Supplemental Information for

A unique set of vesicle fusion machinery defines an activity-dependent AMPAR exocytosis pathway distinct from LTP pathway

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Supplemental Experimental Procedures

RARα conditional knockout mouse and genotyping information

The RARα floxed mouse (C57BL/6 background) is a gift from Drs. Pierre Chambon and Norbert Ghyselinck (IGBMC, Strasbourg, France) (Chapellier et al., 2002). Breeding colonies are maintained in the animal facility at Stanford Medical School. Genotyping of the mice was achieved by PCR with the following primers: Primer 1 Fwd 5′-GTGTGTGTGTGTATTCGCGTGC-3′, Primer 2 Rev 5′-ACAAAGCAAGGCTTGTAGATGC-3′ and compared with wild-type (WT) C57BL/6. Following infection with a lentiviral vector expressing Cre recombinase or a truncated and inactive version of Cre, successful lox-P mediated recombination in organotypic hippocampal slice cultures was assayed by PCR with Primer 1 and Primer 3: 5′-TACACTAACTACCCTTGACC-3′. Conditions were 30 cycles for 30 s at 92°C, annealing at decreasing temperatures in the range from 62°C to 56°C to increase product specificity and elongation for 30 s at 72°C.

Organotypic Hippocampal slice cultures

Organotypic slice cultures were prepared from young RARα floxed mice (postnatal day 6 to 7) and placed on semiporous membranes (Milipore) for 5 to 7 days prior to recording (Aoto et al., 2008; Arendt et al., 2013). Briefly, slices were maintained in an MEM based culture media comprised of 1 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM L-glutamine, 1mg/L insulin, 0.0012% ascorbic acid, 30mM HEPES, 13 mM D-glucose,

and 5.2 mM $NaHCO_3$. Culture media was adjusted to a pH of 7.25 and the osmolarity to 320 mOsm. Cultures were maintained in an incubator with 95% O_2/ 5% CO_2 at 34 degrees C. For experiments with shRNA expression, cultures were injected on DIV 0 and maintained for 10 days prior to recording.

Viral vectors and viral infection

Recombinant lentiviruse was produced as previously described (Aoto et al., 2008). Briefly, using the calcium phosphate method, HEK293 cells were transfected with four plasmids, the lentiviral shuttle vector, pVSVG, pRRE and pREV. The HEK293 culture media was collected 40-44 h after transfection and filtered with 0.45 µm PVDF filter (Millipore) to remove cellular debris followed by centrifugation at 50,000 x g to concentrate the virus. Concentrated virus was dissolved in a small volume of medium, aliquoted and stored frozen at - 80°C. Virus expressing ShRNA, Cre-recombinase (tagged with GFP), or a truncated and inactive version of Cre (mCre-GFP) (Kaeser et al., 2011) was injected into the CA1 region of organotypic hippocampal slice cultures at DIV0 and expressed for 10 days. Recordings from infected neurons were carried out at DIV10.

Generation of ShRNAs for SNARES

SNAP47, Syntaxins 1, 3, and 4 knockdown and knockdown rescues were generated as previously described in (Jurado et al., 2013). Complexin1-2 knockdown was generated as previously described in (Ahmad et al., 2012). Tetanus toxin expression via lentivirus was as previous described in (Xu et al., 2012). Syntaxin 3/4 and Syntaxin 4/3 rescue chimeras were generated as previously described in (Jurado et al., 2013). To identify infected cells, the lentiviral shuttle vector containing the shRNA also expressed mCherry under the control of the ubiquitin promoter. Since all the shRNA's were made against the UTRs of the cognate genes, for molecular replacement experiments we used only the coding regions of the wild type or mutant proteins driven by the human synapsin promoter followed by IRES-GFP.

Electrophysiology

Voltage-clamp whole-cell recordings were obtained from CA1 pyramidal neurons in organotypic slice cultures treated with either vehicle controls or 10 µM RA for 2-4 hours prior to recording, under visual guidance using transmitted light illumination. For experiments testing the requirements for RA-mediated scaling, RA was co-applied with either 1 µM TTX or 100 µM D-APV for 4 hours prior to

recording. Vehicle control and RA treated cells were obtained from the same batches of slices on the same experimental day.

