Neuron, Volume *61*

Supplemental Data

Temporal Phases of Activity-Dependent Plasticity and Memory Are Mediated by Compartmentalized Routing of MAPK Signaling in *Aplysia* Sensory Neurons

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Supplemental Experimental Procedures

Behavioral Procedures

Briefly, wild-caught *Aplysia* (250-400 g; supplied by Marinus, Long Beach, CA) were anesthetized by injection of isotonic $MgCl_2$ (approximately 100 ml/100 g body weight). The tail and mantle were surgically removed along with the ring ganglia, abdominal ganglion, and their peripheral connections. The siphon artery was cannulated with silastic tubing (ID = 0.25 in) and perfused at ~ 5 ml/min with cooled seawater (Instant Ocean, 15 °C), to keep the siphon inflated throughout the experiment. The tail was bisected and manually perfused (~15 ml) via a 22-guage needle inserted into the internal cavity. The tail and mantle were pinned to the Sylgard-coated floor of a chamber containing circulating tank seawater (15 °C), while the ring ganglia (comprised of the cerebral and two paired pleural-pedal ganglia) were pinned in a separate Sylgard-coated sub-chamber that was independently perfused with room temperature (20-22 °C) artificial seawater (ASW) containing in mM: 460 NaCl, 55 $MgCl_2$, 11 $CaCl_2$ 10 KCl, 10 Tris pH 7.6. (Figure 1A). The P9 nerves and pleural-abdominal nerves exited the sub-chamber through small slits that were sealed with Vaseline. Preparations were allowed at least 60 min to recover prior to pre-test measurements.

Prior to training, 3-4 pre-tests (ITI = 15 min) were conducted to establish a baseline of tail-elicited siphon withdrawal (TSW) duration using a mild shock (12mA - 0.5 s duration) from a hand-held bipolar electrode. Upon achieving a stable baseline, a single strong tail shock (120mA - 1.5 s duration) was delivered to the test site (see Figure 1A). Post-tests of TSW (elicited in the same fashion as the pre-tests described above) were conducted at the indicated times following training.

For statistical analysis, baseline was determined by the average of the last three pre-tests. All post-test scores (at each time) were normalized to this baseline. Scores for each animal were then averaged across time since we did not observe a differential effect of any drug over time. Finally, these averages were compared between groups with an unpaired t-test (the functional equivalent of a one-way ANOVA).

Western Blotting

The SN clusters were harvested in the $MgCl_2$ solution and lysed immediately in 20µl of lysis buffer % [50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 4 mM *para*-nitrophenylphosphate, 2% sodium dodecyl sulfate and a protease inhibitor cocktail (1 tablet of Roche Diagnostics GmbH protease inhibitor cocktail/25 mL)], by mechanical agitation. Samples were then stored at –80 °C until use. Samples were resolved on a 4-12% Bis-Tris Gel (MES buffer) using SDS-PAGE and transferred to nitrocellulose using standard procedures. Initially, blots were probed with phospho-p44/p42 MAPK antibodies (Cell Signaling, 1:2000), and stripped with buffer (62.5 mM Tris-Cl, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS) at 65 °C for 45 min (confirmed by probing the blots without the primary antibody), and finally, reprobed with antibodies to total MAPK (Cell Signaling, 1:1000). Exposure of the film was kept in the linear range and band intensity was quantified using NIH Image software (NIH, Bethesda, Maryland). Approximately equal amounts of tissue were taken for the control and experimental

samples. To account for small differences in the protein amount, the phospho-MAPK signal was normalized to the total MAPK signal in each lane. Although we occasionally detect two bands (as is the typical case in the mammalian CNS) with the phospho-MAPK and total MAPK antibodies, we usually observe only one band migrating at about 43 kDa, which is consistent with other reports of *Aplysia* MAPK (Chin et al., 2002; Michael et al., 1998).

For statistical analysis, we performed a paired t-test on the scores from the raw phospho-MAPK/total MAPK ratios between the treated side (right or left) and control side to assess the significance of MAPK activation above its own baseline (p values listed inside bars). In contrast, when comparing across treatment groups, we performed an unpaired t-test on the difference scores (obtained using above calculation) between groups to determine the effect of a given treatment (bracketed p values). Experimental and controls groups were always run in parallel.

