The 5-HT- and FMRFa-activated signaling pathways interact at the level of the Erk MAPK cascade: Potential inhibitory constraints on memory formation
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Abstract
The sensorimotor synapse of Aplysia exhibits long-term facilitation (LTF) and long-term depression (LTD) elicited by the neuromodulator serotonin (5-HT) and the peptide Phe-Met-Arg-Phe-NH₂, respectively. 5-HT-induced LTF engages extracellular-regulated kinase (Erk) and CREB1, whereas FMRFa-induced LTD engages p38 MAPK (mitogen-activated protein kinase) and CREB2. The interaction of the 5-HT and FMRFa pathways was recently investigated in Aplysia at the level of gene expression. However, little is known about crosstalk of these pathways at the level of the second messenger cascades. We investigated the potential interaction of the 5-HT and FMRFa pathways at the level of the Erk cascade. We found that FMRFa inhibited basal Erk activity through p38 MAPK. FMRFa also inhibited 5-HT-induced phosphorylation of Erk and nuclear accumulation of phospho-ERK, suggesting that FMRFa may place inhibitory constraints on memory formation through regulation of the Erk MAPK cascade.

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The sensorimotor synapse in Aplysia has been used extensively to study cellular and molecular mechanisms of learning and memory. This synapse exhibits both short-term and long-term forms of plasticity, including long-term facilitation (LTF) and long-term depression (LTD). LTF can be induced by repeated application of the neuromodulator serotonin (5-HT), and depends on elevation of cAMP and activation of protein kinase A (PKA) [8]. 5-HT exposure also activates p42/p44 MAP kinase/extracellular-regulated kinase (Erk) [22,25]. Erk is activated when phosphorylated by MAPK/Erk kinase (MEK). 5-HT also promotes nuclear translocation of Erk, and inhibition of Erk blocks LTD [20]. LTD also depends on gene induction mediated by the transcriptional activator cAMP response element binding protein 1 (CREB1) [11,3,23]. PKA and Erk can phosphorylate CREB1 [3]. Phosphorylation of CREB1 promotes induction of genes regulated by cAMP response elements (CREs) [14,23].

LTD induced by application of the peptide Phe-Met-Arg-Phe-NH₂ (FMRFa) involves p38 MAP kinase and a transcriptional repressor, cAMP response element binding protein 2 (CREB2) [14,15]. FMRFa activates p38 MAPK, which phosphorylates CREB2 and enhances CREB2’s repression of CRE-regulated genes such as C/EBP [14]. Erk also phosphorylates CREB2 (probably at a different site than p38 MAPK does). This phosphorylation is hypothesized to relieve repression, enhancing gene induction [1,4].

The interaction of the 5-HT and FMRFa pathways was recently investigated in Aplysia at the level of gene expression [14] as well as p38 MAPK [15]. When FMRFa and 5-HT were co-applied, repression by phospho-CREB2 overrode activation by phospho-CREB1, so that C/EBP induction and LTD were blocked [14]. Moreover, p38 MAPK was bidirectionally regulated, with 5-HT inhibiting, and FMRFa promoting, p38 MAPK activation and consequent CREB2 phosphorylation [15]. To further investigate crosstalk between the 5-HT and FMRFa pathways, we explored their potential interaction at the level of the Erk cascade. In other systems, p38 MAPK indirectly inhibits MEK [31]. Therefore, we investigated the effects of concurrent application of 5-HT and FMRFa on Erk. We tested the hypothesis that FMRFa will override 5-HT-induced Erk phosphorylation and nuclear localization. We also investigated the...
To validate the specificity of commercial anti-Erk antibodies (phospho- and total-) for the Aplysia Erk homolog (ApErk; also termed ApMAPK in [22]), we cloned ApErk from pleural-pedal ganglia via PCR, using the following primers (synthesized by IDT, Coralville, IA): (1)-GAAATCGTGGAGGCCAGACTTTTGA-(26) and (1069)-AAAACGTCCAGTCCTAAGTGCTCC-(1044) (based on the sequence submitted to GenBank by Michael, Baston, and Kandel, 1995; GenBank accession number U40484). Following sequencing (Seqwright, Houston, TX), the PCR product was inserted into a pGEX-4T-1 expression vector at the EcoRI site (Amersham Biosciences Piscataway, NJ). Escherichia coli BL21 (Amersham Biosciences) were transformed with the construct and grown overnight at 37 °C on ampicillin-containing plates. Twelve clones were grown to OD600 = 0.5 and induced with isopropyl-beta-D-thiogalactopyranoside (IPTG; 0.4 mM; Sigma, St. Louis, MO) for 3 h. Aliquots of the cultures were collected and spun at 10,000 × g. SDS-containing sample buffer was added to the pellets, followed by SDS-gel electrophoresis. Coomassie staining indicated that 50% of the clones expressed a product of the expected molecular weight (approximately 69 kDa). Lysates from some of these clones were used in the experiments described in Fig. 1.

