SUPPORTING INFORMATION

Palmitoylation and membrane binding of Arc/Arg3.1: A potential role in synaptic depression

Barbara Barylko, Julia R.Wilkerson, Sheridan H. Cavalier, Derk D. Binns, Nicholas G. James, David M. Jameson, Kimberly M. Huber, and Joseph P. Albanesi*

Experimental Procedures

Materials - Mouse Arc cDNA, cloning reagents and reagents for mutagenesis were from Thermo Scientific. Primers were from Invitrogen. pGST parallel 1 vector was a gift of Dr. Hong Zhang (UT Southwestern). His-TEV protease was a gift of Dr. Elizabeth Goldsmith (UT Southwestern). Monoclonal anti-Myc antibody 9E10 was from National Cell Culture Center (Minneapolis, MN). Polyclonal anti-Arc antibodies were from Synaptic Systems (Gottingen, Germany). Fluorescently labeled secondary antibodies for Infrared Imaging System were from LI-COR. Reagents for electrophoresis and immunoblotting were from Bio-Rad. Glutathione agarose was from Pierce. Ni^{2+} NTA resin was from Roche**.** Lipids were from Avanti Polar Lipids. [9,10-^{3}H]palmitic acid was from PerkinElmer Life Sciences. Q Sepharose, thiopropyl Sepharose, and other reagents were from Sigma-Aldrich.

Generation and purification of constructs – Arc and Arc mutant bacterial expression and purification was as described previously (1). Briefly, mouse Arc cDNA was cloned into pGST.parallel 1 vector to yield Arc with a TEV cleavage site at the N terminus. GST-Arc was expressed in *E.coli* Rosetta 2 cells. Cells were harvested after growing for 20 h at 16°C and GST-Arc was extracted from bacterial pellet with solution A (20 mM Hepes, pH 8.0, 100 mM NaCl, 2 mM DTT, protease inhibitor cocktail consisting of 10ug/ml each of N-p-tosyl-L-lysine chloromethyl ester, N-p-tosyl-L-arginine methyl ester, N-p-tosyl-L-lysine chloromethyl ketone, leupeptin, pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.05 mg/ml lysozyme. The extract was centrifuged at 100,000 x g for 1 hour and the supernatant was incubated with glutathione resin for at least 4 hours at 40C. The resin was washed first with the extraction solution (A), next with A + 0.2 % Triton X100, then with A + 1M NaCl (without detergent). Washed resin was either eluted with glutathione to obtain GST-tagged Arc or incubated with TEV protease to release Arc. Arc was dialysed against 20 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM DTT, and PMFS and, if necessary, was additionally purified on Q Sepharose column. TEV was removed by incubation of Arc solution with Ni^{2+}-NTA**.** Purified Arc was aliquoted and stored at -70^{0}C.

The endophilin A2 construct was generated by subcloning mouse cDNA into a pQE-80-L vector which contains a hex-His tag. His_6–endophilin was purified using Ni^{2+}-NTA resin as described previously (2).

For mammalian expression, Arc constructs were generated by cloning Arc cDNA into pCMV5 myc vector or pcDNA3.1 vector to obtain myc-Arc or non-tagged Arc, respectively. Unpalmitoylatable Arc constructs containing C94S, C96S, and C98S mutations were generated by site-directed mutagenesis using Myc-Arc and non-tagged Arc clones as templates.

MEF2-VP16-GFP, MRE-mCherry, pcDNA3-Arc and pcDNA-mCherry have been described previously (3).

