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Molecular nodes in memory processing: insights from *Aplysia*

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Abstract. Recent research in a variety of systems indicates that memory formation can involve the activation of a wide range of molecular cascades. In assessing this recent work it is clear that no single cascade is uniquely important for all forms of memory, nor is a single form of memory uniquely dependent on a single cascade. Rather, it appears that molecular networks are differentially engaged in the induction of various forms of memory. Despite this highly interactive array of possible cascades, specific 'molecular nodes' have emerged as critical regulatory points in memory formation. Functionally, these nodes can operate in two sequential steps, beginning with a convergence of inputs which coordinately influence the activation state of the node, in which the nature of stimulation determines the dynamics of nodal activity, followed by a divergence of substrate selection, in which the node serves as a gateway that activates specific downstream effectors. Finally, specific nodes can be differentially engaged (i.e. have different 'weights') depending upon the nature and pattern of the activating stimulus. The marine mollusk *Aplysia* has proven useful for a molecular analysis of memory formation. We will use this system to highlight some of the molecular strategies employed by the nervous system in the formation of memory for sensitization, and we will focus on extracellular signal-related kinase as a candidate node integral to these processes.

Keywords. *Aplysia*, ERK, node, memory, facilitation, serotonin.

Introduction

The pursuit of molecular mechanisms responsible for synaptic plasticity and memory has accelerated substantially over the last two decades. This accelerated research has yielded an extensive body of literature highlighting a large number of essential molecular interactions and signal transduction pathways [1, 2]. Literally hundreds of molecules, many of which are kinases, have been identified as necessary components. The further elucidation of distinct forms of plasticity and memory, each with overlapping but discrete molecular requirements, has additionally complicated efforts to identify the cellular correlates of these processes [1, 3]. Taken together, this complexity has led to the daunting task of understanding how and when molecular pathways function independently or coordinately in the induction of long-lasting changes in synaptic strength and behavior.

Given this complexity, how can we understand the unique contribution of a given molecule in the translation of an experience into memory? In this review we propose a strategy to identify 'molecular nodes' within a series of networks. Specifically, we suggest that individual pathways can be integrated into a series of highly interactive molecular networks with points of intersection (nodes) between them. In this context, we will define a molecular node as a point of high convergence and divergence. This definition allows for differentiation between molecular nodes and required molecules. Simply put, the mere requirement of a molecule for plasticity or memory does not make it a node. Many molecules are required for plasticity and memory without high convergence/divergence, and therefore would not be considered nodes. For example, protein kinase A

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(PKA) is responsible for the phosphorylation and concomitant activation (or inactivation) of many molecules, thus demonstrating a high degree of divergence in response to activation. However, in some contexts the activation of PKA is directly due to synthesis of cyclic AMP (cAMP) in response to G-protein signaling following metabotropic receptor activation. Therefore, in these contexts PKA would not be considered a node because it does not demonstrate a high degree of convergence. This example underscores another important aspect of the notion of a node: in one context (e.g., defined temporally, spatially or both) a particular molecule might serve as a node, and in another context the same molecule might not. This introduces an important dynamic feature to nodal processing.

The purpose of this review is to consider how a specific node can function within a molecular network to translate stimulus patterns into different forms of synaptic plasticity and memory, each differentiated by their temporal features and molecular signatures. Toward that end, we will consider ERK (extracellular signal-related kinase), a member of the MAPK (mitogen-activated protein kinase) family, as an exemplar of a candidate node. Numerous reviews highlight the significance of ERK signaling in synaptic plasticity and memory, in both vertebrate and invertebrate systems [4–7]. Thus, we will focus specifically on how convergent factors (those that activate ERK) and subsequent substrate divergence are regulated and may modulate memory formation. A useful model for these kinds of questions is the invertebrate *Aplysia californica*. The requirements for both plasticity and memory for sensitization in *Aplysia* have been well described and are thus amenable to nodal analysis. From this perspective we will consider several questions: How does ERK activation in response to a behaviorally relevant sensitizing stimulus (e.g. tail shock), or tail shock-evoked serotonin (5-HT) release, fulfill the requirements for a node? How can a specific node (e.g., ERK) become differentially engaged with different training patterns? How can differential engagement influence substrate selection and downstream cellular consequences? We will also explore how a nodal analysis may allow for a conceptual and experimental strategy to consider interactions that may not be intuitively obvious, but that can emerge from a consideration of interactions funneling through a common molecular node.

ERK in plasticity and memory in *Aplysia:* spatial and temporal constraints

The simplicity of the *Aplysia* nervous system allows for investigation of direct links between molecular, synaptic and behavioral levels of analysis, thereby making it a particularly useful model system for consideration of nodal points within molecular networks. The well-defined *Aplysia* nervous system, combined with its learning capacities, presents the unique opportunity to map molecular events within specific subpopulations of homogeneous neurons onto distinct phases of plasticity and memory [8–10]. Thus, at the outset, a preliminary introduction to plasticity and memory in *Aplysia* will provide a context for consideration of nodes and molecular networks responsible for these processes.