Following established procedures that produce LTD and LTF [21,14], pleural-pedal ganglia were exposed to FMRFa (10 μM; American Peptide Company, Sunnyvale CA) continuously for 90 min, and/or to five 5-min pulses of 5-HT (50 μM; Sigma-Aldrich Inc.; ISI: 20 min). Control ganglia were treated similarly with vehicle (artificial seawater; ASW). The p38 MAPK inhibitor SB203580 was applied at 10 μM 30 min prior to and during FMRFa exposure. After treatment, ganglia were rinsed with five volumes 50% modified L15–50% ASW (for solution composition see [24] and [34]) and rapidly frozen on dry ice.

The pleural ganglia were rapidly isolated, homogenized in lysis buffer [10% SDS, 10 mM Tris, 10 mM EDTA, 1 mM DTT, 1% protease inhibitor cocktail (Sigma-Aldrich Inc.), 10 mM sodium fluoride, 5 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 0.5 mM okadaic acid], boiled for 5 min and centrifuged at 20,000 × g. The supernatant was subjected to SDS-PAGE. Following protein transfer to nitrocellulose membrane, Western blot analysis was performed using anti-phospho-Erk antibody from Promega also recognizes endogenous and recombinant phospho-ApErk. Membranes with various extracts were prepared as in (B). In the last two panels showing anti-phospho-Erk immunoreactivity in Aplysia extract, the signal appeared weak. These two membranes had been treated with phosphatase buffer with or without phosphatase. It is likely that this treatment affected antibody immunoreactivity. (D) Anti-total-Erk antibody (Cell Signaling) recognizes ApErk independently of phosphorylation state. Membranes with various extracts were prepared as in (B). Multiple bands with apparent molecular weights <69 kDa are evident in lanes 3, 5 and 6. They are probably degradation products of GST-ApErk detected by anti-total-Erk antibody but not by anti-phospho-Erk antibodies.
acted predominantly with a 43 kDa band in naling, Fig. 1D lanes 3–5). Moreover, all antibodies immunoreacted with Aplysia Erk [22]. The antibodies also detected a doublet at 42 and 44 kDa in mouse CNS extract (Fig. 1B–D; lane 1), which served as a positive control. The difference in intensity of the signals from mouse and Aplysia proteins immunoreact specifically with the invertebrate homologs (unpublished observations), validation of the specificity of the vertebrate antibodies against Aplysia Erk is necessary. To do so, we cloned the Aplysia Erk isoform (ApErk; also termed ApMAPK in [22]) from pleural-pedal ganglia using PCR. Lysates of bacterial clones expressing either glutathione S-transferase (GST)-ApErk or GST-alone were subjected to electrophoresis followed by gel fixation and Coomassie staining and transblotted to nitrocellulose membranes and treated with anti-phospho-Erk from Cell Signaling, Fig. 1B, lanes 3–4; anti-phospho-Erk from Promega, Fig. 1C, lanes 3 and 4; and anti-total Erk from Cell Signaling, Fig. 1D lanes 3–5). Moreover, all antibodies immunoreacted predominantly with a 43 kDa band in Aplysia pleural-pedal extract (Fig. 1B–D; lane 2). This protein band corresponds to Aplysia Erk [22]. The antibodies also detected a doublet at 42 and 44 kDa in mouse CNS extract (Fig. 1B–D; lane 1), which served as a positive control. The difference in intensity of the signals from mouse and Aplysia extracts does not reflect potential differences in antibody avidity because different amounts of total protein were loaded and Western blot conditions were optimized for Aplysia.