Synaptosome preparation - Mouse brains were homogenized with a Dounce homogenizer in 0.32 M sucrose, 20 mM Hepes, pH 7.5. The homogenate was centrifuged for 10 min at 1,000 x *g* and the resulting pellet was washed and centrifuged again at 1,000 x *g*. Supernatants from the two spins were combined and centrifuged for 55 min at 17,000 x *g*. The pellet (P2; crude synaptosomes) was resuspended in 0.32 M sucrose, 20 mM Hepes, pH 7.5 and 3 ml of this suspension were loaded on a sucrose gradient consisting of 4.5 ml 1.2 M sucrose overlaid with 4.5 ml 0.8 M sucrose. The gradients were centrifuged at 100,000 x *g* for 1 h in a Beckman SW41 rotor, the 0.8-1.2 M sucrose interfaces, which contain purified synaptosomes, were collected and diluted with 0.1 M sucrose to achieve a final sucrose concentration of 0.32 M, and then centrifuged for 1 h at 100,000 x *g*. The pellet was resuspended in synaptosome buffer (20 mM Hepes, pH 7.5, 128 mM NaCl, 3 mM KCl, 1.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 11 mM glucose and protease inhibitors(as above).

Analysis of Arc solubilization from synaptosomes - Synaptosomes were "opened" by short sonication (5 min at medium setting) using Bioruptor UCD-200 (diagenode) and the suspension was divided into 4 aliquots. The solution in each aliquot was adjusted to obtain the following conditions: (a). 120 mM NaCl; (b). 1 M NaCl; (c). 120 mM NaCl and 0.2 M Na_2CO_3; and (d). 120 mM NaCl and 1% Triton X-100. The samples were centrifuged at 245,000 x g for 15 min. in a TLA-100.1 rotor in a tabletop ultracentrifuge. Supernatants (solubilized fraction) and pellets (membranous fraction) resuspended to the initial sample volumes were electrophoresed, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Primary antibodies were detected by fluorescently labeled secondary antibody using the LI-COR Odyssey system.

Cell fractionation - HeLa cells transfected with myc-Arc were lysed in a solution containing 20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 5 mM EGTA, 1 mM PMSF and protease inhibitor cocktail. They were "open" by 5 min sonication at medium setting in cell disruptor and centrifuged for 10 min at 1000 x g to get post-nuclear supernatant (PNS). Supernatant was divided into 4 aliquots, 1. No treatment, 2. Resuspension solution containing 1 M NaCl, 3. Resuspension solution with 0.2 M Na_2CO_3, and 4. Resuspension solution supplemented with 1 % Triton X-100. The samples were centrifuged at 245,000 x g for 15 min.as described above. The supernatants and the pellets resuspended in the initial volume of the sample were used for analysis as described above.

Preparation of membrane rafts - Low buoyant density "membrane rafts" were prepared from Triton-insoluble pellets of purified synaptosomes (Figure 1C). Triton-insoluble pellets were obtained after centrifugation of synaptosome suspension in 1% Triton X-100 for 1 h at 165,000 x *g*. Rafts were also prepared from crude synaptosome suspensions (P2 fraction) in 1% Triton X-100 (Figure S1). Samples were adjusted to 42.5% sucrose (0.6 ml of fraction was mixed with 0.6

ml of 85% sucrose), overlaid with 35% sucrose (2 ml) and then with 16% sucrose (1.2 ml) in the synaptosomal resuspension buffer described above but without detergent (4). Following centrifugation at 210,000 x *g* for 20 h at 4°C in an SW60 rotor, 0.4 ml fractions were collected from the bottom of the tubes using Fraction Recovery System (Beckman) and equal aliquots were subjected to SDS-gel electrophoresis and immunoblotting as described above.

Liposome binding assay - Liposomes were prepared according to a procedure described by Lee and Lemmon (5). Brominated phosphatidylcholine (PC) or total lipid extract from brain (Folch fraction1 (6)); was dissolved in chloroform and dried under a stream of nitrogen followed by overnight drying under vacuum. Dried lipids were resuspended in 20 mM Hepes (pH 7.4) and 100 mM NaCl, followed by 10 freeze/thaw cycles in liquid nitrogen and bath sonication. To remove aggregated lipids, liposomes were extruded 10 times through 0.1 μm filters using a Mini–Extruder (Avanti Polar Lipids). Immediately prior to incubation with liposomes, Arc solution was centrifuged for 1 h at 265,000 x *g* at 25^0C. Binding assays were carried out by incubating 4 μM Arc with liposomes for 15 min at 25^0C in 20 mM Hepes (pH 7.4) and 100 mM NaCl. Samples (80 μl) were then centrifuged at 214,000 x *g* for 1 h at 25^0C in a TL-100 tabletop ultracentrifuge. Supernatants were removed and pellets were resuspended in initial sample volumes. Equal volumes of supernatants and pellets were electrophoresed, proteins were visualized by Coomassie blue staining and quantified by scanning in LiCOR Odyssey system.