The recording chamber was perfused with 119 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 0.1 mM picrotoxin, and 4 μM 2-chloroadenosine, at pH 7.4, gassed with 5% CO_2/95% O_2 and held at 30°C. Patch recording pipettes (3–6 MΩ) are filled with 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phosphocreatine, and 0.6 mM EGTA at pH 7.25. Stable recording were ensured by on line monitoring of Rm, Ra, and Cm. Only neurons with stable Rm, Ra and Cm were kept for further analysis.

Spontaneous miniature transmission was obtained in the presence of 1uM TTX in the external solution. Ranked plots were achieved by pooling first 50 responses from 20-30 randomly selected cells from each group. For dual component mESPCs, taken from spontaneous miniature transmission, recordings were performed in the same recording solutions minus $MgCl_2$ using a previously described method (Arendt et al., 2013; Gomperts et al., 1998; Tracy et al., 2011). The NMDAR component was measured by subtracting the AMPAR-only component of mEPSCs in the presence of the NMDAR antagonist APV from the dual component mEPSCs taken before APV addition for each neuron. Synaptic responses were evoked with bipolar electrodes using single-voltage pulses (200 μs, up to 20 V or 10mA). The stimulating electrodes were placed over Schaffer collateral fibers between 300 and 500 μm from the recorded cells. Synaptic AMPAR-mediated responses were measured at -60 mV and NMDA receptor (NMDAR)-mediated responses at +40 mV, taken at a latency at which AMPAR responses had fully decayed (60 ms). Synaptic responses were averaged over 50– 100 trials. Failure rate experiments were performed using minimum stimulation. Failure rate was calculated as percentage of failed evoked responses over 50 sweeps at the holding potential of -60mV and again at +40mV. For slices previously exposed to TTX and/or RA, slices were washed out prior to recording evoked responses.

LTP was induced using a pairing protocol by stimulating Schaffer collateral fibers at 3 Hz (540 pulses) while depolarizing the postsynaptic cell to 0 mV. Slices previously exposed to TTX and/or RA for scaling were first washed out of TTX prior to whole-cell patch. Slices previously exposed to RA or RA in the presence of anisomycin or cycloheximide were washed out prior to whole-cell patch and induction of LTP. Magnitude of LTP was quantified by averaging percentage of EPSC enhancement during the time

window between 55 and 60 minutes after LTP induction. All electrophysiological recordings were carried out with Multiclamp 700B amplifiers (Axon Instruments) and analysis was completed in Clampfit (Axon), MiniAnalysis (Synaptosoft), Excel (Microsoft), and Prism (GraphPad).

Statistical analyses

All graphs represent average values ± s.e.m. Statistical differences were calculated according to nonparametric tests. Comparisons between multiple groups were performed with the Kruskal-Wallis ANOVA. When significant differences were observed, p values for pairwise comparisons were calculated according to two-tailed Mann-Whitney tests (for unpaired data) or Wilcoxon tests (for paired data). Comparisons between cumulative distributions were performed with two-sample Kolmogorov–Smirnov tests. p values were indicated in each figure.

Supplemental Figure Legends

Figure S1 (related to Figure 1). Acute RA treatment impairs hippocampal LTP.

(A) Scatter plots of LTP obtained from individual experiments in DMSO- or RA-treated hippocampal CA1 pyramidal neurons (summarized in Figure 1A; ***, $p < 0.0001$). (B) Scatter plots of LTP obtained from individual experiments with RARα deletion (summarized in Figure 1B; ***, $p < 0.005$). (C) Scatter plots of LTP in neurons treated with anisomycin, RA or both (summarized in Figure 1C; *, $p < 0.05$; ***, $p < 0.005$). (D) Summary graph of LTP in CA1 pyramidal neurons treated with cyclohexmide, RA or both. (E) Scatter plots of LTP obtained from individual experiments in cyclo-, RA- or RA+cyclo-treated hippocampal CA1 pyramidal neurons (***, $p < 0.0005$). All bar graphs represent average values ± s.e.m.

Figure S2 (related to Figure 2). Acute RA treatment potentiates mEPSC in does not change paired pulse ratio or passive membrane properties.

(A) Ranked DMSO-mEPSC amplitudes (black dots) or RA-mEPSC amplitudes (gray dots) were plotted against ranked DNSO-mEPSC amplitudes. Black line indicates a slope of 1. Red line indicates multiplicative scaling with a slope matching the increase in average mEPSC amplitude. The zoomed in plots for small and medium amplitude ranges (blue boxes in A) are shown at the bottom (A1, < 30 pA; A2, 30-60 pA). (B) Paired pulse ratio of eEPSC was measured at four different intervals from DMSO- or RA-treated slices ($p > 0.5$). (C) Membrane resistance and capacitance were measured from CA1 pyramidal neurons treated with DMSO or RA ($p > 0.5$).

