Table S3. Data Acquired from AMPAR Biotinylation Assays in Hippocampal Neurons and RFP Reporter Assays in HEK293T Cells, Related to Figures 4 and 6

Experiment	Rationale	Results	Related Figures
Examination of the surface population of AMPAR, their trafficking and endocytosis in cultured hippocampal neurons overexpressing Neurl1 or CPEB3-Ub_{KO}	In addition to regulating AMPAR levels and synapse formation, we wondered whether Neurl1 might also be implicated in AMPAR trafficking. We raised this question because ubiquitination has a well-known role in inducing endocytosis of transmembrane receptors (Hicke, 2001), and in regulating AMPAR trafficking (Mabb and Ehlers, 2010). Despite the fact that we found that E-LTP was not altered in the Neurl1DN mice, findings that suggest that Neurl1 is likely not involved in AMPAR trafficking, we cannot rule out a non-specific effect of Neurl1 overexpression. To address this possibility, we studied the basal and activity-induced surface expression and internalization of GluA1 receptors in cultured hippocampal neurons overexpressing Neurl1 and in control neurons. Because Neurl1 overexpression and Neurl1-dependent ubiquitination and ubiquitin activate CPEB3 with consequent increase of AMPA receptors, we would expect that activated CPEB3 by ubiquitin to have similar effects. Therefore, we also examined the effect of the overexpression of on AMPAR trafficking.	Consistent with our observations in the adult hippocampus, Neurl1 overexpression increased the total levels of GluA1 receptors. We found that this increase was accompanied by an increase of the surface population of GluA1 receptors. However, the relative amount of the surface GluA1 receptors compared to the total levels of GluA1 in the Neurl1 overexpressing neurons and in control neurons was similar. The activity-induced changes of the surface GluA1 receptors were also unaffected by Neurl1 overexpression. The basal and the activity-induced internalization of surface GluA1 receptors were also not altered. Overexpression of CPEB3-Ub_{KO} acted similar to Neurl1 overexpression; it increased the surface population of GluA1 receptors but did not alter their relative amount compared to the total GluA1 as well as their activity-induced changes.	**S4B&C**
RFP-reporter assays in HEK293T cells	CPEB3 is an RNA binding protein that binds the 3'-UTR of GluA1 and GluA2 mRNAs (Huang et al., 2006 & figure S7A&B). Therefore, we would expect that the translational regulation of GluA1 and GluA2 by Neurl1 overexpression and by Neurl1-mediated ubiquitination of CPEB3 is dependent on the 3'-UTRs of the mRNAs of GluA1 and GluA2. To address this question, we turned to HEK293 cells and examined the levels of DsRed reporter protein, the cDNA coding sequence of which was fused to either the SV40 poly(A) signal or the 3'UTR of GluA1 and GluA2 mRNAs (DsRed-SV40, DsRed-R1UTR and DsRed-R2UTR, respectively). HEK293T cells do not express endogenous CPEB3, which also allowed us to test further the specificity of Neurl1 action on CPEB3-dependent translation of GluA1 and GluA2.	CPEB3 by itself significantly reduced the levels of DsRed-R1UTR and DsRed-R2UTR proteins but not DsRed-SV40. Remarkably, coexpression of Neurl1 completely reversed the effect of CPEB3 despite that Neurl1 expression alone did not alter the levels of the reporters compared to control. This strongly supports the finding that Neurl1 modulates GluA1 and GluA2 translation specifically through CPEB3. Neurl1 was incapable of reversing the inhibitory action of CPEB3ΔNter on DsRed-R1UTR and DsRed-R2UTR translation and $Neurl1^{Rm}$ did not reverse the inhibitory action of CPEB3. Finally, CPEB3-Ub_{KO} expression led to a pronounced increase of DsRed-R1UTR and DsRed-R2UTR protein levels without affecting DsRed-SV40.	**S6C**

Table S4. Detailed Statistical Analysis of the Indicated Figures, Related to Figures 4–7