Analysis of palmitoylation - Palmitoylated endogenous Arc from mouse brain or crude synaptosomes (P2 fraction) and expressed Myc-Arc from HeLa cell were detected using an adaptation of the Acyl-Resin Assisted Capture (Acyl-Rac) method (7). Briefly, the tissue or cells were solubilized with 2.5% SDS in 100 mM Hepes (pH 7.5), 1 mM EDTA, protease inhibitors, 50 mM DTT and incubated at 40^0C for 0.5 h (to reduce potential S-S bonds), then for additional 4 h with methyl methanethiosulfonate (MMTS) to block free thiols. Proteins were precipitated with cold acetone, and the pellet was extensively washed with 70% acetone to remove excess of MMTS. Dried pellet was re-solubilized in 100mM Hepes (pH 7.5) ,0.1 M EDTA and 1% SDS, an aliquot was taken as "Total" and the rest was divided into two equal portions; one was incubated with freshly prepared hydroxylamine (pH 7.5) to remove palmitoyl groups, the other was incubated with NaCl as a negative control. Proteins with free thiols (i.e., from cysteines which were originally palmitoylated) were captured on thiopropyl Sepharose resin. After extensive washing with solubilization solution, the proteins were released from the resin with SDS PAGE loading buffer supplemented with 100 mM DTT, analyzed by SDS-PAGE and identified by immunoblotting.

The ratio of Arc/PSD95 palmitoylation was estimated by comparing the amount of protein (Arc or PSD95) released from thiopropyl resin (labeled NH_2OH in Figures 2A and S1) vs. total protein (Arc or PSD95) prior to NH_2OH treatment.

To monitor palmitate incorporation directly, HeLa cells expressing Myc-Arc were incubated with ^{3}H-palmitate (2.5 mCi/ml) for 3-4 h, solubilized in RIPA buffer (consisting of 0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P40, 0.5% deoxycholate, 0.05% SDS, 2 mM EDTA, 0.2 mM PMSF, protease and phosphatase inhibitors described above), and Arc was immunoprecipitated with anti-Myc antibodies chemicaly crosslinked to protein G-Sepharose with DMP as previously described (8). The immunoprecipitate was subjected to SDS-PAGE, stained with Coomassie Blue, and [^{3}H]palmitate incorporation was detected by autoradiography.

Circular dichroism (CD) - CD spectra were obtained on a Jasco J-851 CD Spectrometer using a Jasco J/0556 quartz cuvette (1 mm pathlength). The concentration of each protein was determined by Bradford Assay, and 0.3 mg/ml was used for each CD spectrum. Proteins were dialyzed into buffer containing 10 mM HEPES/NaOH, pH 7.5, 25 mM NaCl, and 0.5mM TCEP and centrifuged (213000 x *g*, 15 min, 4°C) to remove potential aggregates.

Organotypic Hippocampal Slice Culture & Transfection - Organotypic hippocampal slice cultures were made from postnatal day (P) 6-7 WT or *Arc* KO mice bred from the congenic C57BL/6CR mouse strain as described (10). WT C57Bl/6/CR mice were maintained in an onsite breeding colony and new breeders were obtained from Charles River Laboratories. *Arc*-D2EGFP (*Arc* KO) mice were generously provided by Dr. Kuan Hong Wang (NIMH/NIH) and have been described (11). Organotypic hippocampal slice cultures were biolistically transfected at 5-6 DIV as indicated. Biolistic transfection and bullet preparation were performed with the Helios Gene Gun system as described in (12, 13**).**