Physiology in Intact Ganglia

Pleural-pedal ganglia were desheathed in a 1:1 solution of ASW and $MgCl_2$ and perfused with ASW for at least 15 minutes before each experiment. SNs and MNs were impaled with glass microelectrodes containing 3M KCl and monosynaptic connections were identified. Signals were amplified with an Axoclamp 2B intracellular amplifier (Axon Instruments). Three pretests (15 min ITI) were taken to determine the baseline MN EPSP amplitude in response to a single action potential elicited in the SN by a 4ms suprathreshold stimulus. Only synapses that displayed a stable baseline (all pretests within 20% of the mean) were included in the analysis. Fifteen minutes after the third pretest, a single 5 min pulse of 50 μM 5HT was perfused, and four 2s trains of activity (suprathreshold, 4ms @10Hz) were delivered to the SN 1, 2, 3, and 4 min after 5HT onset. After 5 minutes of 5HT exposure, perfusion of normal ASW resumed. The EPSP amplitude in the MN was measured at 15 min intervals starting 25 min after 5HT offset

and continuing 55 min post-5HT. The MN membrane potential was held at approximately -70 mV during the training and testing with constant negative current to allow detection of the EPSPs in the absence of MN spiking.

Cell Culture

Culture dishes and media were prepared as previously described (Montarolo et al., 1986; Schacher and Proshansky, 1983). Detailed methods for culture preparation are also available at http://www.gonda/ucla.edu/researchlabs/martin/. Data were acquired and analyzed using Clampex 8 pClamp (Axon Instruments).

Physiology in Cultured Cells

Sensory-motor cultures on day 4, excitatory postsynaptic potentials (EPSPs) were recorded using intracellular glass micropipettes (20-30mΩ,filled with 0.5 M potassium chloride and 2 M potassium acetate) under current clamp with the SN and MN held at -50mV and -80mV, respectively. Data were acquired and analyzed using Clampex 8 pClamp (Axon Instruments). Data are expressed as mean ± SEM. Student's unpaired t-tests were performed between experimental and control groups using Prism GraphPad software (San Diego, CA). Plasmids were pressure injected [pNEX-eGFP-PKC Apl I or pNEX-eGFP-PKC Apl II (200 ng/µl)] into the nucleus one day after culturing (30-50ms duration, 10 PSI, PLI-90, Harvard Apparatus, MA)

Image Acquisition and Analysis

Neurons expressing eGFP-PKC were imaged on a Zeiss Pascal scanning laser microscope using a 63x water immersion objective and 10% of a 25 mW argon laser. Optical sections (Z-stack interval of 0.8 µm) were acquired, and the gain was held constant during each experiment. To detect Apl I translocation following KCl+5HT stimulation, baseline images were acquired while cultures were incubated with ASW, and the media was then replaced with ASW containing KCl (100mM) and 5HT (10µM), and cells were imaged 5 min later. In studies using kinase inhibitors, cultures were

preincubated with U0126 (20 μM) , U0124 (20 μM), genistein(100 μM), SSQ22536 (1.5 mM), Rp-isomer (500 μM) for 30min in ASW, and images were acquired before and after stimulation (Apl I: KCl + 5HT; Apl II: 5HT alone).

To quantify membrane translocation of Apl-eGFP, the pixel intensity of the eGFP signal within concentric rings one pixel in width from the periphery to the center of the cell body was measured using IPLab software (Scanalytics, Fairfax VA), as described in Zhao et al., 2006. We counted the outmost 3μm as membrane, the inner 4-10 μm as cytoplasm, then measured the mean fluorescence intensity of membrane and cytosol, calculated the ratio of mean intensity between the two subcompartments (M/C), and determined the percent change of ratio before and after stimulation as a measure of membrane translocation.

FIGURE LEGENDS

Figure S1. MEK activity is required for ongoing MAPK activity.

Isolated pleural-pedal ganglia were separated and both were stimulated with the KCl+5HT protocol (as described). Immediately following treatment, either the right or left pair (randomly chosen) received 10μM U0126 while the other received 10μM U0124. After 1 hour, sensory clusters were harvested and subjected to western analysis for P-MAPK and T-MAPK. The ratio represents the value of P-MAPK normalized to T-MAPK. A paired t-test revealed a significant difference (t-test, n=4, $P<0.05$).

Figure S2. SN depolarization induced by high KCl-ASW.

Intracellular recording were taken from cultured SNs to measure the change in resting potential during the 5min application of high KCl-ASW (100mM). We observed a mean depolarization of 20 ± 1 mV (n=6, $p<0.0001$ paired t-test).

Figure S3. KCl-ASW induces robust Apl I translocation in SNs.

SNs expressing GFP-PKC Apl I before and after treatment with the associative analog, high KCl-ASW (KCl: 100mM) + 5HT (10 μM) for 5 min (top). Quantification of mean pixel intensity reveals that the GFP signal in the outermost 3 μm of plasma membrane (Mem, M) increased following 5min KCl+5HT stimulation while the GFP signal in the adjacent 4–10 μm (Cyt, C) decreased. (Zhao, et al. 2006).