One of the best characterized forms of non-associative memory is sensitization, in which the animal learns to strengthen its inherent withdrawal reflexes in response to a noxious stimulation (such as a tail shock). Underlying this modest form of memory is a surprising array of synaptic and molecular mechanisms that occur with spatial and temporal specificity. The duration of memory for sensitization varies considerably, lasting from minutes to days. Mechanistically, the longer lasting phases become progressively more reliant upon new protein and RNA synthesis, and the neuromodulator 5-HT is a major regulatory component of virtually all of the underlying plasticity. At the synaptic level, sensitization training and exogenous 5-HT application increase the synaptic efficacy of sensory neuron (SN) to motor neuron (MN) communication. This SN-MN facilitation corresponds both temporally and mechanistically to the phases of behavioral sensitization; thus, 5-HT-induced synaptic facilitation is often used as a cellular analog for sensitization.

Each form of synaptic facilitation has unique molecular requirements that occur within distinct temporal phases. Short-term facilitation (STF), induced by a single pulse of 5-HT, and short-term memory for sensitization (STM), induced by a single shock, are independent of translation and transcription and thus only require modification of pre-existing substrates. Intermediate-term facilitation (ITF), induced by multiple pulses of 5-HT, and intermediate-term memory for sensitization (ITM), induced by repeated tail (RT) shocks, are independent of transcription but do require new protein synthesis. Finally, long-term facilitation (LTF) and long-term memory for sensitization (LTM) have the same induction requirements as intermediate term, but they rely on both transcription and translation [11–16].

Further, there is clear evidence that the amount of stimuli affects both synaptic facilitation and memory formation for sensitization in *Aplysia*. For example, at the synaptic level, Mauelshagen et al. (1998) showed that five pulses of 5-HT led to both ITF and LTF, whereas fewer pulses do not [17]. At the behavioral level, Sutton et al. (2002) showed that two spaced tail shocks led to STM, while four or five spaced trials led to ITM and LTM. However, four or five massed trials led only to a short-lasting form of ITM (E-LTM), but not ITM or LTM [18].

There are also different types of facilitation and memory within the same temporal domain. For instance, there are two kinds of intermediate-term memory RT-ITM (discussed above) and site-specific intermediate-term memory SS-ITM, and each has a unique induction requirement. Whereas RT-ITM requires multiple shocks, SS-ITM is induced by a single shock to the test site. In this latter case testing occurs in the same receptive field as the shock, which introduces the opportunity for activity-dependent modulation. In a similar manner, there is also a synaptic counterpart to SS-ITM called activity dependent facilitation (AD-ITF), which is induced by pairing a single pulse of 5-HT with a bout of activity delivered to the SN [13, 14, 19–21]. Not surprisingly, these activity-dependent forms of facilitation and memory have distinct underlying mechanisms that differentiate them from their repeated trial counterparts.

These separate temporal phases mechanistically map onto a distinct set of neuronal compartments. For instance, short and intermediate forms of facilitation can be induced locally at the synapse, indicating that nuclear events (such as gene expression) are not necessary for this plasticity. Long lasting facilitation, however, requires the cell body [13, 22]. Thus, it is evident that signaling cascades that occur within separate compartments (e.g. cell body vs. neurites) can be differentially utilized in the service of different forms of plasticity.

It has been unambiguously demonstrated that ERK is required for several forms of facilitation and memory for sensitization [23–25]. Moreover, ERK is differentially activated by distinct patterns of 5-HT stimulation and interfaces with several fundamentally distinct downstream cascades. These observations collectively suggest that some critical properties of ERK cascades align with those of plasticity and memory; thus a comparison of ERK activation under different stimulus patterns allows us to identify areas of intersection between behavioral systems and their underlying molecular pathways. From this perspective we will address whether unique ERK signaling pathways map onto distinct phases of synaptic plasticity and memory in *Aplysia*.

Thus, ERK is uniquely suited to govern these processes because (i) it is located in all the relevant cellular compartments, (ii) it regulates a variety of spatially constrained downstream pathways, and (iii) it is critically required for several forms of facilitation and memory for sensitization [4]. We propose that ERK is in the right place at the right time to serve as a molecular node that functions to induce three mechanistically distinct forms of plasticity and memory that are characterized by either modification of pre-existing substrates, regulation of translationally sensitive proteins or regulation of new genes. In the sections that follow we will consider each of these distinct mechanistic roles of ERK.

Short-lasting forms plasticity and memory: modification of preexisting effectors

The hallmark of short-term term plasticity and memory is that the underlying changes do not rely on the synthesis of new proteins or RNA. Therefore, plasticity is only achieved by the modification of pre-existing proteins. Numerous studies in both mammalian and invertebrate research have demonstrated that kinases play a critical role in this process via the phosphorylation of cytoplasmic effectors such as ion channels and release machinery. In *Aplysia* a single pulse of 5-HT activates PKA and protein kinase C (PKC) in the SNs, and enhances synaptic strength for about 15 min (STF). This STF requires PKA and PKC to a degree that varies depending on the initial state of the synapse [26]. For a non-depressed synapse the plasticity produced by the 5-HT acts primarily through PKA via two main factors: spike broadening and presumed covalent modification of the release machinery. Spike broadening is a process by which PKA-dependent phosphorylation of K^+ channels amplifies Ca^{2+} influx at the terminals during depolarization.