Electrophysiology - Dual whole-cell patch recordings were obtained from CA1 pyramidal neurons in slice cultures using IR-DIC and GFP fluorescence to identify non-transfected and transfected neurons as described (9). Recordings from slice cultures were made at 30°C in a submersion chamber perfused at 2.5-3 ml/min with artificial cerebrospinal fluid (aCSF) containing 119 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM D-Glucose, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0.1 mM picrotoxin, 0.002 mM 2-chloro-adenosine, 1uM TTX; pH 7.28, 310 mOsm and saturated with 95% O_2/5%CO_2. Whole cell recording pipettes (~4-7 MΩ) were filled with intracellular solution containing 0.2 mM EGTA, 130 mM K-gluconate, 6 mM KCl, 3 mM NaCl, 10 mM HEPES, 10 mM sucrose, 4 mM ATP-Mg, 0.4 mM GTP-Na, 14 mM phosphocreatine-Tris; pH 7.2, 285 mOsm. For all recordings, input and series resistances were measured in voltage clamp with a 400-ms, –10 mV step from a –60 mV holding potential (filtered at 30 kHz, sampled at 50 kHz). Cells were only used for analysis if they met the following criteria: series resistance < 25 MΩ and stable throughout the experiment, resting membrane potential < -35mV; input resistance >75 MΩ. Waveforms were filtered at 3 kHz, acquired and digitized at 10 kHz on a PC using custom software (LabView; National Instruments). Data analysis was performed using custom-designed software in LabView (12). mEPSCs were detected off-line using an automatic detection program (MiniAnalysis; ºSynaptosoft Inc.) with a detection threshold set at a value greater than at least 2 standard deviations of the noise values, followed by a subsequent round of visual confirmation. The detection threshold remained constant for the duration of each experiment.

Immunocytochemistry and Imaging - Organotypic hippocampal slice cultures were transfected at DIV3 with pcDNA3-Arc (WT or C94,96,98S) and pcDNA3-mCherry (marker). Immunocytochemistry was performed 7-10 days post-transfection as described (13), with minor modifications**.** Slices were quickly rinsed twice in cold phosphate buffered saline (PBS), then fixed for 3 h in 2.5% PFA/4% sucrose at 40 C. Following five 30 min washes in cold PBS, slices underwent an antigen retrieval protocol by submersion in 950 C ddH_2O for 5 min (14). After cooling to room temperature, slices were blocked and permeabilized overnight at 4ºC (10% normal donkey serum, 0.6% Triton X-100 in 0.1 M PBS). Slices were washed twice for 20 min each in cold PBS, then incubated overnight at 4ºC in primary antibodies for Arc (1:1000, polyclonal rabbit, kind gift of Dr. Paul Worley) (15) and mCherry (1:1000, polyclonal chicken,

Novus Biologicals) diluted in blocking solution. Following four 30 min washes in cold PBS, slices were incubated overnight at 4ºC in secondary antibodies (1:1000 each, AlexaFluor 488 goat anti-rabbit and AlexaFluor 555 goat anti-chicken) diluted in blocking solution. Following four 30 min washes in cold PBS, slices were mounted on pre-cleaned microscope slides (Fisherbrand, Cat No. 12-550-343) with Aqua Poly/Mount (Polysciences Inc., Cat#18606), cover-slipped, and dried overnight at room temperature in a light-protected environment.

Hippocampal CA1 neurons were selected for imaging based on mCherry expression. Transfected neurons were imaged with a Zeiss LSM 510 confocal microscope using a 63X1.40 n.a. oil objective lens. Stacked images (1024x1024 pixel resolution, x=y=142.7µm, z=1.98µm, 3-9 steps) Quantification of green (Arc) somal and dendritic fluorescence was performed using Metamorph software as in previously described studies (10, 16). Arc signal intensity over background intensity was compared between the two groups.

Statistical Analysis - For electrophysiology assays, a paired t-test or Wilcoxon matched pairs signed rank test was used depending on whether data were normally distributed (Table S1). For immunocytochemistry data in Fig. 4, data were compared with a student's t-test. In all figures, error bars represent SEM and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Other methods – HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics. To express Arc, they were transfected with myc-Arc using Lipofectamine 2000 according to manufacturer. Protein concentration was determined using the modified Lowry method (17) according to Peterson (18) with BSA as a standard. SDS–PAGE was carried out according to Laemmli (19).

