Transcriptional Regulation Underlying Long-term Sensitization in Aplysia

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1 Abstract

The induction of a long-term memory requires both transcriptional change and neural plasticity. Many of the links between transcription and memory have been revealed through the study of long-term sensitization in the *Aplysia* genus of marine mollusk*s*. Sensitization is an evolutionarily conserved non-associative form of pain memory in which a painful stimulus (e.g. a strong electrical shock) produces an increase in arousal and defensive behavior. *Aplysia* have proven useful for studying sensitization because it has been possible to trace the neural circuits that help encode sensitization memory and to simulate sensitization in neuronal cell cultures.

One notable feature of sensitization in *Aplysia* is that only some training protocols initiate transcription and produce long-term memory; others fail to activate transcription and produce only short-term memories. This occurs because the induction of long-term sensitization requires activation of two signal-transduction pathways that regulate transcription: 1) a fast but transient activation of the cAMP/PKA pathway that activates the transcription factor CREB1, and 2) delayed activation of the ERK isoform of MAPK that de-activates the transcriptional repressor CREB2. The effectiveness of different training protocols is based on the degree to which activation of these pathways is synchronized. The cAMP/PKA and MAPK pathways are complex, involving extracellular and trans-synaptic signaling, feedback loops, and cross-talk. It has proven possible, though, to model transcriptional activation with enough fidelity to generate *in silico* predictions for optimized learning that have been validated in cell culture and intact animals.

Training protocols that successfully activate CREB1 while de-activating CREB2 produce a complex transcriptional cascade that helps encode long-term sensitization memory. The transcriptional cascade involves a focused wave of immediate-early transcriptional activations. This includes activation of additional transcription factors, such as C/EBP, as well as effector genes like *uch*, *sensorin*, and *tolloid*. These early transcriptional changes close feedback loops that help extend and stabilize the early wave of transcriptional changes, triggering a much broader late wave of transcriptional changes that develops within 1 day of training. The late wave involves transcripts likely to alter neural signaling, increase protein production, transport mRNAs, and induce meta-plasticity. A small number of transcripts participate in both the early and late waves of transcriptional change, and several of these have been shown to play essential roles in completing the induction of long-term sensitization; this includes *creb1*, *syntaxin*, and *eIF4*. Most transcriptional changes fade as sensitization memory is forgotten, but some changes persist beyond forgetting, including a long-lasting up-regulation of an inhibitory peptide transmitter that could foster forgetting.

The maintenance of long-term sensitization may involve self-sustaining transcriptional feedback loops. In particular, CREB1 binds to its own promoter, producing a long-lasting increase in CREB1 mRNA, protein, and gene activation that is essential for sustaining cellular correlates of sensitization for at least 1 day after induction. Multiple puzzles about maintenance remain to be solved though, including how long transcriptional loops might play a role in maintenance, how they might interact with other maintenance mechanisms, and how transcriptional states relate to long-term sensitization memory during forgetting.

Many aspects of the induction, stabilization, and maintenance of sensitization memory in *Aplysia* are conserved, suggesting it will continue to be a fruitful simpler system for understanding the physical basis of lasting memory.

Preprint

This is a preprint of *"Transcriptional Regulation Underlying Long-term Sensitization in Aplysia"* by Robert J. Calin-Jageman, Theresa Wilsterman & Irina Calin-Jageman.

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2 Background: Sensitization Memory in *Aplysia*

2.1 Sensitization is a conserved and adaptive form of non-associative pain memory

Sensitization is a non-associative form of memory for painful experiences; it is expressed as a generalized increase in responsiveness that persists after exposure to a noxious stimulation (Thompson and Spencer 1966). Sensitization memory helps organisms that have experienced pain re-allocate their behavioral repertoire to avoid additional injury, aiding the chances of survival (Crook et al. 2014). Sensitization is observed across the animal kingdom (Abramson 1994) and has been hypothesized to represent basal memory mechanisms from which more complex forms of memory evolved (Robert D. Hawkins and Kandel 1984). Mechanistically, sensitization shares many characteristics with clinical dysregulation of pain processing, including allodynia and hyperalgesia (Edgar T. Walters et al. 2023).