In an effort to better understand the role of ERK in the modulation of cytoplasmic effectors, we recently characterized the role of MAPK in the activity-dependent form of ITM, SS-ITM (described briefly above). SS-ITM lasts several hours but does not require new protein or new RNA synthesis. Therefore, SS-ITM is mechanistically similar to short-term plasticity and memory. Initial work by Sutton et al. [19] indicated that SS-ITM requires PKC activity during its induction (initial memory formation) and expression (the maintenance of memory phase). In contrast, PKA activity is not required. Shobe et al. (2004) now find that MAPK is critically required for the induction but not the expression of SS-ITM [27]. Moreover, they have developed a molecular analog of site-specific training which produces robust MAPK activation in SNs. Interestingly, using this preparation, Shobe et al. identified a novel form of plasticity that requires cAMP, but not PKA, suggesting the possibility of a cAMP-dependent activation of one or more exchange factors that in turn activates ERK [28–33]. In sum, these results suggest that SS-ITM training activates MAPK, leading to the phosphorylation of pre-existing effectors which ultimately underlie SS-ITM.

Intermediate-term plasticity and translationally dependent cascades

In *Aplysia,* it was initially thought that synaptic plasticity and memory existed in two main phases: short-term and long-term. However, as first described by Ghirardi et al. (1995), it became apparent that there was an intermediate phase that was a blend of both [12]. This phase displays intermediate temporal and mechanistic characteristics. Repeated trial ITF [12, 13] and repeated trial ITM [14]

last for several hours and require translation but not transcription. In an effort to further define the role of new protein synthesis, a subsequent study by Yanow et al. showed that ITF was not affected by rapamycin, suggesting that regulation of translation does not involve mammalian target of rapamycin (mTOR) [34]. These translational studies suggest that all the transcripts necessary to induce the memory are extant before the behaviorally relevant training episode. Where are these transcripts located within the neuron? Although the answer remains largely unknown, several lines of evidence suggest that they reside in neurites and perhaps even at the synapse. First, isolated SN neurites are capable of ITF formation [35]. Second, evidence from mammalian systems indicates that translational machinery resides near synapses [36, 37]. Third, local application of 5-HT to the synapse increases synaptic CPEB in a translationally dependent manner [38], and induces ITF [39]. Collectively, these studies suggest that transcripts are competent and synaptically localized, and can be translated in response to appropriate activation patterns to give rise to long-lasting plasticity.

Kinases also play a critical role in regulating the induction and expression of RT-ITM and ITF. For example, Sutton et al. found that PKA activity is required for the expression of ITM and ITF. However, PKC activity is not involved. Recently, Sharma et al. (2003) have found that ERK activation is also required for the induction but not the expression of ITM. In support of these observations, induction of ITF also requires ERK activity [25]. Moreover, synaptically and behaviorally relevant stimuli, such as multiple spaced pulses of 5-HT and repeated tail shock, produces robust ERK activation. Taken together, these findings suggest that ERK regulates the induction of ITF and ITM.

The fact that both kinase activity and translation are required for ITF and ITM raises an obvious question: Are these upstream kinases tapping into the translational machinery, and if so, how? Since both PKA and ERK are required for full expression of ITM and ITF, they are the most likely candidates. In *Aplysia* the evidence linking these kinases to the translational machinery is mixed. In one report, there is evidence that PKA participates in S6 kinase activation. Specifically, Khan et al. [40] found that 5-HT-induced S6 kinase phosphorylation requires PKA activity. In addition, S6 kinase phosphorylation induced by 5-HT requires mTOR activity. Thus, in Khan and colleagues' model, PKA acts upstream of mTOR to the activate S6 kinase [40]. However, since ITF formation is independent of mTOR, this would suggest that PKA activation of mTOR does not play a role in intermediate-term plasticity. Clearly more work is necessary to elucidate the molecular interactions contributing to intermediate processing. But even at this point, important and intriguing insights are at hand.

Long-term plasticity and genetic control

To retain memories for days, months and even years is no small feat. This enterprise requires long-term plasticity within specific neuronal structures such as the synapse. However, these changes can demand a high energetic price, so from a cell biological perspective, the neuron must 'be certain' that a stimulus warrants this form of plasticity. Once a threshold for long-term change is exceeded, the cell executes a series of temporally choreographed genetic manipulations. Initially, pre-existing transcription factors, such as CREB, are activated by stimulated signaling cascades [41–43]. This triggers the first wave of gene expression, the immediate early genes (IEGs), such as c-fos and C/EBP [44]. These IEGs are thought to then upregulate effector genes that function to carry out the structural changes necessary for long-term plasticity. *Aplysia* is a particularly useful system to study the molecular mechanisms that underlie long-term changes in synaptic plasticity and memory. Although this system is simple in design (relative to mammals), it retains all the elements required for long-lasting synaptic and behavioral changes. For instance, memory for sensitization, habituation and classical conditioning can last for days or weeks, and this finding correlates well with the physiological changes of the underlying circuits. Also it has been shown that 5-HT application induces synaptic restructuring such as neurite outgrowth and vesicle docking [45]. Finally, it is well established that 5-HT induces these long-lasting modifications via the activation of specific signaling cascades [9, 46].