Related figure	Experiment	Analysis
Figure 4A	Golgi stain and tracing of CA1 apical dendrites of Neurl1 overexpressing mice and controls	Neurl1 overexpressing mice (DT off dox) displayed highly significant increase of spine density compared to all the other groups of animals (mean spine number/µm ±SEM; DT off dox: 1.01±0.05; controls off dox 0.64±0.03; DT on dox: 0.62±0.07; controls on dox: 0.59±0.05; $F_{(3,58)}$=16.5, p<0.0001). There was significant doxycycline treatment effect between the two groups of DT mice (p=0.0002). Spine density of DT mice on dox and controls on and off dox was similar (ANOVA; $F_{(2,41)}$=0.381, p=0.6855). DT off dox: n=3, 18 neurons; DT on dox: n=2, 10 neurons controls off dox: n=6, 18 neurons; controls on dox: n=5, 16 neurons
Figure 4B	mEPSCs recorded in CA1 pyramidal neurons of Neurl1 overexpressing mice and controls	miniEPSCs amplitude was identical in both genotypes (controls: 24.27 ± 0.46 pA vs. DT: 23.26 ± 0.85 pA, p = 0.28). mEPSC the frequency was significantly increased in DT mice compared to controls (1.65 ± 0.18 Hz vs. 1.17 ± 0.13 respectively, p = 0.036) Controls: 21 neurons (6 mice); DT: 17 neurons (5 mice)
Figure 4C	Protein levels of PSD95, Shank and Synaptophysin of Neurl1 overexpressing mice and controls	Comparisons are made with control mice off dox. Control mice for each treatment showed similar protein levels and were pooled (p>0.27 and p>0.65 for off dox and on dox, respectively). ANOVA showed significant increase of protein levels in the DT mice off dox compared to controls (either off or on dox) and DT mice on dox (p<0.0001). There was no difference between DT mice on dox and control animals (p>0.3). controls off dox: single tetO, single tTA and wild type mice, n=3 per genotype controls on dox: n=3 for single tetO and wild type mice and n=2 for single tTA animals DT: n=6 for each treatment
Figure 4D	Protein levels of GluA1 and GluA2 in the hippocampus of adult Neurl1 overexpressing and control mice.	DT mice off dox showed significantly higher levels of GluA1 and GluA2 compared to DT animals on dox and controls either on dox or off dox (*p<0.0001). The levels of GluA1 and GluA2 were not different between control mice (for both treatments) and DT animals on dox (p>0.5). Number of mice and genotypes are described in figure 4C (see above).
Figure 4E	Quantitative real time PCRs on reverse transcription products of total RNA isolated from the hippocampus of adult Neurl1 overexpressing and control mice.	Averaged fold difference ± SEM of GluA1 and GluA2 mRNA levels between DT animals (Neurl1 overexpression) and controls. The control genotypes showed similar levels of GluA1 and GluA2 transcripts (p>0.2) and were pooled. No differences were observed for the GluA1 and GluA2 mRNA levels between controls and DT mice (p>0.3). control mice: single tTA, n=4; single tetO, n=4; wild type, n=2 DT mice: n=6
Figure 4F **&** **S4Diii**	Averaged half-life of GluA1 and GluA2 in Neurl1 overexpressing mice and controls (3 independent experiments) Time course for ^{35}S-GluA1 and ^{35}S-GluA2 degradation in control and Neurl1-overexpressing hippocampal cultures (16DIV) after a 1hr pulse of ^{35}S-methionine/^{35}S-cysteine (same groups as in figure 4F)	No differences were observed (mock: 31.67±2.02 hrs and 42.78 ±4.48hrs; Neurl1: 32.64±1.88 hrs and 41.27 ±4.37 hrs; for GluA1 and GluA2, respectively; GluA1: p=0.9935, GluA2: p=0.8128). The rate of decrease of ^{35}S-GluA1 and ^{35}S-GluA2 in mock and Neurl1 overexpressing neurons was similar (repeated-measures ANOVA; no significant genotype or genotype*time-point effects; GluA1 and GluA2, respectively: Genotype: $F_{(1, 10)}$=0.358, p=0.5630 & $F_{(1, 10)}$=0.157, p=0.6987; genotype*time-point: $F_{(3, 30)}$=0.273, p=0.8444 & $F_{(3, 30)}$=0.590, p=0.6256).
Figure 5E	Averaged relative levels of monoubiquitinated CPEB3 in the hippocampus of either Neurl1 overexpressing or Neurl1DN expressing mice and controls	Averaged data from 3 independent experiments (1 mouse per genotype, treatment and experiment; controls for Neurl1 DT: single tetO-Neurl1-Flag, single CaMKIIa-tTA; controls for Neurl1DN DT: single tetO-Neurl1DN-Flag, single CaMKIIa-tTA; n=3 per genotype; Neurl1 DT on dox and Neurl1DN DT on dox: n=3 for each transgene). The relative levels of monoubiquitinated CPEB3 were significantly increased in Neurl1 DT mice compared to controls and Neurl1 DT mice on dox (p<0.0016), while they were significantly reduced in the Neurl1DN DT mice (comparison with respective controls and DT on dox mice; p<0.0003). The relative levels of monoubiquitinated CPEB3 in the adult hippocampus in DT animals on dox (either Neurl1 or Neurl1DN) and controls were similar (for Neurl1: p=0.9973; for Neurl1DN: p=0.9844).
Figure 6A	Averaged protein levels of GluA1 and GluA2 in cultured hippocampal neurons	Collected data from four independent experiments. CPEB3-Ub_{KO} significantly increases the levels of GluA1 and GluA2 compared to mock (p<0.0001). CPEB3-EGFP and CPEB3-SUMO significantly decreased the protein levels of AMPA receptors compared to mock (p<0.0007). The effects of CPEB3-EGFP and CPEB3-SUMO were similar to CPEB3 (p>0.9).
Figure 6B	Averaged levels of newly synthesized GluA1 and GluA2 in cultured	Newly synthesized GluA1 and GluA2 proteins were significantly increased in neurons overexpressing Neurl1 compared to mock (p<0.0001 for GluA1 and p=0.0073 for GluA2). Significant increase was also observed in neurons overexpressing Neurl1 and CPEB3 together (p<0.0001 and p=0.0091, for

