Published in final edited form as:

Neurobiol Learn Mem. 2015 November ; 125: 47–54. doi:10.1016/j.nlm.2015.07.017.

Synaptic generation of an intracellular retrograde signal requires activation of the tyrosine kinase and mitogen-activated protein kinase signaling cascades in *Aplysia*

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Abstract

Cellular changes underlying memory formation can be generated in an activity-dependent manner at specific synapses. Thus an important question concerns the mechanisms by which synaptic signals communicate with the cell body to mediate these cellular changes. A monosynaptic circuit that is enhanced by sensitization in *Aplysia* is well-suited to study this question because three different subcellular compartments: (i) the sensorimotor SN-MN synapses, (ii) the SN projections to MNs via axonal connections, (iii) the SN cell bodies, can all be manipulated and studied independently. Here, we report that activity-dependent (AD) training in either the entire SN-MN circuit or in only the synaptic compartment, activates MAPK in a temporally and spatially specific pattern. Specifically, we find (i) MAPK activation is first transiently generated at SN-MN synapses during training, (ii) immediately after training MAPK is transiently activated in SN-MN axonal connections and persistently activated in SN cell bodies, and finally, (iii) MAPK is activated in SN cell bodies and SN-MN synapses 1hr after training. These data suggest that there is an intracellularly transported retrograde signal generated at the synapse which is later responsible for delayed MAPK activation at SN somata. Finally, we find that this retrograde signal requires activation of tyrosine kinase (TK) and MEK signaling cascades specifically at the synapses.

Keywords

retrograde signal; MAPK; tyrosine kinase; intracellular communication; long-term memory

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The authors cite no conflicts of interest.

1. Introduction

The formation of a long-term memory (LTM) requires transcription of new gene products in the nucleus that can be utilized in distal neuritic processes to maintain synapse-specific experience-dependent modifications of synaptic strength. The importance of rapid, local protein synthesis at synapses for enhancement of synaptic strength and memory formation has been well established (Steward and Levy 1982; Miller et al. 2002; Liu et al. 2003; Hu et al. 2006; Martin and Zukin 2006; Wang et al. 2009), suggesting that signals that engage transcription may be initiated at synapses. Therefore, an important area of investigation has been to elucidate the events that occur in these two general compartments of the neuron (the cell body containing the nucleus and the distal processes containing synapses) and how these neuronal compartments communicate during LTM formation.

The invertebrate *Aplysia* is a particularly useful model system in which to study these questions for two reasons. First, the neural circuitry that supports memory for sensitization of a withdrawal reflex in *Aplysia* is relatively simple and well-understood. Second, a critical site for plasticity underlying memory formation is the sensorimotor synapse (SN-MN) mediating the reflex (Kandel 2001). The SN cell body is located in one anatomical compartment, the pleural ganglion, and the synaptic connections to the MNs are located in another compartment several millimeters away in the pedal ganglion. This distance permits the construction of a barrier that allows independent pharmacological manipulation of the two cellular compartments, referred to as a split-bath preparation (Sherff and Carew 1999; Sherff and Carew 2004). Thus, this preparation can be used to investigate molecular signaling events in individual subcellular compartments of the intact SN-MN circuit responsible for the induction of synaptic plasticity and memory in *Aplysia*.

As has been demonstrated in mammalian systems, multiple lines of evidence suggest the importance of communication between the synaptic and somatic compartments in long-term synaptic plasticity and memory in *Aplysia*. For example, repeated applications of serotonin (5HT) to SN-MN synapses in bifurcated cell cultures induces long-term facilitation (LTF) at the stimulated synapses 24hrs later, while no significant enhancement is observed in the non-treated synapses (Martin et al. 1997a). This form of LTF requires CREB-mediated transcription in the SN soma. Additionally, local translation at synapses occurs after 5HT exposure, and translational inhibitors applied to stimulated synapses prevent the induction of LTF (Martin et al. 1997a; Liu et al. 2003; Hu et al. 2006; Wang et al. 2009). These results demonstrate that the SN-MN synapse is a critical site for the induction of molecular signaling to the soma which results in the induction of LTF, and thus support the hypothesis that trans-compartmental communication is mediated by a retrogradely transported signal from the synapse to the soma during the induction of LTF and LTM.