Supplementary References:

1. Byers CE, Barylko B, Ross JA, Southworth DR, James NG, Taylor CA 4th, Wang L, Collins KA, Estrada A, Waung M, Tassin TC, Huber KM, Jameson DM, and Albanesi JP. (2015) Biochim Biophys Acta. 1850, 1310-1318.
2. Ross JA, Chen Y, Muller J, Barylko B, Wang L, Banks HB, Albanesi JP, and Jameson DM. (2011) Biophys J 100, 729-737.
3. Wilkerson JR, Tsai NP, Maksimova MA, Wu H, Cabalo NP, Loerwald KW, Dictenberg JB, Gibson JR, and Huber KM. (2014) Cell Reports. 7, 1589-1600.
4. Gil C, Falqués A, Sarró E, Cubi R, Blasi J, Aguilera J, Itarte E. (2011) FEBS Lett. 585, 414-420.
5. Lee A and Lemmon MA. (2001) Methods Enzymol. 329, 457-468.
6. Folch J, Ascoli I, Lees M, Meath JA, leBaron N. (1951) J Biol Chem. 191, 833-841.
7. Forrester MT, Hess DT, Thompson JW, Hultman R, Moseley MA, Stamler JS, and Casey PJ. (2011) J Lipid Res. 52, 393-398.
8. Barylko B, Gerbers SH, Binns DD, Grichine N, Khvotchev M, Sudhof TC, Albanesi JP. (2001) J Biol Chem. 276,7705-7708.
9. Bagatolli LA, Binns DD, Jameson DM, and Albanesi JP. (2002) J Protein Chem. 21,383-391.
10. Pfeiffer, B. E., Zang, T., Wilkerson, J. R., Taniguchi, M., Maksimova, M. A., Smith, L. N., Cowan, C. W., and Huber, K. M. (2010) Neuron 66, 191-197.
11. Wang, K. H., Majewska, A., Schummers, J., Farley, B., Hu, C., Sur, M., and Tonegawa, S. (2006) Cell 126, 389-402.

12. McAllister, A. K. (2004) Methods Mol Biol 245, 197-206.
13. Pfeiffer, B. E., and Huber, K. M. (2007) J Neurosci 27, 3120-3130.
14. Huang, F., Chotiner, J. K., and Steward, O. (2007) *J Neurosci* **27**, 9054-9067.
15. Vazdarjanova, A., Ramirez-Amaya, V., Insel, N., Plummer, T. K., Rosi, S., Chowdhury, S., Mikhael, D., Worley, P. F., Guzowski, J. F., and Barnes, C. A. (2006) The Journal of comparative neurology 498, 317-329.
16. Pulipparacharuvil, S., Renthal, W., Hale, C. F., Taniguchi, M., Xiao, G., Kumar, A., Russo, S. J., Sikder, D., Dewey, C. M., Davis, M. M., Greengard, P., Nairn, A. C., Nestler, E. J., and Cowan, C. W. (2008) Neuron 59, 621-633.
17. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951). J Biol Chem. 193, 265-275.
18. Peterson GL. (1979) Anal. Biochem. 100, 201-220.
19. Laemmli UK. (1970) Nature 227, 680-685.

Supplementary Figures:

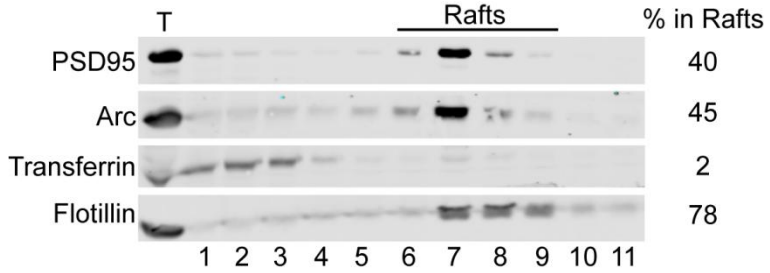

Figure S1. Association of Arc with Lipid Rafts. Crude synaptosomes (P2 fraction) were used for making rafts. T (total) represents 2% of sample loaded on gradient (12 μl of 600 μl); 5% of each gradient fraction (20 μl of 400 μl) was loaded on gel. Transferrin and flotillin were added as negative and positive controls, respectively. Percentages in rafts represent averages of two separate preparations.