2.2 Aplysia are marine mollusks especially suitable for exploring the neurobiology of memory

Aplysia is a genus of marine gastropod useful for linking behavioral and neural plasticity (Figure 2.1). Studies have primarily focused on *Aplysia californica*, which is found throughout much of the western coast of North America and *Aplysia kurodai,* which is found in South-East Asia. *Aplysia* have several advantages for linking behavior to neural function: 1) they have a relatively simple CNS consisting of about 10,000 neurons (Cash and Carew 1989), 2) the neural circuitry underlying several ethologically-relevant behaviors has been mapped at the single-cell level (L. J. Cleary, Byrne, and Frost 1995a), and 3) many *Aplysia* neurons are large, enabling repeated physiological measurements, single-cell manipulation of gene expression, and single-cell qPCR (Lovell and Moroz 2006).

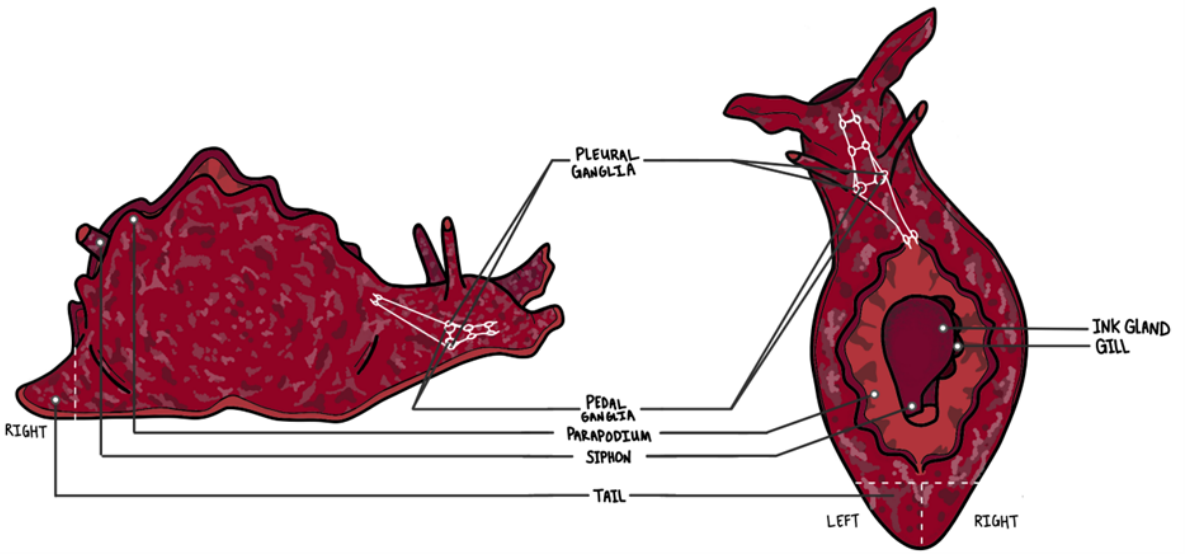

Figure 2.1: **Lateral and dorsal views of an Aplysia**. Studies of sensitization often examine defensive reflexes of the tail, the siphon (a respiratory structure), or the gill. The nervous system is internal, but this figure shows its general location, highlighting the pleural and pedal ganglia. The pleural ganglia contain many of the nociceptors that innervate the body; the pedal ganglia contain many motor neurons that contribute to defensive withdrawal. Reprinted with permission from Wilsterman, 2023.

2.3 Painful shocks increase the duration of defensive withdrawal reflexes in Aplysia, a form of sensitization memory

In *Aplysia,* sensitization memories can be induced by attack from their natural predators, including pinching from lobsters (Mason et al. 2014; Watkins et al. 2010) and strikes from the carnivorous gastropod *Navanax* (Pepino et al. 2022). For experimental control, though, sensitization training typically consists of the application of one or more strong electrical shocks (Pinsker et al. 1973a; Scholz and Byrne 1987a). The level of shock is calibrated to produce pain-related behaviors (withdrawal, inking, and escape locomotion) without causing notable tissue damage. This ensures that any behavioral changes observed after training are due to changes in the nervous system.