	hippocampal neurons	GluA1 and GluA2, respectively) and neurons expressing CPEB3-Ub_{KO} alone ($p<0.0001$). The levels of ^{35}S-GluA1 and ^{35}S-GluA2 in neurons overexpressing CPEB3, CPEB3 and $Neurl1^{Rm}$-F together, CPEB3ΔNter, CPEB3ΔNter and Neurl1-F together and CPEB3-SUMO were similar ($p>0.25$) and significantly lower compared to mock ($p<0.0001$ for GluA1 and $p<0.009$ for GluA2).
Figure 7	Averaged density of dendritic protrusions of hippocampal neurons	The density of dendritic protrusions was significantly different in neurons expressing the indicated proteins compared to control neurons ($p<0.0004$). The density of dendritic protrusions was similar between neurons overexpressing CPEB3, CPEB3ΔNter +Neurl1-F, CPEB3+$Neurl1^{Rm}$-F, CPEB3-SUMO and CPEB3-Ds-Red (ANOVA did not reveal significant difference between these groups, $p=0.6165$) and significantly lower compared to control neurons ($p<0.0003$). The spine densities of neurons either overexpressing Neurl1 alone or together with CPEB3 as well as the spine density of neurons expressing CPEB3-Ub_{KO} alone were significantly increased compared to mock (CPEB3+Neurl1-F: $p=0.041$; all the others: $p<0.0001$). The difference between neurons overexpressing CPEB3 alone and neurons overexpressing CPEB3+Neurl1 was highly significant ($p<0.0001$).
Figure S4Dii	Analysis of GluA1 and GluA2 protein levels 90 minutes after subcutaneous injection of the protein synthesis inhibitor anisomycin	Controls injected with anisomycin (single tetO, single tTA and wild type mice; n=3 per genotype) showed similar change of GluA1 and GluA2 levels ($p>0.8$) and were pooled. In the presence of anisomycin, GluA1 and GluA2 were significantly decreased in both controls and Neurl1DT mice ($p<0.0001$). The decrease of GluA1 and GluA2 proteins in DT (n=4) and controls was also similar (DT: 25.82%±2.43% decrease of GluA1 and 40.72%±4.1% of GluA2; controls: 26.66%±1.88% for GluA1 and 43.93%±2.83% for GluA2; ANOVA did not reveal significant genotype effect, $F_{(1,44)}=0.057$, $p=0.8120$ and $F_{(1,44)}=0.362$, $p=0.5505$ for GluA1 and GluA2, respectively). Controls/saline: n=3 per genotype. DT/saline:n=4.
Figure S4Eii	Analysis of the protein levels of CPEB3 and its targets GluA1 and GluA2 in the adult hippocampus of mice expressing Neurl1DN and their control littermates	DT mice off dox showed significantly lower levels of CPEB3, GluA1 and GluA2 compared to DT animals on dox and controls on dox or off dox (*$p<0.0001$). The levels of CPEB3, GluA1 and GluA2 were not different between controls (for both treatments) and DT on dox ($p>0.5$). Controls for each treatment had similar levels of CPEB3, GluA1 and GluA2 and were pooled ($p>0.5$). DT: n=4 per treatment. Controls: single tetO-*Neurl1DN-Flag*, single *CaMKIIα-tTA* and wild type mice (n=3 per genotype and treatment).
Figure S4Eiii	Quantitative real time PCRs on reverse transcription products of total RNA isolated from the hippocampus of adult Neurl1DN expressing (DT) and control mice	Averaged fold difference $\pm$ SEM of mRNA levels between DT mice (n=5) and controls (single tTA, n=3; single tetO, n=3; wild type, n=3). Control genotypes showed similar levels of CPEB3, GluA1 and GluA2 transcripts ($p>0.4$) and were pooled. No differences were observed for the CPEB3, GluA1 and GluA2 mRNA levels between controls and DT ($p>0.5$).