LTF can also be generated in an activity-dependent (AD) fashion, in which a single pulse of 5HT paired with either SN activity or KCl gives rise to long-lasting synaptic changes (Hu et al. 2007; Shobe et al. 2009; Fischbach et al. 2014). Here, we report that AD training (consisting of a 5HT pulse combined with KCl) gives rise to an intracellular, retrogradely transported signal in the intact SN-MN circuit. Specifically, we find that mitogen activated protein kinase (MAPK) (i) is activated during AD training at SN-MN synapses, (ii) is

activated in SN-MN connectives and SN cell bodies immediately after the training trial, and (iii) is again activated at a later time at the SN-MN synapses, while remaining persistently activated in SN somata. Interestingly, this temporal profile of MAPK activation is apparent both when the entire circuit receives AD training (wholebath application), and when the training is restricted to the SN-MN synaptic compartment. Finally, the synaptically generated retrograde signal resulting in persistent MAPK activation at the soma requires tyrosine kinase (TK) and MEK activity (the upstream activator of MAPK) in the synaptic compartment. Collectively the data support the view that synapses may not only respond rapidly to AD stimuli, but may also coordinate synaptically-derived somatic signaling that is critical for long-lasting plasticity and memory formation.

2. Methods

2.1 Ganglia Dissections

All experiments were performed on adult wild-caught *Aplysia californica* (150–300g), housed in aquariums maintained at 15°C. On the day of the experiment, animals were anesthetized by injection of isotonic MgCl₂, and pleural-pedal ganglia pairs (containing tail SNs and MNs, respectively) were removed from the animal. The isolated ganglia were pinned in a sylgard coated dish and desheathed in a 1:1 solution of MgCl₂ and artificial seawater (ASW; containing in mM: 460 NaCl, 55 MgCl₂, 11 CaCl₂, 10 KCl, and 10 Tris, pH 7.6) to minimize neuronal activity during dissection.

2.2 Split-bath Preparation

After desheathing, a vaseline barrier was constructed between the pleural and pedal ganglia and sealed around the pleural-pedal connective to allow application of AD training and/or pharmacological inhibitors to be restricted to either the somatic or synaptic cellular compartment (Fig. 1). The integrity of the barrier was tested prior to experimentation, and the split-bath preparation has been used by our laboratory previously to manipulate the molecular environments of cellular compartments independently and reveal differential consequences of drug applications in one chamber versus another (Sherff and Carew 1999; Sherff and Carew 2004). MgCl₂ was washed from preparations with ASW for at least 30 mins prior to AD training.

2.3 Activity-dependent (AD) Training

To study MAPK activation in response to AD training, one isolated pleural-pedal pair from each animal received AD training, while the remaining untrained pair served as a within-animal control. The training consisted of combined 5 minute application of neuromodulation via 5HT (50 µM) and depolarization via high KCl (100mM) ASW. Neither 5min 5HT nor 5min high KCl ASW alone are sufficient to produce MAPK activation 1hr after training (Philips et al. 2007; Shobe et al. 2009; Philips et al. 2013). To study activity-dependent plasticity initiated by the synapse alone, we restricted training (KCl+5HT; AD training) to the pedal ganglion compartment containing the SN-MN synaptic connections. Samples were collected at three different times after AD training onset: 1 min (during training), 5 min (immediately after training), and 1hr after training.

In experiments testing the requirement of TK or MAPK signaling, drug or vehicle was applied before, during, and/or after training. Ganglia were pretreated with the general tyrosine kinase inhibitor Genistein (100 μ M; Sigma or Cayman Chemical), or its vehicle (0.33% DMSO, 0.43% ETOH in ASW) for 30min, or with the MEK (the upstream activator of MAPK) inhibitor U0126 or its inactive analog U0124 (20 μ M in 0.1% DMSO; Sigma) for 1 hr.

At the appropriate time points during or after the 5min AD training trial, the subcellular compartments of the SN-MN circuit were collected (Fig. 1), including (i) the SN-MN neuropil, a region under the MN cell bodies that is enriched in SN-MN synapses, (i) the pleural-pedal connective, which contains the SN-MN axonal connections, and (iii) the SN cell bodies, and prepared for Western blot analysis.