Figure S4. MAPK activity is not required for PKC Apl II translocation and short-term facilitation on a depressing baseline.

A. Facilitation induced by 5HT (10 μM, 5 min) after depression by low frequency stimulation (20 stimuli, 0.05Hz) was not affected by U0126. $\mathbf{A_1}$. Sample traces showing

that 5HT (10μM) dramatically increased EPSP amplitude in the presence of either the inactive analog U0124 or the MEK inhibitor U0126. Scale bar: 20mv, 25ms. **A_2.** Data summary (t-test, U0124: n=4; U0126: n=5, P>0.05). **B.** Translocation of GFP-PKC Apl II in SNs by 5HT stimulation was not affected by U0126. **B_1.** Sample images showing GFP-PKC Apl II translocation before and after 5HT stimulation in the presence of either U0124 or U0126. Scale bar: 20μm. **B_2.** Quantification of translocation by comparing the percentage change in M/C ratio (as defined in materials and methods and in figure legend S3) after stimulation. Neither U0126 nor U0124 significantly altered 5HT-induced Apl II translocation to the plasma membrane (t-test, U0126, n=3; U0124, n=4, P>0.05,).

Figure S5. Double pull-down assay to measure G-protein activation.

A. Diagram depicting the pull-down assay and parallel analysis of the activation of ApRas, ApRap, and MAPK by Western blot. **B.** To verify the effectiveness of the assay, equal amounts of Aplysia CNS extract were pre-treated with GDP or GTP, and then loaded on the column with either (i) no recombinant proteins (ii) GST only or (iii) GST-RBDs. The eluent from the columns was analyzed by Western blot, which shows activated ApRas and ApRap (GTP-bound) interact specifically with the binding domains. **C**. Representative Western blots showing that the associative analog, high KCl-ASW with 5HT (K+5) activates the G-proteins: ApRap and ApRas collectively. In all cases, active signal was normalized to its respective input control.