Table S5. Identified Proteins from Mass Spectrometric Sequencing of Protein Bands Isolated from Neurl1 Adult Hippocampal Immunoprecipitants Separated by SDS-PAGE, Related to Figure 5

Name	Score	Identified Peptides	Accession #
Neuralized1	1113	21	gi|15128197
Cytoplasmic polyadenylation element-binding protein 3	1106	19	gi|81912025
heat shock protein 1, beta	1058	17	gi|40556608
Ubiquinol-cytochrome-c reductase complex core protein I, mitochondrial precursor	245	7	gi|14548301
beta subunit of Ca2+ /calmodulin dependent protein kinase II	153	3	gi|50276
serine/threonine specific protein phosphatase	117	3	gi|4584820
hexokinase	116	2	gi|309289
intersectin-EH binding protein Ibp2	75	1	gi|3063649

Table S6. Mass Spectrometric Sequencing Results Identified CPEB3 in the Pool of Proteins Coimmunoprecipitated from Adult Hippocampal Homogenates Using Neurl1-Specific Antibody, Related to Figure 5

Nineteen tryptic peptides were identified covering 43% of CPEB3 protein sequence. The sequencing search results and the identified peptides are shown.

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **42%**

Start-End	Observed	Mr(expt)	Mr(calc)	Delta	Sequence
22 - 74	1349.8306	5395.2933	5395.6444	-0.3511	R.QQQQQQQQLQPEPGAAEAPSTPLSSEIPKPEDSSAVPALSPASAPPAPNGPDK.M
193 - 206	758.3394	1514.6642	1514.7590	-0.0947	R.RSPASPSQAPYAQR.S
193 - 206	505.9125	1514.7157	1514.7590	-0.0433	R.RSPASPSQAPYAQR.S
193 - 206	506.2357	1515.6853	1514.7590	0.9263	R.RSPASPSQAPYAQR.S
194 - 206	680.3293	1358.6440	1358.6579	-0.0138	R.SPASPSQAPYAQR.S
194 - 206	680.7990	1359.5834	1358.6579	0.9256	R.SPASPSQAPYAQR.S
272 - 294	1057.0284	2112.0422	2112.2307	-0.1885	R.AVGVGVGVGVGVPSPLNPISPLK.K
295 - 306	428.8968	1283.6686	1283.7238	-0.0552	K.KPFSSNVIAPPK.F
295 - 306	642.8455	1283.6764	1283.7238	-0.0473	K.KPFSSNVIAPPK.F
295 - 306	643.3396	1284.6646	1283.7238	0.9409	K.KPFSSNVIAPPK.F
317 - 325	586.2077	1170.4008	1170.4764	-0.0755	K.SWMEDNAFR.T
317 - 338	885.9957	2654.9653	2655.1772	-0.2119	K.SWMEDNAFRTDNGNNLLPFQDR.S
317 - 338	1328.5149	2655.0152	2655.1772	-0.1620	K.SWMEDNAFRTDNGNNLLPFQDR.S
392 - 410	724.2983	2169.8731	2170.0048	-0.1317	R.MGINFHHPGTDNIMALNTR.S
419 - 447	1028.3900	3082.1482	3082.4156	-0.2674	R.SSLFPFEDAFLDDSHGDQALSSGLSSPTR.C
461 - 480	1088.9268	2175.8390	2176.0688	-0.2298	K.VFVGGLPPDIDEDEITASFR.R
481 - 492	484.2417	1449.7033	1449.7881	-0.0848	R.RFGPLVVDWPHK.A
482 - 492	432.2036	1293.5890	1293.6870	-0.0980	R.FGPLVVDWPHK.A
529 - 539	640.8060	1279.5974	1279.6846	-0.0872	K.LYLCVSSPTIK.D
568 - 579	437.9052	1310.6938	1310.7823	-0.0885	K.TIFVGGVPRPLR.A
580 - 589	590.7459	1179.4772	1179.5628	-0.0855	R.AVELAMIMDR.L
580 - 589	590.8040	1179.5934	1179.5628	0.0307	R.AVELAMIMDR.L
590 - 606	935.8906	1869.7666	1869.8819	-0.1152	R.LYGGVCYAGIDTDPELK.Y
614 - 629	863.3557	1724.6968	1724.8846	-0.1877	R.VAFSNQQSYIAAISAR.F
614 - 629	863.3975	1724.7804	1724.8846	-0.1041	R.VAFSNQQSYIAAISAR.F
630 - 640	452.8576	1355.5510	1355.6834	-0.1324	R.FVQLQHNDIDK.R
630 - 640	678.8063	1355.5980	1355.6834	-0.0853	R.FVQLQHNDIDK.R
630 - 640	453.2007	1356.5803	1355.6834	0.8969	R.FVQLQHNDIDK.R
641 - 661	650.2584	2597.0045	2597.1673	-0.1628	K.RVEVKPYVLDDQMCDECQGTR.C