As is the case for short- and intermediate-term plasticity, long-term plasticity critically relies on the function of several kinases, including PKA and ERK. In fact, more experiments have been conducted to investigate the role of PKA in long-lasting synaptic plasticity than any other kinase. For example, Müller and Carew (1998) found that multiple pulses of 5-HT activate sustained and translationally sensitive PKA activity in SNs [47]. This persistent activity is required for LTF and presumably LTM [48]. Moreover, significant strides have been made in elucidating the transcriptional mechanisms by which the PKA cascade mediates LTF. The current model is as follows. Once sufficiently activated, PKA translocates to the nucleus where it phosphorylates the transcription factor, CREB1, which is considered an essential factor for three main reasons: (i) CREB1 is activated by plasticity-inducing stimulation (such as 5×5-HT), (ii) CREB1 is required for LTF and (iii) activated CREB is sufficient to induce LTF [43]. In the nucleus phospho-CREB1 binds to CRE sequences, producing upregulation of IEGs, such as Apuch and C/EBP, which are also required for the induction of LTF [49, 50]. These IEGs can function as either effectors or regulators. Those that serve a regulatory function do so via induction of late genes which are at present largely unknown. However, the identification of *Aplysia*

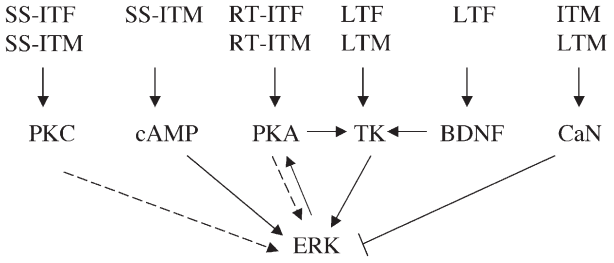

Figure 1. Summary of convergence of inputs to ERK in *Aplysia*. Different training patterns result in the formation of site-specific (SS) ITF and ITM as well as repeated-trial (RT) ITM, ITM, LTF and LTM. These different training patterns recruit the participation of molecules from different signaling pathways necessary for the activation and/or translocation of ERK. Dashed lines indicate pathways of activation which, in some cases, may not be required (see text for discussion). Activation of ERK in turn leads to prolonged activation of PKA, due to the translation of IEGs such as Ap-uch, responsible for the degradation of PKA regulatory subunit [50, 61]. Further, activation of tyrosine kinases upstream of ERK are modulated by BDNF and PKA [54, 57], as indicated by the horizontal arrows. Moreover, calcineurin exerts an inhibitory influence on ERK activation (which must be functionally overcome to induce ITM and LTM) [72], thus contributing to the complex nature of convergence of inputs.

eukaryotic translation elongation factor 1α, Ap-eEF1A, suggests that hours after the initiating stimulus (e.g. 5-HT pulses) is terminated, the translational machinery itself is upregulated in order to maintain the later phases of LTF [51]. Interestingly, inhibition of Ap-eEF1A during this time period blocks the late but not the early phase of facilitation.

In this brief review we have identified some of the key molecular steps involved in diverse forms of synaptic plasticity and memory in *Aplysia*. This background now permits us to consider how ERK becomes activated in response to 5-HT or sensitizing stimuli, and in turn, how ERK initiates a variety of downstream effects. These dual facets of ERK-dependent processing allow us to use it as an exemplar of a molecular node within the series of pathways activated during synaptic facilitation and memory formation.

Convergence: mechanisms of ERK activation

Introduction to the idea of convergence of inputs

Our two primary criteria for a molecular node are a high degree of convergent inputs and divergence of outputs. The idea of convergence of inputs, in this case activation of ERK, allows for a point of intersection among multiple signaling pathways (see Fig. 1). In *Aplysia*, activation of ERK occurs predominantly in response to release of 5-HT onto the sensory and motor neurons, and their affiliated synapses. However, it has become clear that there exist multiple cellular responses to 5-HT upstream of ERK activation, some or all of which are activated, depending upon stimulus patterns.

Molecular mechanisms of ERK activation in response to 5-HT

Compelling evidence shows that 5-HT is released in response to sensitizing stimuli such as tail shock [52, 53]. A number of studies have further demonstrated that ERK becomes activated in response to multiple pulses or prolonged application of 5-HT (see e.g. [23–25, 54, 55]). The potential significance of ERK activation and nuclear translocation in response to 5-HT was first demonstrated in two studies by Martin et al. and Michael et al. [23, 24]. Martin et al. (1997) first demonstrated that ERK is required for LTF at SN-MN synapses. In the SNs, ERK is translocated to the nucleus from the cytoplasm in response to 5-HT. Michael et al. (1998) showed that ERK is activated in response to five pulses of 5-HT (stimuli capable of producing LTF), but not one pulse (a stimulus capable of producing only STF), and once activated in response to 5-HT, ERK can then phosphorylate targets including C/EBP and CREB2. Both of these studies indicated that cAMP plays an important role in ERK activation and translocation in response to 5-HT. There is also a precedent for this in vertebrates [56]; however, whether cAMP and PKA are in all cases necessary for the activation of ERK following 5-HT application is unclear.