Supplemental References

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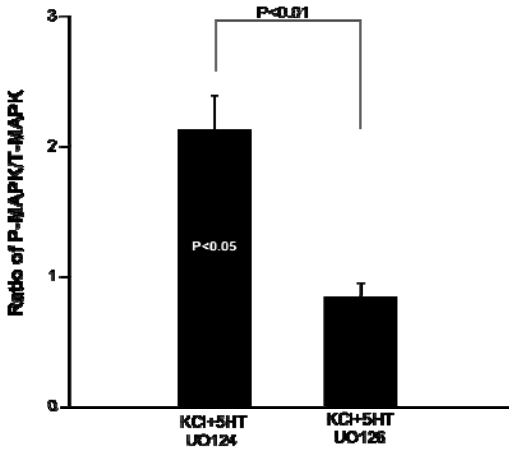
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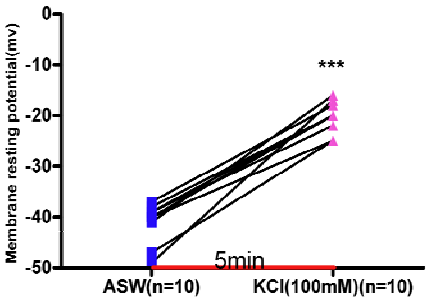
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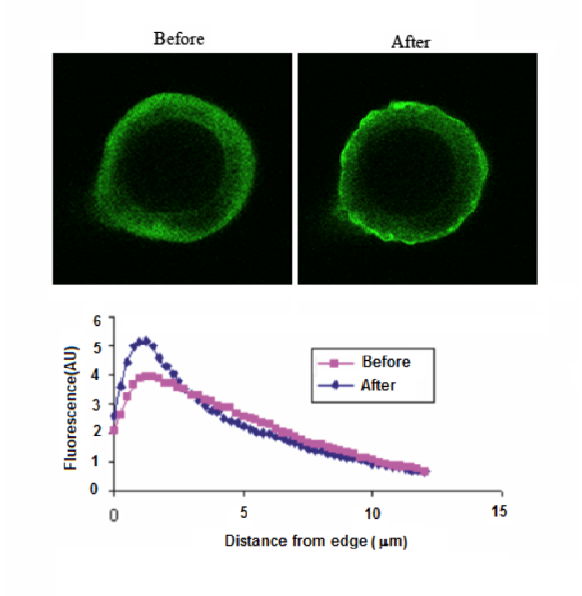
S2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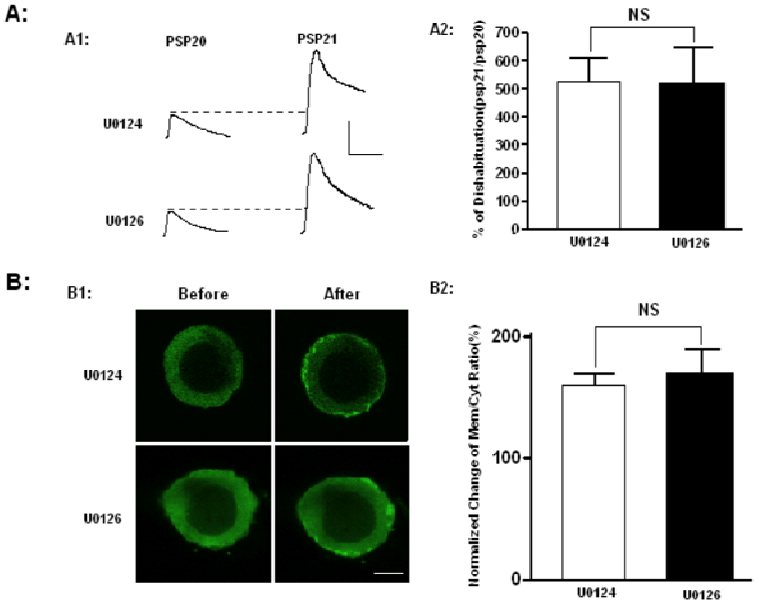
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U0124
U0126
B:
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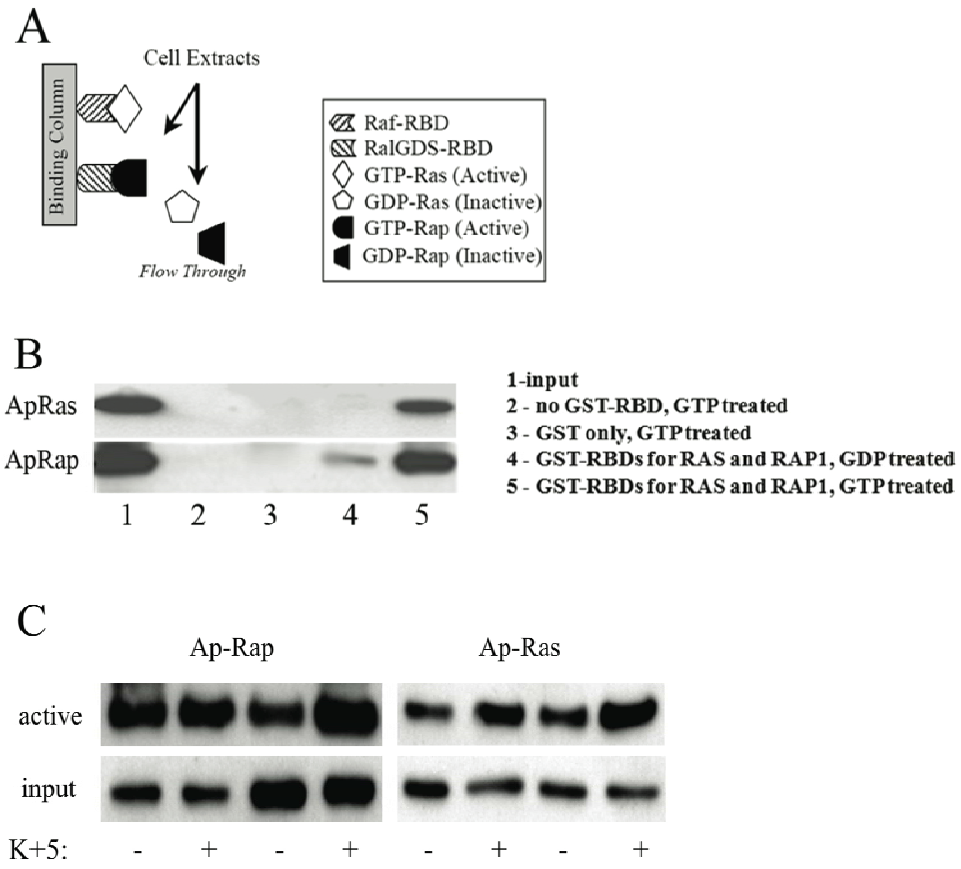
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GDP-Rap (Inactive)
B
ApRas
ApRap
1 2 3 4 5
1-input
2 - no GST-RBD, GTP treated
3 - GST only, GTP treated
4 - GST-RBDs for RAS and RAP1, GDP treated
5 - GST-RBDs for RAS and RAP1, GTP treated
C
Ap-Rap
Ap-Ras
active
input
K+5: - + - + - + - +