Sensitization training produces an altered behavioral state in *Aplysia:* they become more likely to exhibit escape locomotion (Stopfer and Carew 1988), heart rate becomes elevated (Krontiris-Litowitz 1999; Marinesco et al. 2004a), and food-seeking behavior is temporarily suppressed (Acheampong et al. 2012). The most studied behavioral phenotype, however, is the duration of defensive withdrawal reflexes (Carew, Castellucci, and Kandel 1971), behaviors in which an innocuous stimulus (light touch, weak electrical shock, mild water pressure) triggers a protective retraction of sensitive body parts. A number of different withdrawal reflexes have been studied, including the gill-withdrawal reflex, the tail-withdrawal reflex, and the siphon-withdrawal reflex (the siphon is a respiratory structure). While details vary somewhat, each of these defensive behaviors provides a clear and robust index of sensitization memory: after sensitization training a typical animal will maintain a withdrawal reflex 2-3 times as long as before training (Figure 2.2). The strength of sensitization memory can then be tracked over time, as measurement of defensive withdrawals can be made fairly frequently without notable fatigue or habituation. Sensitizing shocks can be applied to just one side of the body. This produces sensitization of tail-evoked reflexes only on the side of training (Scholz and Byrne 1987b), allowing within-subjects experiments comparing the trained and untrained sides of the nervous system.

2.4 Serotonergic neuromodulation is a key factor in the induction of sensitization in Aplysia

The development of the sensitized state in *Aplysia* depends on shock-induced serotonin release. Sensitization training produces prolonged activation of serotonergic fibers and increases serotonin concentrations at synapses involved in modulated behaviors (Marinesco et al. 2004b). Depletion of serotonin prevents the development of a sensitized state (Glanzman et al. 1989), and soaking animals in serotonin produces sensitization (Bonnick et al. 2012; Levenson et al. 2000). The close linkage between sensitization training and serotonin has enabled serotonin exposure to serve as a substitute for sensitization training not only in whole animals but also in cellular models of sensitization (see below).

Figure 2.2: **Sensitization in Aplysia**. In a typical sensitization experiment, defensive reflexes are measured before (top) and after (bottom) sensitization training (middle). In this example, baseline measures are made of the tail-elicited withdrawal reflex (top). To elicit the reflex, a light stimulus is applied to the tail. This produces a brief contraction of the tail, siphon, and gill. The duration of contraction is used as an index of reflex strength. After baseline measures, animals undergo sensitization training (middle). In this example, training consists of a single painful shock applied to one side of the body (lightning bolts). This produces inking and escape locomotion, consistent with the aversive nature of the stimulus. Post-tests (bottom) reveal an increase in the duration of the withdrawal reflex; the increase in reflex duration is used as an index of the sensitization memory. In this specific form of sensitization training, sensitization is only expressed on the side of the body that received training. Training protocols applied bilaterally or at the body midline produce bilateral sensitization. Reprinted with permission from Wilsterman, 2023.

2.5 Sensitization in Aplysia has short-, intermediate- and long-term phases that are distinct in mechanism and induction requirements

The seemingly unitary process of sensitization memory has been dissociated into different phases, each with distinct mechanisms operating at different time scales (Figure 2.3). Three different phases of sensitization have been well-characterized (Vincent F. Castellucci et al. 1989; Sutton et al. 2002; Sutton et al. 2004):

- Short-term sensitization, which develops rapidly after training but lasts less than 1 hour; this phase of memory does not require changes in translation or transcription
- Intermediate-term sensitization, which develops within 30 minutes of training and lasts less than 3 hours; this phase requires changes in translation but not transcription
- Long-term sensitization, which develops slowly ($>$ 6 hours) but can last for weeks; long-term sensitization requires changes in both translation and transcription

These phases of memory have been dissociated not only by their molecular requirements but also by their induction requirements. Very limited training (a single shock) induces only short-term sensitization that rapidly fades. Moderate levels of training (3 spaced shocks) produces short- and intermediate-term sensitization that last several hours. Extensive training (5 spaced shocks) induces short-, intermediate-, and long-term phases of sensitization, producing a sensitization memory expressed for several days. This different engagement of memory mechanisms depends not only on the quantity of training but also on the pattern: massed training (5 shocks delivered in immediate sequence) fails to produce long-term sensitization (Sutton et al. 2002; Wainwright et al. 2002), and intermittent spacing can produce especially robust long-term sensitization (G. Zhang et al. 2012a). One of the key questions addressed in the Induction section is why only some patterns of training induce long-term sensitization.

2.6 Long-term sensitization is encoded via long-term cellular, synaptic, and structural plasticity in the defensive withdrawal circuits of Aplysia

Long-term sensitization training produces a marked and long-lasting increase in the duration of defensive reflexes in *Aplysia* (Pinsker et al. 1973b). Careful work has traced the neural circuitry underlying these defensive behaviors and revealed that sensitization memory is expressed in these circuits through long-lasting changes in excitability, synaptic strength, and growth.