2.4 Western Blotting

Western blotting was performed as previously described (Purcell et al. 2003; Sharma et al. 2003). Briefly, samples harvested from each pleural-pedal pair were boiled, loaded in separate lanes of a 4–12% Bis-Tris gel and transferred to nitrocellulose membranes (Invitrogen). MAPK protein was detected by total- and phospho-MAPK antibodies (Cell Signaling), followed by either HRP-linked anti-rabbit secondary antibody (Cell Signaling) or Li-Cor IRDye secondary antibodies (Li-Cor). Data were quantified using NIH Image software, and later Image J software, after confirming that the two programs yielded similar measurements for the same data set. Data points were excluded as outliers if they were more than two standard deviations from the mean (Dixon and Tukey 1968). This resulted in 6 data points (<3%) being removed from analyses, which is indicated in the text.

2.5 Data Analysis

To determine the level of MAPK activation in response to training, phospho-MAPK levels were normalized to total-MAPK in each sample, and then a difference score was calculated (experimental less within-animal control levels). Data for each subcellular compartment at three different time points (during training [1min], immediately after training [5min], 1hr after training [1hr]) were analyzed with a one-way ANOVA, and significant results were followed by planned paired t-test comparisons. Specifically, the level of phospho:total MAPK was compared between experimental ganglia that received AD training and within-animal control ganglia that received ASW treatment to determine if there was significant activation of MAPK relative to control levels. In experiments with only two groups (e.g. inhibitor vs. vehicle at one time point and in one subcellular compartment), independent-samples t-tests were performed to assess differences between groups, and paired t-tests were performed to determine if there was significant MAPK activation relative to within-animal control levels. In all cases, histograms display mean $\pm$ SEM, and $p < 0.05$ were considered statistically significant. Significant results are indicated by asterisks in the figures.

3. Results

3.1 Activity-dependent stimulation of the SN-MN circuit induces a spatially and temporally regulated profile of MAPK activation

We first sought to determine the spatial and temporal profile of MAPK activation in response to AD stimulation of the SN-MN circuit (whole-bath stimulation; see Fig. 2A). Specifically, we examined three spatial compartments (synapse, connective, and SN somata) and several temporal domains (during or after AD training). Ganglia were stimulated with a high KCl (100mM) and 5HT (50 μ M) artificial sea water (ASW) solution (AD training) to induce neuronal depolarization and neuromodulation, respectively. Subcellular components of the SN-MN circuit, including (a) SN-MN synapses, (b) the SN-MN connective, and (c) SN somata (see Fig. 1), were collected either (i) at 1min during the training trial, (ii) at 5mins immediately after the training trial, or (iii) 1hr after the training trial.

One-way ANOVAs indicated that each individual subcellular compartment showed significant differences in MAPK activation over time (synapse: $F(2, 26)=6.9$, $p<0.005$; connective: $F(2, 24)=3.6$, $p<0.05$; soma: $F(2, 29)=4.0$, $p<0.05$). Subsequent planned paired t-tests revealed a temporal pattern in activation between the subcellular compartments. At SN-MN synapses (Fig. 2D), MAPK was significantly activated relative to within-animal controls during training at 1min (mean $\pm$ SEM: 0.37 ± 0.08 ; $t(8)=4.8$, $p<0.005$, $n=9$, 1 outlier), which was transient and decreased back to baseline immediately after training (0.05 ± 0.06 , $n=10$). Interestingly, MAPK was again activated 1hr after training at SN-MN synapses (0.23 ± 0.05 ; $t(9)=4.9$, $p<0.001$, $n=10$). In the SN-MN connective (Fig. 2C), MAPK was not activated during training (0.02 ± 0.14 , $n=10$) or 1hr after training (-0.08 ± 0.10 , $n=9$), but was significantly activated in a delayed and transient manner immediately after training (0.34 ± 0.07 ; $t(7)=4.7$, $p<0.005$, $n=8$, 1 outlier). Finally, MAPK was activated in SN somata (Fig. 2B) in a delayed and persistent manner: there is no activation during training (0.12 ± 0.10 , $n=12$), but there was significant activation immediately (0.36 ± 0.07 ; $t(9)=4.9$, $p<0.001$, $n=10$) and at 1hr (0.41 ± 0.04 ; $t(9)=10.4$, $p<0.0001$, $n=10$) after training. Taken together, these data reveal a spatial and temporal profile of MAPK activation, in which activation is induced at synapses during training, in the connective and SN somata immediately after training, and at synapses and SN somata 1hr after training. These results suggest that the synapses may initiate a functional response to AD training, even when the entire circuit is exposed to the training simultaneously.

