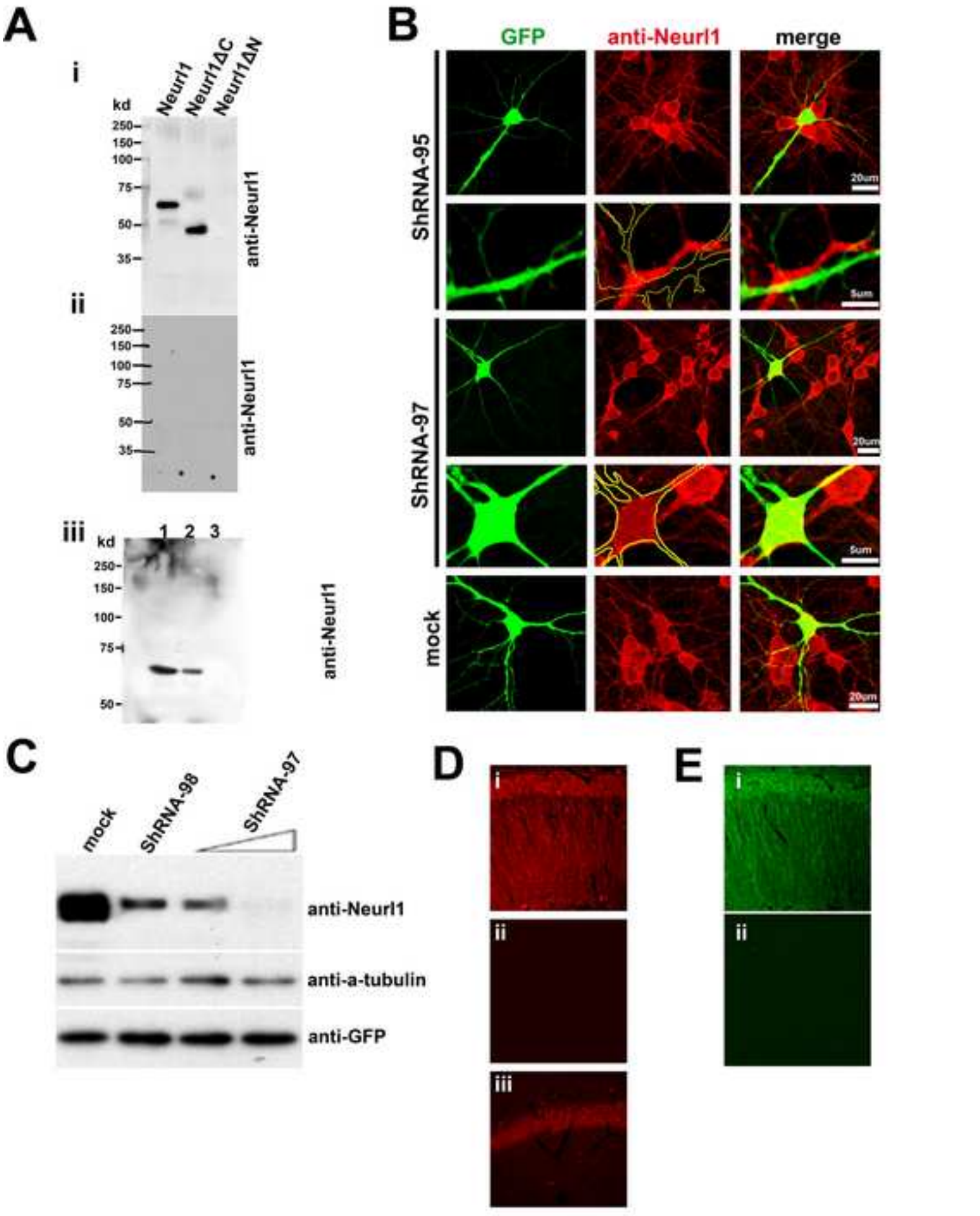
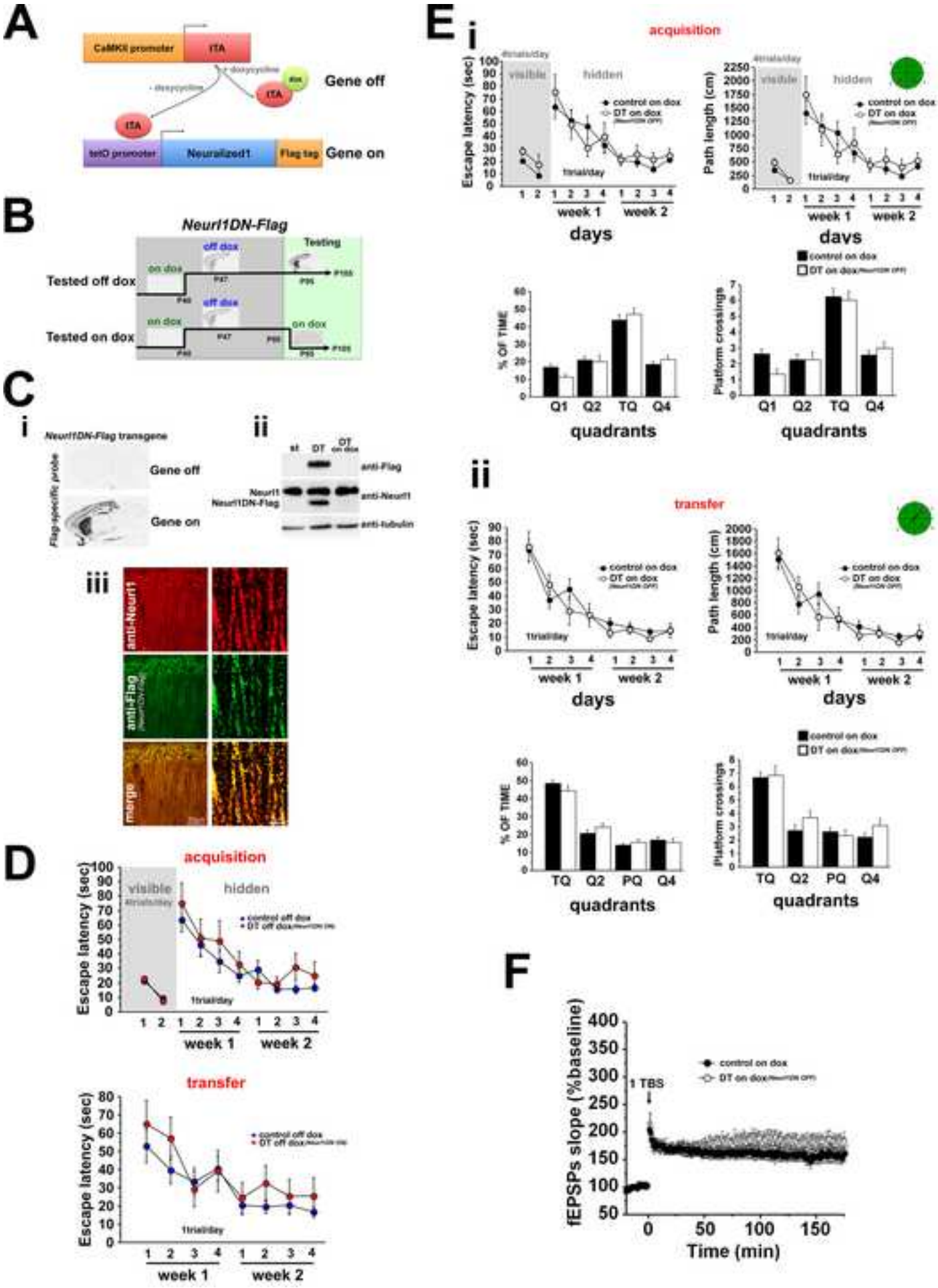