To further investigate the specificity of the anti-phospho-Erk antibodies, the effect of protein phosphatase treatment on phospho-Erk immunoreactivity was investigated (Fig. 1B and C; lanes 4 and 5). Recombinant proteins produced in bacteria can undergo extensive phosphorylation [18]. Taking advantage of this discovery, GST-ApErk was analyzed through SDS-PAGE, transflected to nitrocellulose membranes and treated with an antibody against phospho-Erk (1:2000; Cell Signaling, Beverly, MA, or Promega, Madison, WI). Following rinses with Tris-buffered solution supplemented with 0.1% Tween, 10 mM sodium fluoride and 1 mM sodium orthovanadate [27], the membrane was incubated with an HRP-conjugated secondary antibody (1:100,000; Zymed, San Francisco, CA). Signal detection was performed through Enhanced Chemiluminescence (ECL, Amersham Biosciences). Caution was taken to avoid film saturation by keeping exposures brief (a few seconds). Subsequently, the membrane was stripped and reprobed with the antibody against total-Erk (1:100; Cell Signaling). For quantification, films were scanned and signals were analyzed using ImageQuant software (Molecular Devices, Sunnyvale, CA). For statistical comparisons, the phospho-Erk signal was normalized to that of total Erk, which was not affected by the treatments (see Fig. 2A1, B1 and C1).

Isolated sensory neurons from pleural ganglia were plated on glass cover slips and maintained as described previously [2]. Treatments consisted of five 5-min pulses of 5-HT (50 μM) alone or in the presence of 10 μM FMRFas (continuous application). Controls were treated with ASW. Immediately after treatment, cells were fixed with 4% paraformaldehyde as described elsewhere [10,2] and processed for immunofluorescence using a polyclonal phospho-Erk antibody (1:500; Promega) followed by an Alexa 596-conjugated secondary antibody (Molecular Probes, Eugene, OR). Images were obtained with a BioRad 1024 MP confocal microscope using a 60× oil immersion lens (NA 1.4) and consisted of a single optical section through the center of the cell body. Images were analyzed with Metamorph Offline software (Universal Imaging Corporation, Downingtown, PA) by an experimenter who was “blind” to the treatment condition. For each cell, the outline of the nucleus was traced and average fluorescence intensity was recorded.

The regulation of Aplysia Erk has been previously studied using commercial antibodies against mammalian Erk (e.g., [10,28,16]). Because not all antibodies raised against vertebrate proteins immunoreacted specifically with the invertebrate homologs (unpublished observations), validation of the specificity of the vertebrate antibodies against Aplysia Erk is necessary. To do so, we cloned the Aplysia Erk isoform (ApErk; also termed ApMAPK in [22]) from pleural-pedal ganglia using PCR. Lysates of bacterial clones expressing either glutathione S-transferase (GST)-ApErk or GST-alone were subjected to electrophoresis followed by gel fixation and Coomassie staining according to standard procedures [26]. Two clones were randomly chosen (Fig. 1A), to investigate the ability of the antibodies to recognize recombinant ApErk. Clone #2 expressed GST-ApErk after induction with IPTG whereas clone #1 expressed GST-alone and served as control. As shown in Fig. 1, all three antibodies recognized recombinant ApErk with no apparent immunoreactivity to the GST moiety (anti-phospho-Erk from Cell Signaling, Fig. 1B, lanes 3–4; anti-phospho-Erk from Promega, Fig. 1C, lanes 3 and 4; and anti-total Erk from Cell Signaling, Fig. 1D lanes 3–5). Moreover, all antibodies immunoreacted predominantly with a 43 kDa band in Aplysia pleural-pedal extract (Fig. 1B–D; lane 2). This protein band corresponds to Aplysia Erk [22]. The antibodies also detected a doublet at 42 and 44 kDa in mouse CNS extract (Fig. 1B–D; lane 1), which served as a positive control. The difference in intensity of the signals between mouse and Aplysia extracts does not reflect potential differences in antibody avidity because different amounts of total protein were loaded and Western blot conditions were optimized for Aplysia.
protein phosphatase (10 units/µl in 1 x supplied buffer: 2 mM MgCl₂, 10 mM Tris–HCl, 100 mM NaCl, 2 mM DTT, 0.1 mM EGTA, 0.01% Brij 35, pH 7.5; New England Biolabs, Beverly, MA) or with just buffer, for 1 h at 30 °C. Western blot analysis using anti-phospho-Erk antibody (either from Cell Signaling (Fig. 1B) or Promega (Fig. 1C)) indicated that treatment with phosphatase reduced immunoreactivity of both anti-phospho-Erk antibodies (Fig. 1B and C, compare lanes 4 and 5), whereas the total Erk signal was unaffected (Fig. 1D, compare lane 5 and 6). Similar results were obtained when Aplysia pleural-pedal ganglia extract was used as the source of ApErk (Fig. 1B and C, compare lanes 6 and 7; Fig. 1D, compare lanes 7 and 8). These findings suggest that the anti-phospho-Erk antibodies react specifically to the phosphorylated form of Erk, and further suggest that the anti-total-Erk antibody is not sensitive to the phosphorylation state of ApErk.