3.2 AD training restricted to the synaptic compartment induces the generation of a retrogradely transported signal in SNs

We next tested the hypothesis that synaptic signaling regulates the spatiotemporal profile of MAPK activation seen in response to wholebath stimulation of the SN-MN circuit (Fig. 2). Thus, we restricted AD training of the SN-MN circuit to the synaptic compartment using a split bath preparation (Fig. 3A; Sherff and Carew 1999). As in the previous wholebath experiments, one-way ANOVAs indicated that each individual subcellular compartment showed significant differences in MAPK activation over time in response to synaptic AD training (synapse: $F(2, 28)=4.1$, $p<0.05$; connective: $F(2, 30)=3.6$, $p<0.05$; soma: $F(2,$

28)=3.6, $p<0.05$), and subsequent planned paired t-tests revealed an identical temporal pattern in activation between the subcellular compartments.

MAPK was significantly activated at SN-MN synapses (Fig. 3D) during training (0.28 ± 0.10 ; $t(10)=2.7$, $p<0.05$, $n=11$) and 1hr after training (0.20 ± 0.04 ; $t(8)=5.6$, $p<0.001$, $n=9$, 1 outlier), but not immediately after training (-0.01 ± 0.06 , $n=11$). Conversely, MAPK was significantly activated in the SN-MN connective (Fig. 3C) immediately after training (0.19 ± 0.08 ; $t(10)=2.5$, $p<0.05$, $n=11$), but not during (-0.03 ± 0.03 , $n=11$, 1 outlier) or 1hr after training (0.05 ± 0.06 , $n=11$, 1 outlier). In SN 1 outlier and 1hr after training (0.14 ± 0.03 ; $t(9)=4.4$, $p<0.005$, $n=10$), but not during somata (Fig. 3B), MAPK was activated immediately (0.12 ± 0.05 ; $t(9)=2.3$, $p<0.05$, $n=10$, training (-0.02 ± 0.05 , $n=11$).

Collectively, the data support two conclusions: i) activity-dependent stimulation of the SN-MN circuit as a whole can be recapitulated by stimulation restricted to the synaptic compartment, suggesting that synaptic signaling drives the spatiotemporal profile of MAPK activation observed in Fig. 2, and ii) because stimulation isolated to the synaptic compartment results in persistent MAPK activation at SN somata at a later point in time, the data strongly suggest that the synapse is able to generate an intracellular, retrogradely transported signal that travels to the SN somata to support MAPK activation in that compartment. It is important to note that although the pattern of MAPK activation is identical in wholebath and synaptic training, the magnitude of activation is relatively larger when AD training is applied to the entire SN-MN versus when it is applied only to the synaptic compartment (Fig. 2 vs. Fig. 3), suggesting that there may be additional interactive processes occurring when the rest of the circuit is exposed to AD training.