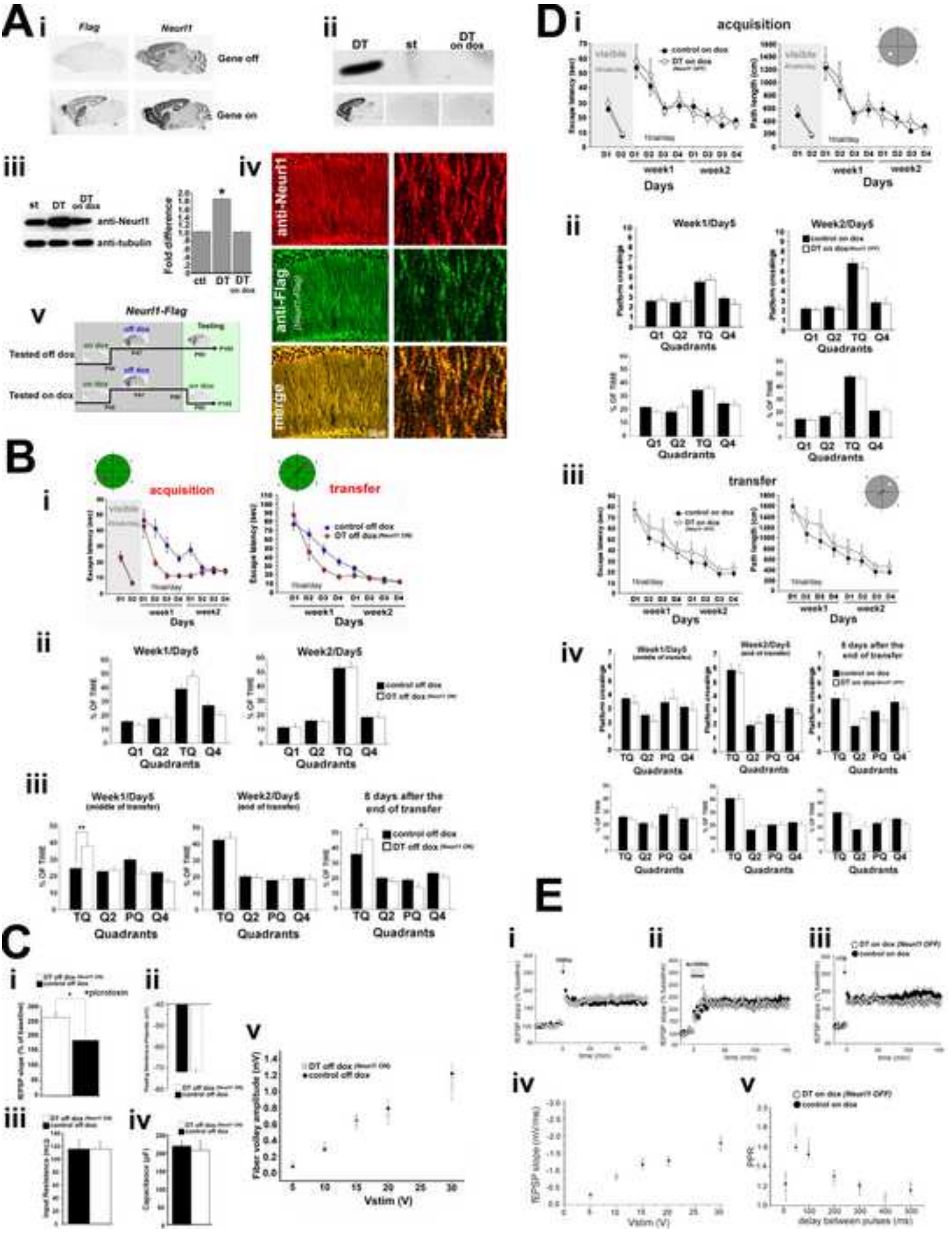