For example, Dyer et al. (2003) have demonstrated that a 90-min application of 5-HT results in prolonged ERK activation that is independent of PKA or PKC [55]. This finding appears to contrast with the observations of Michael et al. and Martin et al. who showed that activation and translocation of ERK in response to 5-HT is dependent upon cAMP, and can be observed in response to application of forskolin. In both of these latter studies, 5-HT was applied in 5-min spaced pulses, in contrast to the 90-min bath application by Dyer et al., suggesting that prolonged static bath application of 5-HT may lead to mechanistically distinct ERK activation. The idea that massed versus spaced application of 5-HT may lead to activation of distinct cellular mechanisms is relevant to the context of differential engagement of a node in response to training.

In pursuit of the mechanism(s) responsible for ERK activation and facilitation and memory in response to 5-HT, Purcell et al. (2003) demonstrated that 5-HT-induced activation of ERK is blocked by the tyrosine kinase inhibitor genistein [54]. Further, application of the tyrosine phosphatase inhibitor bpV, in combination with one pulse of 5-HT, leads to ERK activation and LTF. Moreover, LTF induced in this fashion is blocked by the MEK inhibitor U0126. A similar gain of function was observed when human recombinant brain-derived neurotrophic factor (BDNF) application was paired with one pulse of 5-HT,

and this BDNF-induced gain of function also required ERK [54]. These findings indicated the likely presence of a receptor tyrosine kinase (RTK) in *Aplysia* necessary for ERK activation and ERK-dependent long-term plasticity. Further evidence for an RTK in *Aplysia* was provided by the finding that the neuropeptide sensorin is released by SNs in response to 5-HT, leading to ERK activation in response to signaling through autoreceptors [57]. This activation was blocked by application of an anti-sensorin antibody, as well as inhibitors of PKA, RTK or ERK. Moreover, a single application of 5-HT coterminating with bath-applied sensorin produced ERK activation and translocation. These results suggest that ERK activity in response to 5-HT can be mediated by sensorin release, causing activation of tyrosine kinase-like receptors and subsequent activation and translocation of ERK.

Ormond et al. have since isolated and cloned an RTK in *Aplysia*, ApTrkl [58]. ApTrkl is activated, as measured by a phosphospecific antibody directed against the likely signaling tyrosine found within the intracellular NPxY motif, by a 1-h bath application, or one or more 5-min pulses of 5-HT. Following injection of double-stranded RNA (dsRNA) of ApTrkl, ERK activation is no longer observed in response to stimulation with 5-HT. Whether the autoreceptor necessary for sensorin-mediated signaling is ApTrkl or another RTK remains unclear.

Very recently Sharma and colleagues showed that application of the extracellular portion of mammalian TrkB as an immunoglobulin G (IgG) fusion protein (TrkB-Fc) blocks ERK activation in response to 5-HT, as well as LTF and LTM [59], demonstrating that a TrkB ligand, possibly a BDNF-related molecule, is secreted in response to 5-HT and is required for subsequent ERK activation and, ultimately, long-term memory formation.

Differential engagement of ERK

An important aspect of convergence of inputs is that the nature and pattern of a stimulus can provide different degrees of nodal activation – not just off or on, but in a graded fashion. This graded response may allow for control of interactions among networks and may have implications for divergence of signaling downstream of the node (i.e. substrate selection, discussed in more detail below). The stimulus pattern responsible for activation of a node may therefore govern downstream cellular events. This supposition suggests that context can regulate ERK activation in response to 5-HT in *Aplysia*. For example, ERK activation and translocation are dependent upon degree and pattern of 5-HT stimulation. Martin et al. (1997) demonstrated that, while one pulse of 5-HT slightly increases the nuclear:cytoplasmic ration of ERK, three pulses leads to a greater ratio, and five pulses leads to even greater translocation, indicating there is a graded response of ERK translocation in response to degree of 5-HT pulses [23]. It has also been demonstrated that there is a graded response of CRE-dependent transcription following application of 5-HT [60]. While this effect is not known to be dependent upon ERK, this finding provides support for the idea that repeated pulses of 5-HT lead to a graded response of cellular activity.

Byrne and colleagues have developed a computational model to examine the nonlinearity of PKA and ERK activation in response to 5-HT [61]. This model predicts that zero-order hypersensitivity is responsible for this nonlinearity. Zero-order ultrasensitivity is a biochemical mechanism first described by Goldbeter and Koshland to explain a large change in output response (in this case, ERK activation) in response to small changes in stimulus input [62–64]. The Byrne model also provides a possible mechanism for the observation that a massed 5-HT application of 25 or 90 min can lead to LTF, but less reliably than five spaced pulses.