3.3 Molecular mechanisms underlying the synaptically generated intracellular retrograde signal

Persistent, somatic MAPK activation is required for LTF in SN-MN coculture (Martin et al. 1997b), thus we next explored the molecular mechanisms that result in a retrograde signal and MAPK activation in SN somata 1hr after training. Tyrosine kinase (TK) activity is often required for MAPK activation (Reissner et al. 2006; Kopec and Carew 2013). Moreover, we have previously demonstrated the importance of a TK-MAPK signaling cascade in the induction of synaptic plasticity and memory, both by AD training (Shobe et al. 2009) and by repeated 5HT training (Purcell et al. 2003; Sharma et al. 2006). Thus, we next asked whether TK activity is required for the retrograde signal resulting in MAPK activation in SN somata 1hr after training. There are two mechanisms by which TK is known to be involved in retrograde signaling in other systems: (i) as part of a long-lived signaling endosome that is transported from the synapse to the soma (Riccio et al. 1997; Watson et al. 1999), and (ii) by lateral propagation of the signal through transactivation of receptor TKs along the cell membrane (Lee et al. 2002). The mechanisms by which TK acts as a retrograde signal by transactivation of receptor TKs along the cell have been most extensively studied in the context of neurotrophin signaling (for review see How and Mobley 2005; Ibanez 2007), and studies of neurotrophin-TK signaling have demonstrated that ongoing TK activity is required in both the distal neurites and the cell body for activation of downstream signaling cascades (Riccio et al. 1997; Ye et al. 2003).

To specifically test whether ongoing TK signaling is required for MAPK activation, we generated a retrograde signal by administering synaptic AD training (as in Fig. 3) while applying the TK inhibitor, genistein, to either the synaptic (Fig. 4A₁) or somatic compartment (Fig. 4A₂) before, during, and after training, and measured MAPK activation in SN somata 1 hr after training. A one-way ANOVA revealed a difference between groups ($F(3, 35)=3.0, p<0.05$), and subsequent planned independent t-tests indicated that TK inhibition in the synaptic compartment blocked activation of somatic MAPK 1 hr after training (vehicle: $0.47\pm0.10, t(9)=4.9; p<0.001, n=10$; genistein: $0.11\pm0.06, n=10$; between groups: $t(18)=3.2, p<0.01$; Fig. 4A₁). However, blocking TK signaling in the somatic compartment did not block MAPK activation 1 hr after training in SN somata (vehicle: $0.20\pm0.06, t(8)=3.1; p<0.05, n=9$; genistein: $0.20\pm0.04, t(9)=5.7; p<0.0005, n=10$; Fig. 4A₂). Thus, TK activity is required in the synaptic compartment for the generation of a retrograde signal, but does not appear to be involved at SN somata for MAPK activation 1 hr after training. These data do not support the hypothesis that either a retrogradely transported TK signaling endosome or laterally propagated receptor TK activation along the membrane gives rise to direct activation of local pools of MAPK in the SN somata.

Another potential mechanism responsible for the retrograde signal is that MAPK is activated in synapses downstream of TK activity, and then the activated MAPK itself translocates to SN somata to contribute to the persistently elevated levels of active MAPK 1 hr after training. To test this hypothesis, we applied the MEK inhibitor U0126, or its inactive analog U0124, to the synaptic compartment (Fig. 4B) before, during, and after synaptic AD training. We found that blocking MAPK activation at the synapse blocked the retrograde signal presumed to underly persistent activation of MAPK in SN somata 1 hr after training (U0124: $0.34\pm0.05, t(8)=6.9, p<0.0005, n=9$; U0126: $0.03\pm0.11, n=10$; between groups: $t(17)=2.5, p<0.05$; Fig. 4B). These data show that the retrograde signal is dependent on MEK activity at the synapse, and support the possibility that activated MAPK may itself be the retrogradely transported signal.

4. Discussion

There has been an increasing interest in identifying candidate molecules which might mediate signaling between the synapse and soma in *Aplysia*. To date most of this work has been carried out in cell culture using repeated applications of 5HT to induce synaptic plasticity (Martin et al. 1997b; Thompson et al. 2004; Lai et al. 2008; Puthanveetil et al. 2008; Choi et al. 2014). The data presented here extend this growing literature by identifying an AD signal generated synaptically in an intact SN-MN circuit. Taken together, our data support the model illustrated in Fig. 5, in which a TK-induced MAPK signaling cascade is activated locally at the synapse during AD training, after which MAPK translocates in a retrograde fashion through the SN-MN connective to SN somata to contribute to the elevated levels of active MAPK 1 hr after training. Because the synaptic compartment contains both pre-synaptic SN varicosities as well as post-synaptic MN cell bodies and dendrites, our data do not inform the question of whether TK and MAPK signaling is occurring in the SN, in the MN, or both. Similarly, our data do not resolve the question of whether MAPK activation at the synapse 1 hr after training is pre- and/or post-synaptic. Interestingly, because MAPK is not significantly activated at the synapse