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



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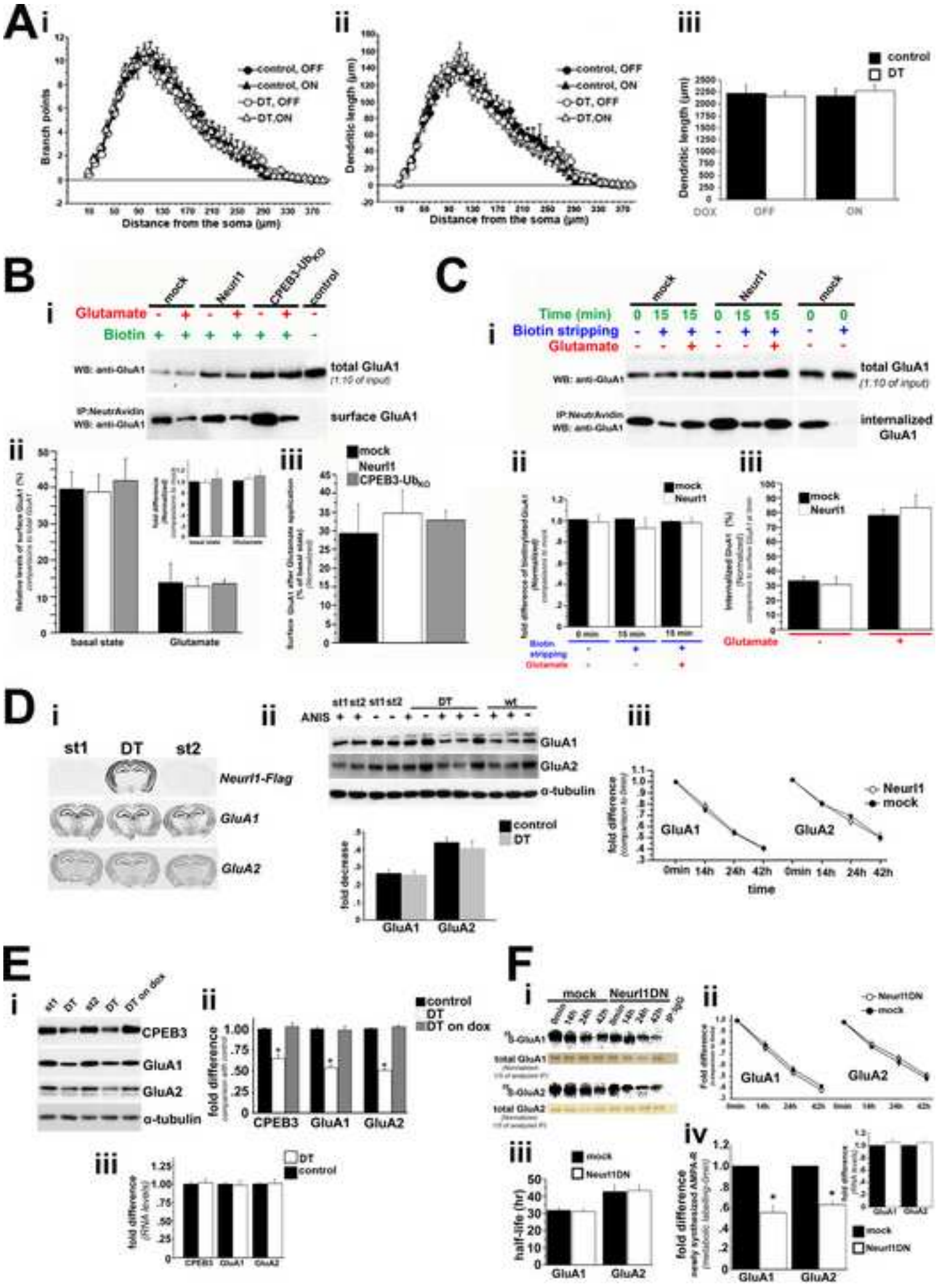