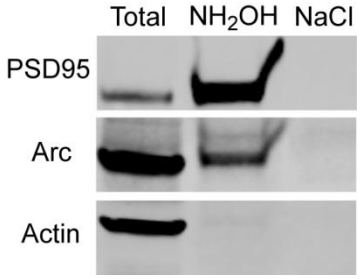

Figure S2. Palmitoylation of Arc in whole brain homogenate. Palmitoylation was detected by Acyl-RAC (see Experimental Procedures). PSD95 and actin are shown as positive and negative controls. The extent of Arc palmitoylation relative to PSD95 was approximately 3.5%.

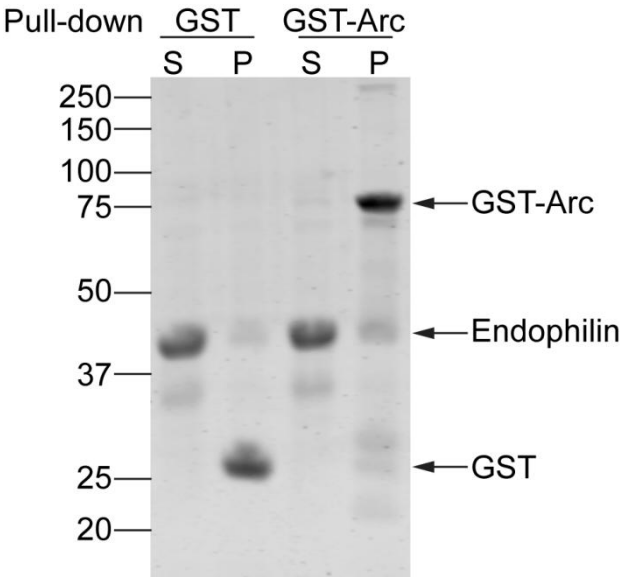

Figure S3. GST-Arc binds weakly to His_6-Endophilin A2. GST-Arc (or GST as negative control) coupled to glutathione resin was incubated with purified His_6-endophilin A2 in a solution containing 20 mM Hepes, pH 7.5, 0.1 M NaCl, and 1 mM DTT for 2 h with constant rotation. Concentration of GST-Arc or GST was 5 µM, endophilin concentration was 10 µM. Samples were centrifuged and supernatants (S) and resins (P) resuspended in the initial sample volumes were electrophoresed and stained with Coomassie blue.

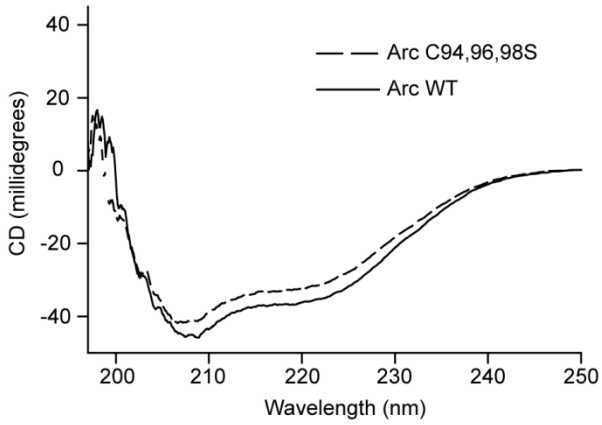

Figure S4. CD spectra for Arc^{WT} and $Arc^{C94,96,98S}$. Five scans were averaged for each CD spectrum. Spectra were acquired between wavelengths 190 nm and 250 nm at a scan rate of 50 nm/min at room temperature. Figure shows the data from 197 nm to 250 nm. The two negative bands at 207 nm and 221 nm are indicative of α-helix conformation.