immediately after training, MAPK activation measured 1hr after training at the synapse may reflect a distinct phase of signaling. In support of this hypothesis, recent work by Kopec et al. (2015) indicates that an initial, early phase of MAPK activation at SN somata requires TK signaling, specifically the receptor TK, TrkB, at the synapse, while a later phase of MAPK activation does not rely on this synaptic signaling. Because GF signaling, particularly by the neurotrophin family and receptor TKs is canonically upstream of MAPK activation (Kopec and Carew 2013), we hypothesize that AD stimuli activate synaptic receptor TKs, which in turn activate synaptic pools of MAPK (Fig. 5A) that then are transported in retrograde fashion to SN somata (Fig. 5B). Furthermore, because drug treatment was present both during and after AD training, the exact time during which TK and MEK activity gives rise to somatic MAPK activation 1hr after training is unclear. However, we hypothesize that TK-MAPK signaling is occurring rapidly during the AD training trial for two reasons: (i) Receptor TK signaling occurs within 15 mins of a training trial to produce LTM in *Aplysia* (Kopec et al. 2015), (ii) we show here that MAPK activation occurs at SN-MN synapses during AD training (at 1 min), but is no longer detected in the synapse immediately after the training trial (5 mins; Fig. 3D), suggesting that MAPK is activated rapidly at synapses at training onset and then is transported into SN-MN axonal connectives (Fig. 3C) and SN somata (Fig. 3B). These hypothesis will be important to test experimentally in future studies.

4.1 Synaptic and nuclear signaling during learning-related plasticity

Our data agree with extensive reports indicating that the synapse is a critical site for the induction of plasticity. Moreover, it is becoming clear that communication between the synapse and nucleus must be highly coordinated in order to produce synaptic plasticity. For example, Sherff and Carew (1999) demonstrated that long-lasting plasticity can be induced in the SN-MN circuit with asymmetric 5HT training to the somatic and synaptic compartments within a restricted temporal window. Specifically, if a 5 min 5HT pulse was delivered to the synaptic compartment coincident with the termination of a 30 min 5HT pulse to the somatic compartment, LTF was induced, while neither the shorter synaptic pulse nor the longer somatic pulse alone induced LTF (Sherff and Carew 1999). This effect required transcription in both pre-synaptic SNs and post-synaptic MNs, as well as immediate protein synthesis in MNs and delayed protein synthesis in SNs (Sherff and Carew 1999; Sherff and Carew 2004). Importantly, if the synaptic pulse was delivered 20 mins before or after the somatic pulse, no LTF was induced (Sherff and Carew 1999), suggesting that signaling between the soma and synapse must be temporally coordinated.

These observations in the present paper and those of Kopec et al. (2015) are in agreement with a study by Casadio and colleagues (1999) reporting that in SN-MN co-cultures, repeated 5HT pulses restricted to the SN cell body or to the SN-MN synapse induce mechanistically distinct forms of LTF (cell wide vs. synapse specific, respectively), and subthreshold training to the SN cell body can induce LTF if a single pulse of 5HT is delivered to the SN-MN synapse immediately after somatic training. Furthermore, synapse specific LTF can be ‘captured’ by another synapse if subthreshold training is delivered to one synapse within a temporally limited window (Casadio et al. 1999). Taken together, these

data suggest that there is dynamic, simultaneous communication between the SN cell body and its synapses with MNs in the induction of long-lasting plasticity.

Given all the data described thus far, an important question arises: What are the retrograde and anterograde signals that allow for temporally restricted communication between the soma and synapse? An important role for anterograde signaling in *Aplysia* has already been demonstrated by Puthanveetil and colleagues (2008; 2013), who showed that components of the anterograde motor protein kinesin are upregulated by sensitizing stimuli and are required for LTF in SN-MN co-culture. Our data further elucidate the nature of retrograde communication between the synapse and the soma and raise the interesting hypothesis that MAPK could serve as one possible messenger to broker communication between both compartments of the SN (Fig. 5).