Figure S3 (related to Figure 3). Activation of silent synapse induced by prolonged TTX treatment impairs subsequent LTP.

(A) Ranked mEPSC amplitude plots between DMSO (black), TTX 60 hr (red) and TTX wash + RA (green) groups. The zoomed in plot for amplitude range 5-15 pA (represent more than 87% response population) is shown at the bottom. (B) Ranked mEPSC amplitude plots between DMSO (black), TTX 60 hr (red) and TTX no wash + RA (blue) groups. The zoomed in plot for amplitude range 5-15 pA (represent more than 84% response population) is shown at the bottom. (C) Summary graph of LTP induced in slices treated with DMSO, TTX (60 hr), TTX (60hr) wash + RA (4 hr), or TTX (60 hr) no wash + RA (4 hr). Black bar indicates the time window for LTP magnitude quantification. (D) Scatter plots of LTP obtained

from individual experiments summarized in (C) with bar graphs representing mean ± s.e.m (*, $p < 0.05$; **, $p < 0.005$).

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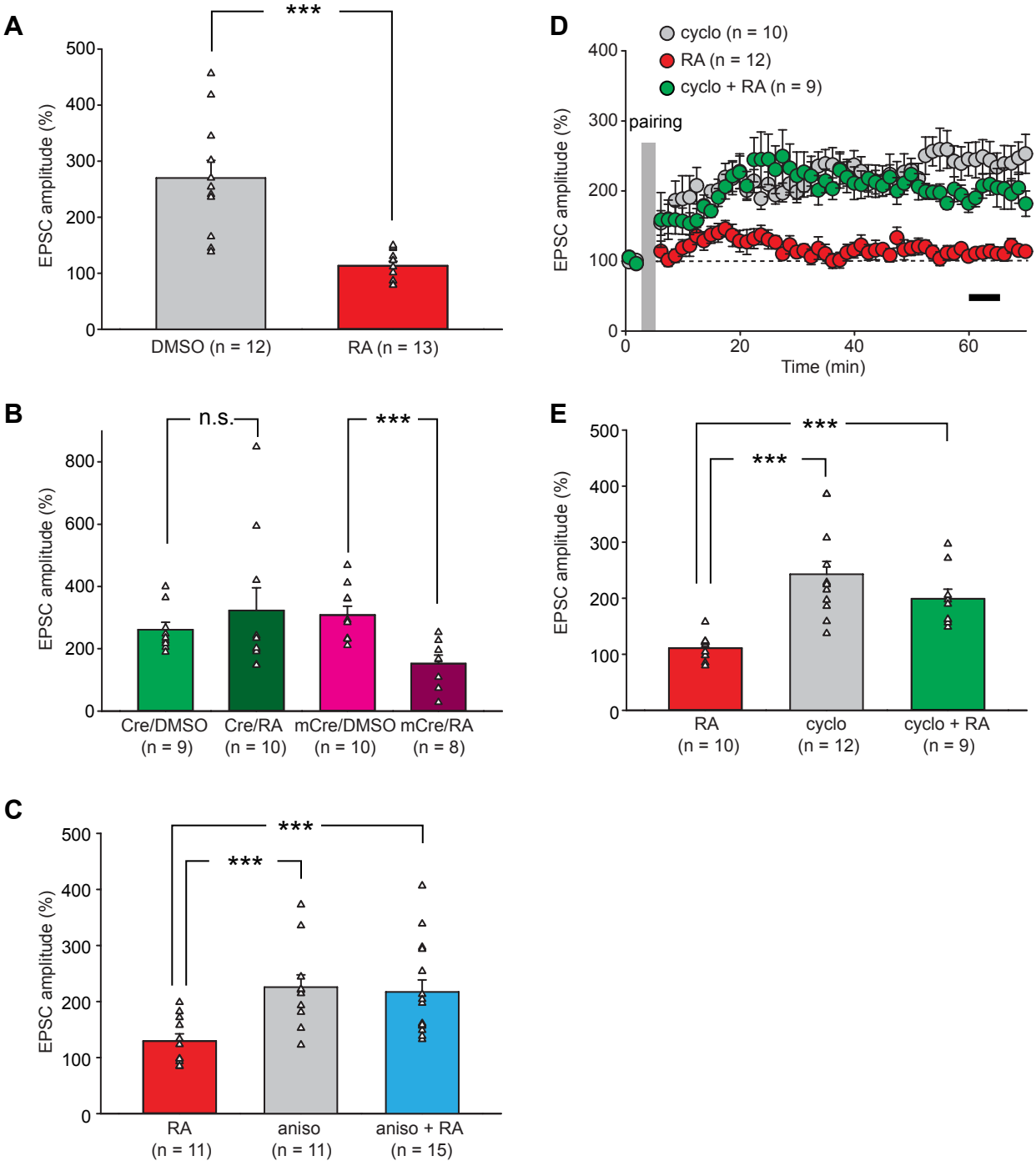
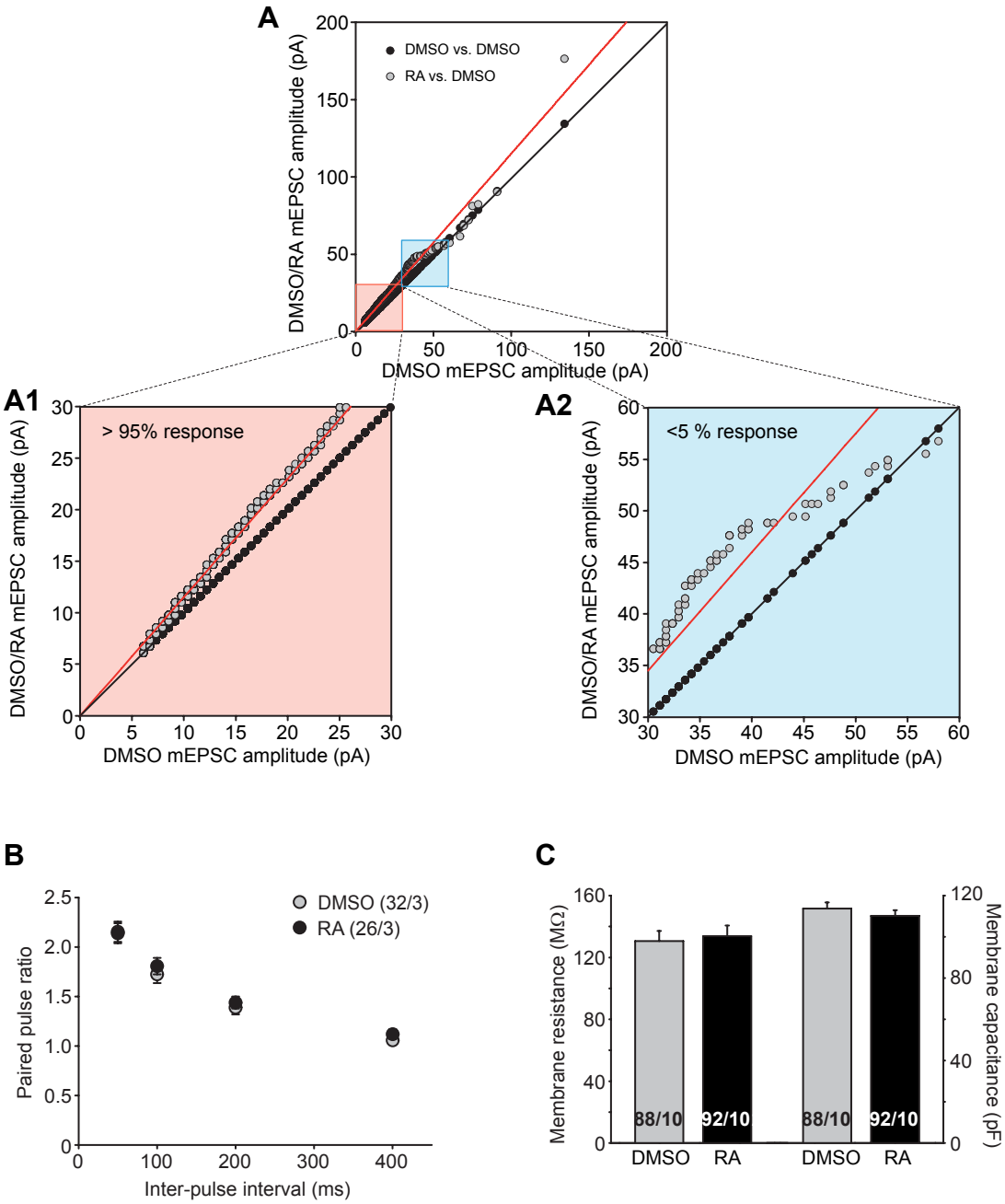