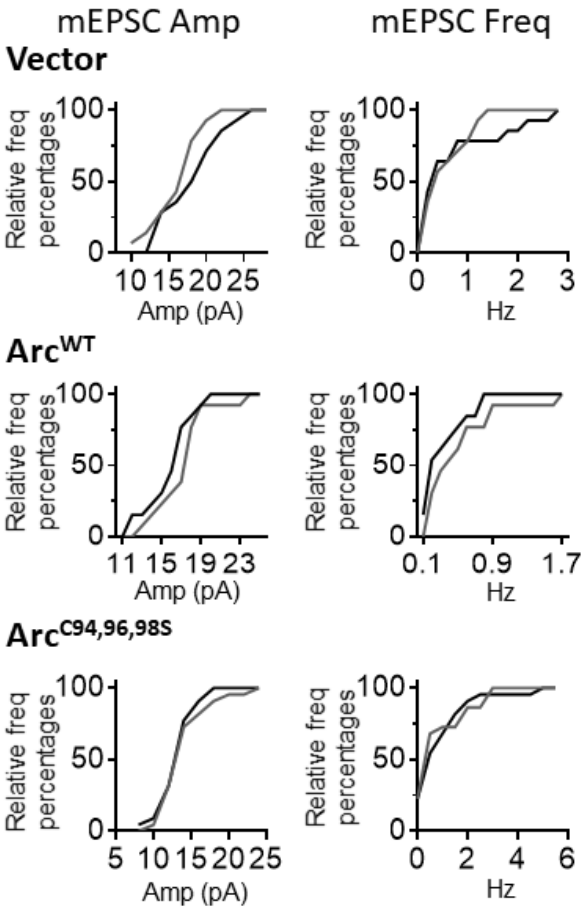

Figure S5. Cumulative distribution graphs of mEPSC amplitude and frequency of data presented in Figure 4. Simultaneous, dual patch mEPSC recordings from Untransfected *Arc* KO neurons and neighboring *Arc* KO neurons Transfected with MEF2-VP16+MRE-mCherry with vector (*top*), the coding region of Arc^{WT} (*middle*) or a nonpalmitoylatable Arc mutant ($Arc^{C94,96,98S}$, *bottom*).

Supplemental Table 1, Related to Figure 4. Raw electrophysiological measurements in untransfected (U) or transfected (T) *Arc* KO hippocampal CA1 neurons

		mEPSC Amp (pA)	mEPSC Freq (Hz)	mEPSC Rise Time (ms)	mEPSC Decay Time (ms)	R_n (MΩ)	V_m[#] (mV)
Vector (pcDNA3)							
with MEF2VP16 (n=14)	U	17±0.8	0.6±0.1	3.2±0.1	5.6±0.2	209±18	-57±1
	T	19±1.0	0.8±0.2	3.1±0.1	5.7±0.3	250±32	-58±1
WT-Arc coding region							
with MEF2VP16 (n=13)	U	18±0.8	0.5±0.1	3.1±0.1	5.0±0.2	181±15	-59±1
	T	16±0.7	0.3±0.1*** [2]p=0.0005	3.3±0.2	5.3±0.4	212±17	-58±1
C94,96,98S-Arc							
no MEF2VP16 (n=20)	U	16±0.5	0.96±0.2	4.4±0.3	6.3±0.4	304±26	-57±1
	T	14±0.6** [1]p=0.0034	0.80±0.2	4.6±0.3	6.2±0.3	317±27	-59±1
with MEF2VP16 (n=22)	U	15±0.7	0.99±0.2	3.3±0.1	4.7±0.2	306±19	-52±1
	T	14±0.5	1.0±0.2	3.5±0.1	5.1±0.2	273±17	-52±1

[#]not corrected for junction potential; data are presented as mean±SEM; **p<0.01, ***p<0.001, [1]paired t-test (Gaussian distribution) or [2]Wilcoxon matched-pairs signed rank test (non Gaussian distribution)