4.2 Identity of the retrograde signal

The recent identification of specific growth factors and receptor tyrosine kinases in *Aplysia*, along with demonstration of their requirement for the induction of synaptic plasticity in culture, has served to significantly inform our understanding of retrograde signaling in *Aplysia* (Ormond et al. 2004; Kassabov et al. 2013; Pu et al. 2014). Here we describe the involvement of synaptic TK and MEK signaling in the activity-dependent generation of a retrograde signal in the intact SN-MN circuit (Fig 5A,B). This retrograde signal results in persistent activation of MAPK in the somatic compartment (Fig. 5C), which, importantly, is a molecular event that is required for LTF (Martin et al. 1997b).

MAPK is well situated to play a critical role as a retrograde signal in the formation of LTM in *Aplysia*, as it can be activated in response to a variety of stimuli and is downstream of multiple signaling cascades (for reviews see Adams and Sweatt 2002; Reissner et al. 2006; Kopec and Carew 2013). Furthermore, it is known to translocate to the nucleus in response to stimuli that induce both synaptic plasticity and LTM, and can regulate the activity of CREB (Martin et al. 1997b; Philips et al. 2013), a transcription factor known to be critical for learning and memory in multiple systems (Dash et al. 1990; Bourtschuladze et al. 1994; Yin et al. 1994).

Although translocation of MAPK from the cytosol in the cell body into the nucleus has been widely studied in many contexts, direct observations of the actual translocation of MAPK from the synapse to the nucleus in the induction of plasticity have been more challenging. There have been several investigations of the question of whether MAPK may signal from distal synapses back to the nucleus to regulate transcription. In the most direct study of this question, Wiegert and colleagues (2007) used a photoactivatable fluorescent tag to study the movement of activated MAPK. They found no evidence of active transport of MAPK in primary hippocampal cultures and the degree of passive diffusion following neurite stimulation was minimal (both in distance and velocity), and thus could not contribute to MAPK signaling from synapses located more than 100µm from the soma. Another study has examined endosomal transport of active MAPK to the nucleus in response to prolonged glutamate application in cultured striatal neurons (Trifilieff et al. 2009). Confocal images suggest the MAPK is translocated from distal neurites. However, wholebath glutamate

application and quantitative comparison of cytosolic to nuclear fraction did not provide definitive evidence to suggest this view.

The compartmental analysis employed in the present study allowed us to draw further conclusions about the role of retrograde signaling, since our data also suggest that the synapse may be the first site of a measurable molecular response despite wholebath AD training. The *Aplysia* pleural-pedal connective is approximately 2mm in length, so transport spanning this distance within the time from training onset to sample collection appears possible given reported rates of active transport (0.5–7 $\mu\text{m}/\text{sec}$; Wang et al. 1995; Watson et al. 1999). Thus the possibility of this novel role for MAPK in *Aplysia* memory warrants further investigation.

Although the retrograde transport of activated MAPK is one possibility that is consistent with our data, it is not the only possible mechanism of an intracellularly transported retrograde signal. During damage to pedal nerves, which induces long-term excitability in SNs and LTF similar to learning-related plasticity, kinases including a MAPK homologue, RISK-1, and PKG have been demonstrated to be retrogradely transported from the site of injury to SN cell bodies (Sung et al. 2001; Sung et al. 2004). Additionally, the classical active nuclear import pathway, previously shown to mediate transport of cargo from the cytoplasm into the nucleus, has been adapted in neurons to mediate retrograde signaling from distal synapses to the nucleus. Martin and colleagues have clearly shown that specific proteins (importins), which are the cargo-carriers in this pathway, are localized to synapses in both *Aplysia* and in mice, and bind to and translocate cargo proteins to the nucleus following activity that induces long-lasting plasticity (Thompson et al. 2004; Lai et al. 2008; Jeffrey et al. 2009). Interestingly, MAPK has been shown to be retrogradely transported via an importin- and dynein-mediated mechanism in mammalian sciatic nerve axons following injury (Perlson et al. 2005). Thus in addition to MAPK, there are several other candidate molecules that could serve as retrograde signals mediating synaptic plasticity and memory formation.

In conclusion, the role of synaptically generated somatic signaling in the induction of long-lasting plasticity remains a fundamental area of inquiry that can critically inform our understanding of memory formation in the nervous system.