Non-5HT-associated mechanisms of ERK activation

The mechanisms of ERK activation described above are all in the context of 5-HT-dependent synaptic facilitation and memory for sensitization. However, it is relevant to mention other stimuli in addition to 5-HT that can result in ERK activation. For example, application of transforming growth factor-β (TGF-β) leads to ERK activation, as well as LTF and long-term excitability [65–67]. While TGF-β application alone leads to LTF, similar LTF is observed in response to 5-HT alone or 5-HT combined with TGF-β, suggesting that these molecules may activate overlapping pathways. In support of this idea, application of a TGF-β inhibitor was effective at blocking 5-HT-induced LTF. In addition, ERK activation and translocation have been demonstrated in response to protein kinase G (PKG) activation and long-term hyperexcitability following nerve injury [68, 69].

The role of ERK in short-term plasticity and memory is much less certain. In fact, the prevailing view is that ERK is only involved in long-lasting forms of facilitation and memory for sensitization. This is well supported by molecular, synaptic and behavioral data. For instance, a single pulse of 5-HT (which is sufficient to induce STF) does not activate ERK [24]. Moreover, STF and STM do not require ERK activity [23, 25]. Consequently, ERK is primarily associated with more enduring changes that require translation- and transcription-dependent mechanisms. However, recent work in *Aplysia* challenges this notion by implicating ERK in several forms of plasticity and memory that only require the modification of pre-existing proteins.

For example, homosynaptic depression, the cellular analog of short-term habituation, is produced by low frequency stimulation of the SN-MN synapse. This plasticity can be modulated by TGF-β which, when applied

during the repeated stimulation, blocks synaptic depression. Interestingly, the evidence indicates that TGF-β activates ERK, which promotes the phosphorylation of synapsin [66]. These combined results suggest that synapsin is a substrate for both PKA and ERK and regulates different forms of short-term synaptic plasticity by virtue of phosphorylation at distinct sites. Synapsin is also phosphorylated during a single pulse of 5-HT (a protocol that produces STF) in an ERK-dependent manner, although the functional significance of this observation is as yet unclear, considering that ERK is not required for STF.

Inhibition

In addition to many forms of ERK activation described above, ERK activation may also be inactivated through direct and indirect inhibitory mechanisms. For example, application of the inhibitory neuropeptide FMRFamide activates p38 MAPK in *Aplysia* [70], which may be inhibitory to ERK [71]. Further, Sharma et al. demonstrated that pharmacological inhibition of the protein phosphatase calcineurin enhances ERK activation in response to one pulse of 5-HT, a protocol normally insufficient to result in ERK activation [72]. Further, the inhibition of calcineurin resulted in ITM and LTM in response to two tail shocks (normally insufficient to lead to LTM) which are also dependent on ERK activation, demonstrating that inhibition of this phosphatase leads to de-repression of ERK-dependent mechanisms for memory.

This brief overview highlights the high degree of convergence of molecular pathways onto ERK in *Aplysia* (see Fig. 1). We now turn to the second major feature of a molecular node: a high degree of divergent output.

Divergence: mechanisms of substrate selection

What are the substrates for ERK in *Aplysia*?

Once ERK is activated by diverse patterns of stimulation and 5-HT application, it in turn gives rise to a wide range of downstream effects. The diversity of these effects is mediated in large part by the wide range of ERK substrates. Once ERK is activated, the most simple question is to ask, What substrates become activated? A number of ERK substrates in *Aplysia* have been reported. Michael et al. (1998) demonstrated that C/EBP, CREB2 and a peptide derived from apCaM are all *in vitro* substrates for ERK [24]. Previously it had been demonstrated that mutation of the MAPK consensus site found in the cytoplasmic tail of apCaM prevented its internalization in response to 5-HT [73], supporting the idea that apCaM is a physiological target of ERK in *Aplysia*. It is also likely that CREB1 becomes phosphorylated in response to ERK activation in *Aplysia*, as in vertebrate systems. However, this has not yet been empirically demonstrated. Nonetheless, Bartsch et al. have shown that injection of phospho-CREB1 into SNs is sufficient to result in LTF [43].

CREB2 is a transcriptional repressor in *Aplysia*, and it is believed that phosphorylation of CREB2 by ERK relieves repression of CREB1-mediated transcription. In support of this hypothesis, injection of an antibody generated against CREB2 into SNs produces LTF in response to a single pulse of 5-HT [74]. This observed LTF is transcriptionally- and translationally-dependent, and is associated with the growth of new synaptic connections. Thus, inhibition of the inhibitory CREB isoform leads to a cellular gain of function. Does phosphorylation of CREB2 by ERK in response to 5-HT cause a similar derepression in *Aplysia*? This question has not yet been experimentally addressed. However, the understanding that ERK is activated in response to 5-HT, and that CREB2 is an *in vitro* substrate for ERK, suggests this may well be the case. Further, CREB2 is a substrate for p38 MAPK in *Aplysia* [70]. Inhibition of p38 leads to a gain of function similar to that observed following injection of an anti-CREB2 antibody [70]. Moreover, 5-HT is inhibitory to p38 activity, indicating an indirect inhibitory relationship between p38 and ERK activation in *Aplysia*. Therefore, a molecular tug-of-war between different MAPKs may be responsible for substrate selection of the node – in this case, competition for the inhibition or activation of the substrate CREB2.