Arendt et al., Figure S1
(related to Figure 1)



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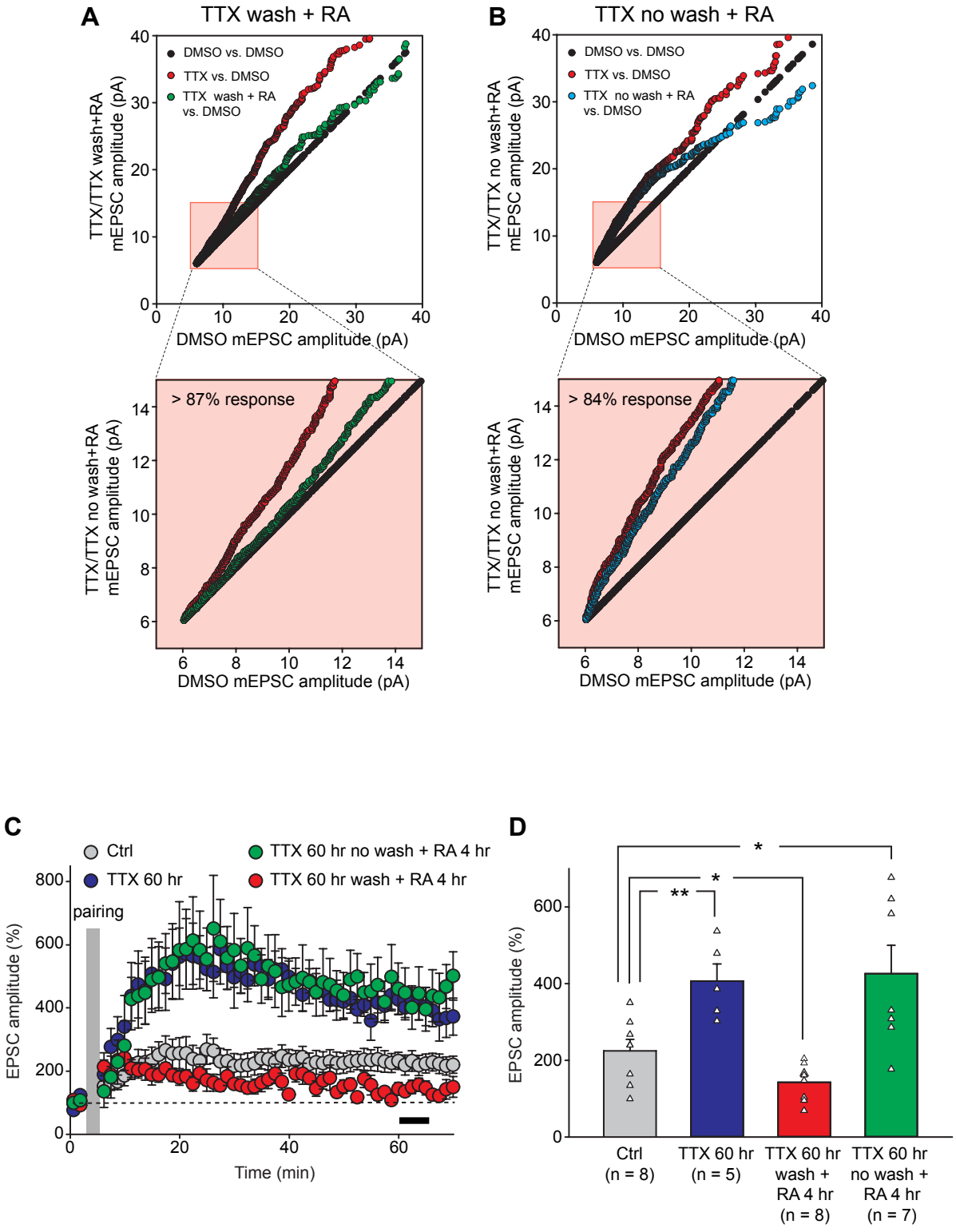

Arendt et al., Figure S3
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