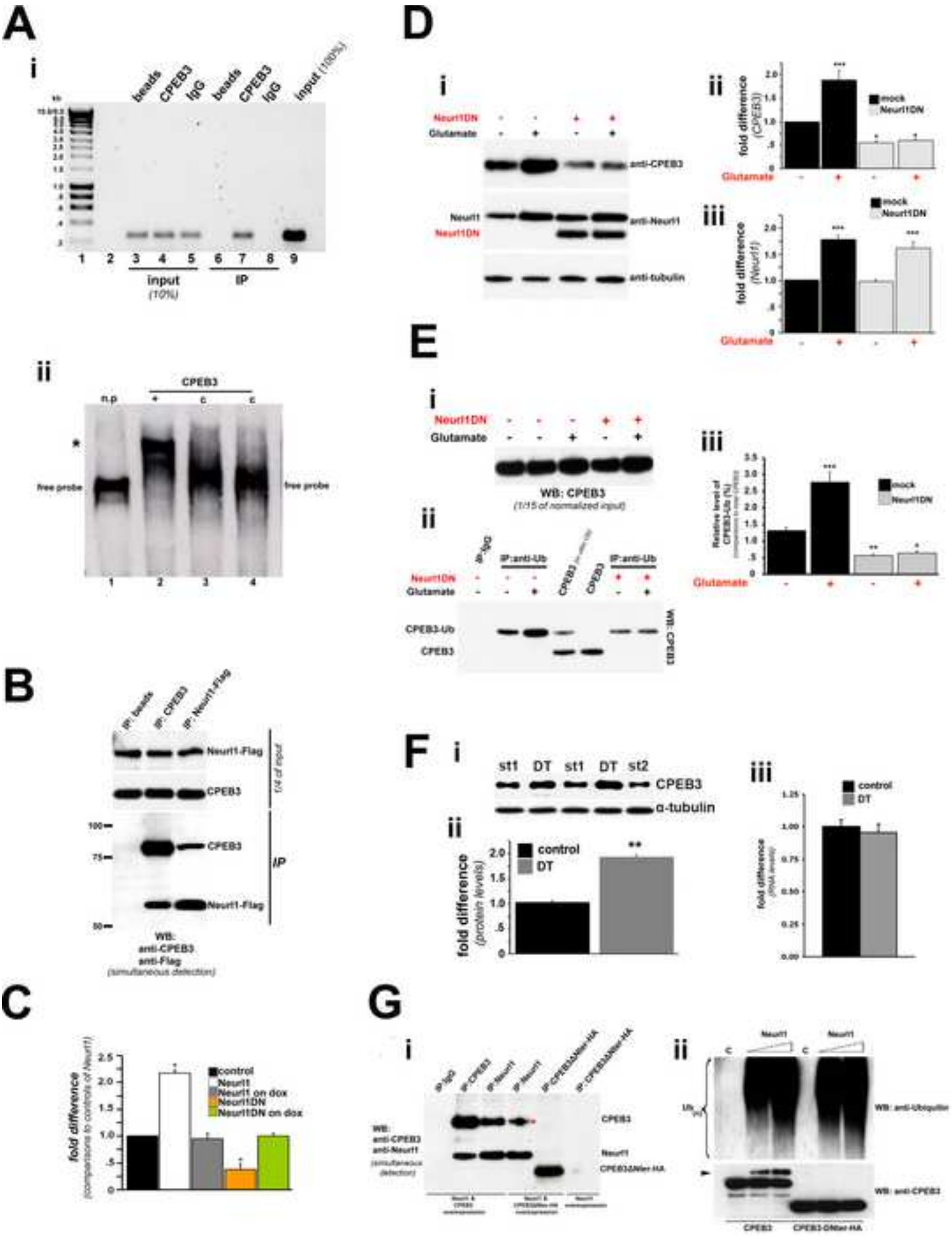
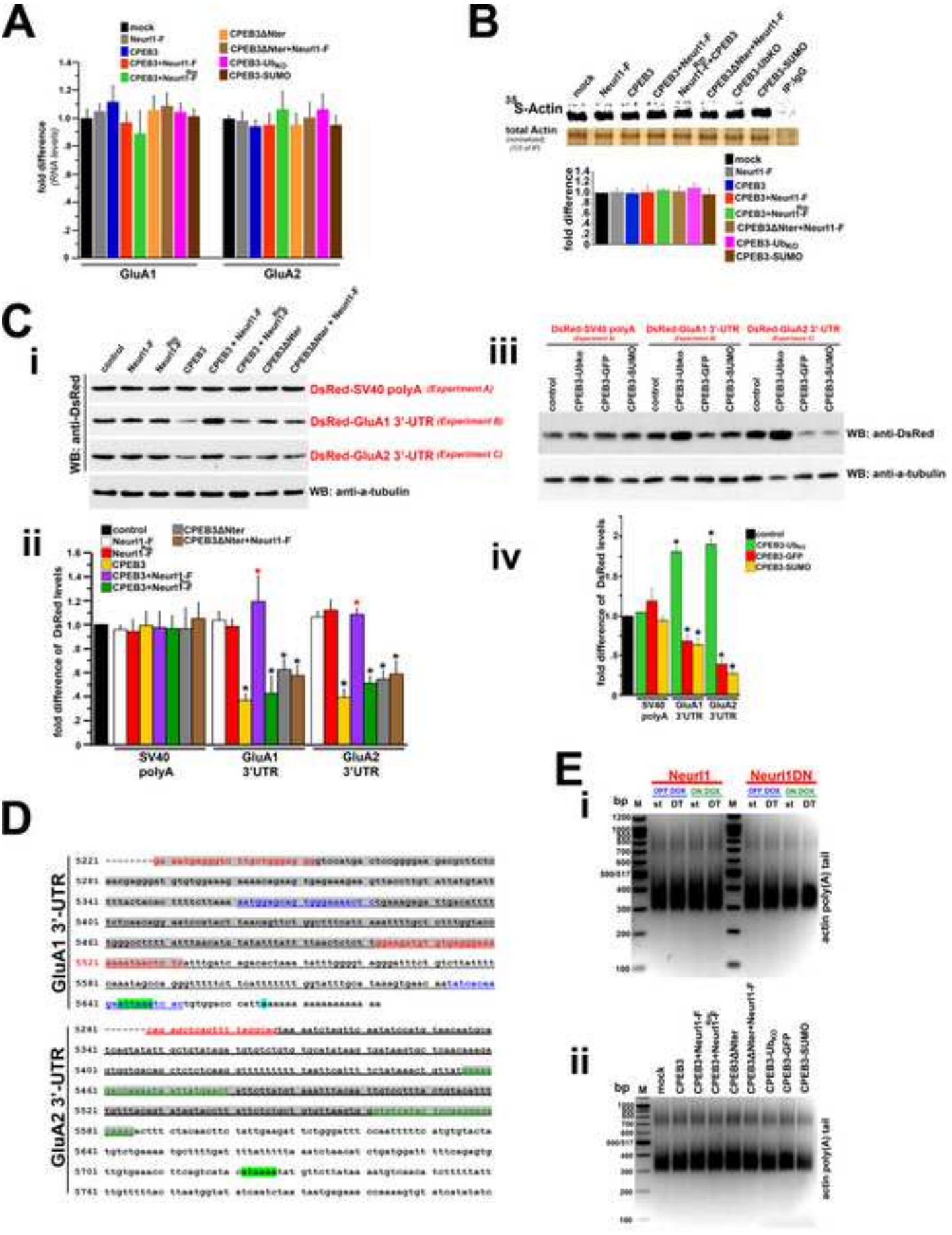


Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7