Acknowledgements

We thank Nate Zarider for technical assistance with experiments. This work was supported by NIMH RO1 MH 041083 and NIMH RO1 MH 094792 to TJC.

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Highlights

- Activity-dependent [AD] training induces temporally regulated MAPK activation.
- MAPK is activated at the synapse during AD training.
- MAPK is activated in SN-MN connectives and SN somata after training.
- AD training restricted to the synapse induces an intracellular retrograde signal.
- Synaptic tyrosine kinase and MEK activity are required for somatic MAPK activation.

Figure 1.

Schematic of the split-bath preparation. Pleural SN somata (top) are physically separated from SN-MN synapses in the pedal ganglia (bottom) by a Vaseline-sealed barrier (gray partition) surrounding the SN-MN connective (middle). Each of the three subcellular compartments of the SN-MN circuit are collected and assayed for MAPK activation.

Figure 2.

Wholebath activity-dependent stimulation induces a temporally and spatially regulated profile of MAPK activation in the SN-MN circuit. (A) High KCl (depolarization) and 5HT (neuromodulation) artificial sea water (ASW; activity-dependent [AD] training) is applied to the entire SN-MN circuit (green), and different subcellular compartments are collected during, immediately after, or 1hr after training. (B) MAPK is significantly activated in SN somata immediately (5min) and persistently (1hr) after training. (C) MAPK is significantly activated in SN-MN connectives immediately after training. (D) MAPK is activated in SN-

MN synapses during and 1hr after training. Data are displayed as mean $\pm$ SEM of the difference score between experimental ganglia (trained) and within animal control ganglia (untrained), and asterisks above histograms indicate significant differences between experimental and control ganglia.

Figure 3.

AD training restricted to the synaptic compartment induces an identical MAPK activation profile to wholebath stimulation. (A) AD training (green) is restricted to the synaptic compartment of a split-bath preparation, and different subcellular compartments are collected during, immediately after, or 1hr after training. (B) MAPK is significantly activated in SN somata immediately (5min) and persistently (1hr) after training. (C) MAPK is significantly activated in SN-MN connectives immediately after training. (D) MAPK is activated in SN-MN synapses during and 1hr after training. Data are displayed as mean $\pm$

SEM of the difference score between experimental ganglia (trained) and within animal control ganglia (untrained), and asterisks above histograms indicate significant differences between experimental and control ganglia.

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Tyrosine Kinase Inhibition

MEK Inhibition

Figure 4.

Tyrosine kinase (TK) and MEK activity is required at the synapse for 1hr MAPK activation in SN somata. (A₁) 100μM Genistein or vehicle (0.33% DMSO, 0.43% ETOH in ASW) was applied to the synaptic compartment (vertical hatching) before, during, and after synaptic AD training (green), and SN somata were collected 1hr after training. TK signaling is required in the synaptic compartment for 1hr MAPK activation in SN somata. (A₂) Genistein or vehicle was applied to the somatic compartment (vertical hatching) before, during, and after synaptic AD training (green), and SN somata were collected 1hr after training. TK signaling is not required in the somatic compartment for 1hr MAPK activation in SN somata. (B) 20μM U0126 or the inactive isomer U0124 (in 0.1% DMSO) was applied to the synaptic compartment (vertical hatching) before, during, and after AD training (green), and SN somata were collected 1hr after training. MEK activity is required in the synaptic compartment for 1hr MAPK activation in SN somata. Data are displayed as mean ± SEM of the difference score between experimental ganglia (trained) and within animal control ganglia (untrained). Asterisks above histograms indicate significant differences between experimental and control ganglia while asterisks between histograms indicate significant differences between groups (vehicle vs. drug).

Figure 5.

Model of retrograde signaling induced by AD training. (A) During synaptic AD training (green), TK-MAPK signaling is initiated at the synapse (pre- and/or post-synaptically) resulting in activated MAPK at SN-MN synapses at 1min. (B) Immediately after training (5min), MAPK is activated in SN-MN connectives and at SN somata. (C) 1hr after training, MAPK remains persistently activated in SN somata and is again activated at SN-MN synapses (pre- and/or post-synaptically).