ERK phosphorylates CBP in vertebrates, thereby leading to an enhancement of histone acetyltransferase activity [75]. The finding by Guan et al. in *Aplysia* that 5-HT leads to recruitment of a CREB1a-CBP dimer at the C/EBP promoter, histone acetylation and expression of C/EBP, suggests that CBP may well also be an ERK substrate in *Aplysia*, and that HAT activity may be modulated by this phosphorylation event [76].

Substrates for ERK have also been revealed in response to stimuli other than 5-HT. A 5-min treatment of ganglia with TGF-β leads to synapsin phosphorylation in SNs that is blocked by the MEK inhibitor U0126 [66]. This phosphorylation leads to dispersion of immunoreactive puncta in SNs. Further, TGF-β application decreases synaptic depression in an ERK-dependent manner, suggesting that an increase in synaptically available synapsin may counter the decrease in available neurotransmitter believed to be responsible for synaptic depression. ERK-dependent (and PKA-dependent) phosphorylation has also been demonstrated in response to 5-HT, leading to dispersion of synapsin within neurites [77].

Regulation of substrate selection

The regulation of substrate selection is an issue for all kinases, not just those that can serve as nodes. For example,

PKA is thought to phosphorylate a channel within moments for the regulation of short-term plasticity and memory, but can also phosphorylate the transcription factor CREB for long-term plasticity and memory. So a fundamental question emerges: In response to a given stimulus (in this case, 5-HT), are all pathways always activated, even those not utilized in a particular form of plasticity? Or are only the necessary pathways activated? Does differential engagement of a node influence substrate selection? Answers to these questions are not known. However, a few educated guesses are possible. The following are several possibilities for how substrate selection may be regulated:

1) The regulation of gene expression/degradation of targets is an example of how substrate selection may be modulated by the nature of the stimulus. Stimulus pattern can affect levels of targets and therefore influence substrate selection at the level of availability. An example of this is the immediate early gene C/EBP. C/EBP becomes expressed soon after exposure to 5-HT, thereby becoming available as a substrate for ERK. Also, combinatorial interactions may be necessary to direct an ERK effect. That is, substrate selection is also dependent on concomitant activation of other molecules. For example, activation of nuclear substrates is dependent upon ERK translocation, which occurs dependently on PKA [23, 56]. In a related way, the combinatorial inhibition of other molecules may be required for the activation of a given ERK substrate.
2) Control of temporal dynamics can influence substrate selection, including negative feedback for regulation. This would predict, for example, that induction of LTM via RTK via ERK can open a temporal window for activation of other pathways by other convergent inputs onto ERK as well. This in turn could predict non-intuitive interactions among different patterns of training. For example, RTK activation of ERK (resulting from LTM training) could in principle facilitate the induction of SS-ITM (see Fig. 2). This prediction requires that the ERK activation by an RTK occurs in the same spatial compartment as that accessed by SS-ITM, as well as in the same temporal window.
3) The node itself can be regulated. For example, if recurrent negative feedback is exerted onto the signaling path of the node, then the time window of that negative regulation may affect other forms of plasticity/memory that run through the node, allowing for possible unforeseen interactions between different types of learning. For example, as stated previously, p38 MAPK becomes activated in response to the inhibitory neuropeptide FMFRa [70]. P38 can activate CREB2, which has been demonstrated to be inhibitory to facilitation [74, 76]. Thus, substrate selection can be a highly dynamic and interactive process. These interactions are schematically illustrated in Figure 2, which is a further elaboration of the model depicted in Figure 1.

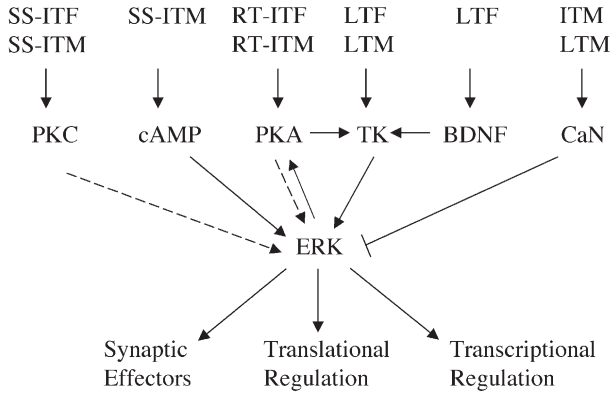

Figure 2. Summary of convergence and divergence through ERK in *Aplysia*. Convergence of inputs leads to a net activation state of ERK. The activation of ERK in turn results in a divergence of outputs onto a variety of targets, including synaptic effectors, and translational and transcriptional machinery. Inhibitory feedback onto ERK, for example by transcription and translation of ERK phosphatases, can influence the ERK activation state and thereby indirectly affect substrate selection.

Additional mechanisms of vertebrate ERK regulation and substrate selection

Extensive research on mammalian ERK pathways has illustrated additional mechanisms of activation, including the Ras > Raf > MEK pathway [78], as well as Epac > Rap1 > B-Raf > MEK [79–82], for example. Ye et al. (2005) recently reported the identification and cloning of Ras and Rap in *Aplysia* [83]. These molecules are each 76 and 86% identical to mammalian Ras and Rap1, respectively.