In Aplysia, levels of constitutively phosphorylated Erk are rather high ([10]; see also Fig. 1B and C) and may contribute to the proper localization of synaptic proteins such as synapsin [2]. To investigate any effects of FMRFαs on basal Erk activity, levels of phosphorylated Erk were assessed immediately after treatment with ASW (control) or FMRFαs, using the Promega phospho-specific antibody. Following incubation with an HRP-conjugated secondary antibody and ECL analysis, the membrane was stripped and re-probed with the total Erk antibody to control for protein load (Fig. 2A1). Subsequent analysis indicated that Erk phosphorylation was significantly reduced in ganglia treated with FMRFα compared to control (mean ± S.E.M.: control: 1.03 ± 0.47 IR; 5-HT: 1.28 ± 0.98 IR; 5HT + FMRFα: 1.03 ± 0.19 IR; p < 0.05, q = 3.5, p < 0.05; control versus 5HT + FM: q = 1.41, p > 0.05), suggesting that FMRFα overrides the effects of 5-HT on both Erk phosphorylation and nuclear accumulation of phospho-Erk.

The sensorimotor synapse in Aplysia exhibits two opposing forms of synaptic plasticity: LTF, which can be elicited by 5-HT, and LTD, which can be elicited by FMRFαs. When applied concurrently with 5-HT, FMRFαs overrides 5-HT at least at two levels: p38 MAPK is activated rather than inhibited [15], and p38 MAPK then phosphorylates CREB2, repressing CRE-regulated gene expression [14,15].

Our results suggest additional crosstalk between the two pathways at the level of Erk regulation. First, we validated the specificity of three commercial antibodies, which are often used to examine the regulation of Aplysia Erk, for the phosphorylated and total forms of Aplysia Erk. All three antibodies recognized recombinant and endogenous ApErk. Furthermore, binding of the phospho-specific antibodies was sensitive to the phosphorylation state of ApErk (Fig. 1). Using these antibodies, we found that FMRFαs blocked the 5-HT-induced phosphorylation of Erk (Fig. 2C). FMRFαs also inhibited the 5-HT-induced increase of phospho-Erk in the cell nucleus (Fig. 3A and B). Both effects of FMRFαs are most probably mediated by p38 MAPK, because this kinase is activated by FMRFαs [15] and is necessary for the inhibitory action of FMRFαs on basal Erk phosphorylation.