Most of mammalian research suggests that ERK regulates translation in an mTOR-independent manner, which is a prerequisite for any factor that regulates translation in the service of ITF and ITM. Moreover, recent work in the rat hippocampus has determined that ERK activation is crucial for maintaining L-LTP and spatial memory, which are both translationally sensitive. Kelleher et al. (2004) showed that ERK inhibition reduces the phosphorylation levels of eIF4E, 4EBP and ribosomal protein S6 [84]. Rapamycin also inhibited phosphorylation levels in these proteins, although with a greater effect on S6 compared with ERK inhibition. This then suggests at least a partial synchronization between mTOR and ERK regulation of the mammalian translational machinery. In *Aplysia*, however, it appears that ERK is not required for maintaining basal levels translation or eIF4E phosphorylation [85]. However, this observation by no means precludes a role

for ERK in ITF and ITM for several reasons. First, there are several eIF4E-independent routes by which ERK could tap into the translational machinery. Second, the above results do not rule out an ERK-eIF4E link, because the role of ERK in *stimulated* translation has not been investigated. Interestingly, Dyer et al. showed that the same 5-HT application, which is sufficient to increase global translation rates, does not produce a net increase in eIF4E-P levels (in fact there is a net reduction across all time points sampled) [85]. However, at one time point (45 min) eIF4E-P levels are significantly higher than the others. These studies raise several important questions. Is ERK activity required for the 45-min increase in eIF4E-P levels? Perhaps even more important, is ERK activity required for the 5-HT-induced increase in global translation rates?

Examples of inhibitory constraints on vertebrate ERK activation have been described as well. For example, a number of vertebrate ERK phosphatases have been described, the activity of some of which is regulated by ERK itself (reviewed in [86, 87]). Further, expression of MAPK phosphatases can be induced in response to stimulation leading to LTP, suggesting a role for these molecules in the regulation of synaptic plasticity [88, 89]. While these pathways have not been demonstrated in *Aplysia*, it is reasonable to hypothesize that some if not all of these pathways have been conserved and represent additional ways to achieve ERK activation in response to 5-HT. Certainly this is a line of research that will continue to develop over time.

Conclusions and future Directions

A major goal of research on molecular correlates of learning and memory is an understanding of how a cell can translate variable experiences into plasticity or memory with distinct cellular requirements and signatures. The idea of molecular networks with sites of interaction (i.e. molecular nodes) provides a way to consider how this translation may occur. The convergence of input at a node, the weighted engagement of the node and subsequent differential selection among an array of targets downstream of the node all provide criteria for consideration of sites of interaction between networks (see Fig. 2). Here we have explored ERK in *Aplysia* as an exemplar of such a node. The idea of molecular nodes can provide a strategy to simplify the study of intersections among signaling pathways.

One example is the intersection between the PKA and ERK pathways. Given that PKA and ERK activation are required for ITF and ITM, do these kinases interact in the service of facilitation and memory? One attractive model is that stimulated ERK maintains persistent PKA activity by activation of translation factors. This model integrates all the key findings. ERK is required for the induction but not the expression of ITF and ITM. In contrast, persistent PKA, which requires multiple pulses and the synthesis of new proteins, maintains the memory by continuous phosphorylation of cytoplasmic effectors, as well as nuclear DNA binding proteins such as CREB.

Clearly, both PKA and ERK are integral players in the formation of LTF and LTM; but what about their relationship? Evidence in *Aplysia* suggests that releasable factors may be involved [54, 57]. For instance, as mentioned previously, Hu et al. showed that a single pulse of 5-HT when combined with sensorin is capable of activating and translocating ERK to the nucleus [57]. Furthermore, the sequence of events is critical in order to produce ERK translocation and LTF induction. In this model, the initial pulses of 5-HT activate PKA, resulting in sensorin release and autocrine stimulation of tyrosine kinase cascades. The PKA activity must precede the tyrosine kinase activity, because PKA somehow 'primes' the tyrosine kinase pathway to produce ERK activation and translocation. In support of their molecular findings, Schacher and colleagues also demonstrate that LTF requires tyrosinse kinase receptor activity following 5-HT stimulation, but not before or during. This study suggests that ERK's function is critically regulated by PKA activity and highlights the importance of additional factors when using nodal analysis. Thus the manner in which PKA and ERK perform their unique functions is not a simple property of the activation state.

An additional aspect of this kind of analysis is that nodes can shift, both with respect to time and space. Under different patterns of training or with different forms of memory, one can easily imagine any of a variety of molecules we have discussed (e.g., PKA, PKC etc.) also serving as nodes. There are, of course, dozens of other molecules not discussed here that are equally good candidates for this type of function as well. Nodes are likely to be dynamic, varying both temporally (some may be active early in processing, others later), and in their spatial distribution within a neuron (different molecules certainly can act in distinct cellular compartments). Thus, a particular molecule may serve as a node in one context, but not another. We propose this notion not as a comprehensive review of all possible molecular interactions (a daunting if not impossible task), but rather as a strategy for identifying key points of intersection that can provide leverage in elucidating complex interactions in molecular networks during memory formation.

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