To investigate any potential interaction of the 5HT- and FMRFα-activated cascades at the level of Erk, levels of phosphorylated Erk were measured immediately after treatment with ASW (control), 5-HT, or 5-HT and FMRFα together, using the Cell Signaling phospho-Erk antibody. After incubation with the HRP-conjugated secondary antibody and ECL analysis, the membrane was stripped and re-probed with the total Erk antibody. Friedman analysis of variance for dependent samples (as described in [33]; see also [21]) showed that an overall difference existed among the three groups [median (and interquartile range); IR]: control: 1.03 (0.47 IR); 5-HT: 1.28 (0.98 IR); 5HT + FM: 0.93 (0.19 IR); n = 4, x² = 6.5, d.f.: 2, p < 0.05] (Fig. 2C). Subsequent Student–Newman–Keuls post hoc tests showed that concurrent application of FMRFαs blocked the 5HT-induced increase in Erk phosphorylation (5HT versus SHT + FM: q = 3.5, p < 0.05; control versus 5HT: q = 3.5, p < 0.05; control versus SHT + FM: q = 4.94, p < 0.05; control versus 5HT + FM: q = 1.41, p > 0.05), suggesting that FMRFα overrides the actions of 5-HT at the level of Erk activation.

5-HT induces nuclear translocation of Erk, where the kinase can regulate gene expression [20,27]. Phosphorylation of Erk is required for its nuclear translocation in other systems [19]. Because FMRFαs blocked the 5-HT-induced phosphorylation of Erk, we hypothesized that it would also block nuclear accumulation of phosphorylated Erk. To address this issue, we quantified levels of phosphorylated Erk in the nucleus of sensory neurons in culture, fixed immediately after treatment with ASW, 5-HT, or 5-HT and FMRFαs (Fig. 3A and B). Kruskal–Wallis analysis of variance followed by Dunn’s post hoc tests (as described in [33]; see [17] and [6]) indicated that treatment with 5-HT induced an increase of nuclear phospho-Erk [from 2 experiments: median (and interquartile range); IR] for control: 82.57 (29.16 IR), 17 cells; 5-HT: 95.59 (51.86 IR), 23 cells; q = 2.94, p < 0.05], confirming previous findings [16,20]. Concurrent application of FMRFαs blocked this effect (from 2 experiments: 5HT: 23 cells (same as above); 5HT + FMRFαs: 62.86 (13.89 IR), 29 cells; q = 6.01, p < 0.05) suggesting that FMRFαs overrides the effects of 5-HT on both Erk phosphorylation and nuclear accumulation of phospho-Erk.

Fig. 3C illustrates a model of the competing effects of 5-HT and FMRFαs on kinase activation and gene expression. 5-HT exposure leads to PKA and Erk activation and consequent phosphorylation of CREB1 and CREB2. Whereas CREB1 is activated by phosphorylation, CREB2 is repressed. These phosphorylations cooperate to induce genes regulated by CREs [14]. FMRFαs, acting through p38 MAPK, inhibits Erk and

blocks phosphorylation of CREB1. Induction of genes necessary for LTF is thereby inhibited. p38 MAPK also phosphorylates CREB2, enhancing CREB2’s repression of transcription, and reinforcing inhibition of genes necessary for LTF. We note that long-term depression induced by FMRFa may depend on induction of another set of genes regulated by p38 MAPK (dashed arrows in Fig. 3C).

The concept of “memory-suppressor genes”, which encode proteins acting to constrain synaptic strengthening and the consequent formation of long-term memory, was previously discussed [1]. CREB2 is one such protein, because inhibition of CREB2 allows a normally subthreshold stimulus (5 min of 5-HT) to induce LTF [4]. We propose a related concept of “memory-suppressor neuromodulators”. The presence of some neuromodulators, such as FMRFa in Aplysia, may maintain activities of intracellular signaling pathways that constrain synaptic strengthening and prevent subthreshold stimuli from inducing memory formation. FMRFa could limit memory formation by maintaining basal levels of p38 MAPK activation, CREB2 phosphorylation, and repression of genes responsible for LTF.

Recently, theoretical and empirical efforts have contributed toward the understanding of bidirectional regulation of synaptic plasticity in mammals [9,32,30,13,29,5]. It is likely that gene induction, synaptic strengthening, and memory formation in mammals, as in Aplysia, is limited by memory suppressor
neuromodulators, and that methods similar to those used here can help elucidate these constraints.

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References