While details vary between the different defensive reflexes that have been studied (siphon withdrawal, tail withdrawal, gill withdrawal), each defensive reflex is mediated by a simple

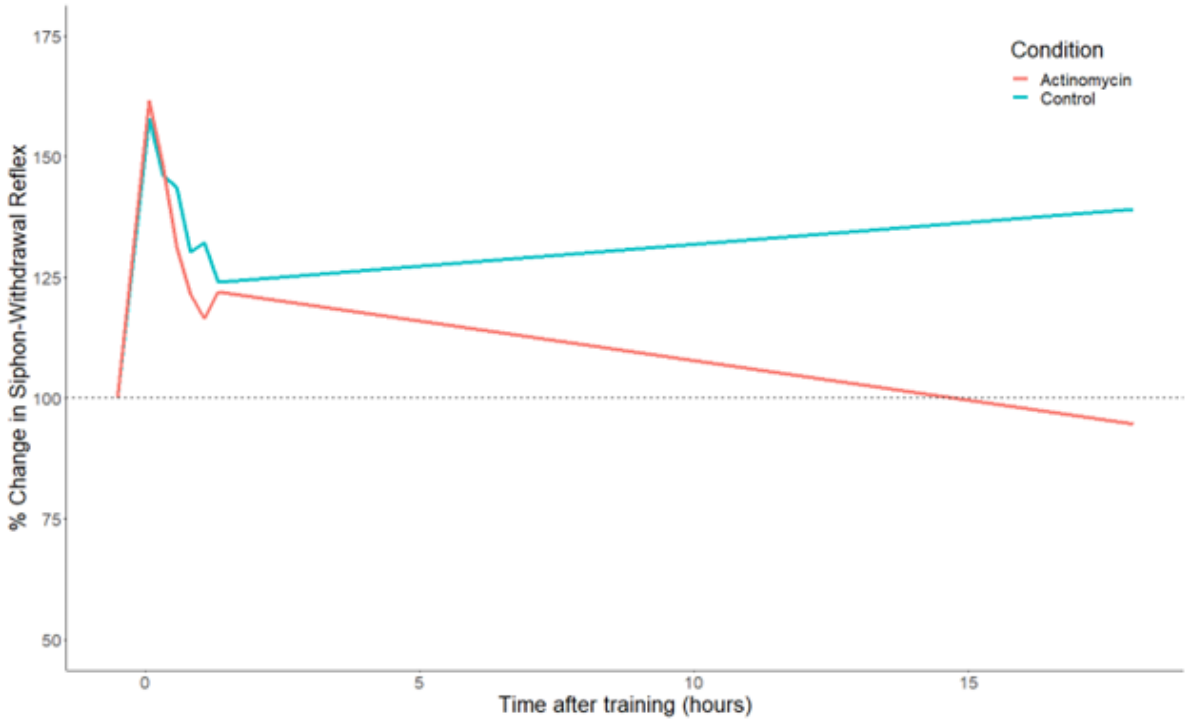

Figure 2.3: **Long-Term Sensitization Requires Changes in Transcription.** This figure shows the effect of inhibiting transcription on sensitization memory, revealing a long-term phase that requires changes in gene expression. Data are replotted from an experiment by Sutton et al. (2001), which tracked the duration of the siphon-withdrawal reflex before and after long-term sensitization training (5 painful shocks) in a reduced preparation consisting of the siphon, tail, and nervous system. The dotted line represents no change in withdrawal duration. Control preparations (n = 7 to 12 per group) showed an immediate increase in withdrawal duration that persisted for 18 hours. Treating the nervous system with the transcriptional inhibitor actinomycin during training did not affect the induction of sensitization memory, but prevented long-term retention, with withdrawal duration returning to baseline 18 hours after training. Treatment with the transcriptional inhibitor alone (without training) did not alter withdrawal durations (data not shown).

two-layer reflex circuit augmented by both polysynaptic pathways and neuromodulatory inputs (Figure 2.4) (L. J. Cleary, Byrne, and Frost 1995b). Input to defensive withdrawal circuits is carried by low-threshold mechanoreceptors with cell bodies in the peripheral nervous system (Calin-Jageman and Fischer 2007; L. Frost et al. 1997) and high-threshold nociceptors with cell bodies in the central nervous system that innervate the siphon (John H. Byrne, Castellucci, and Kandel 1974), gill (V. Castellucci, Pinsker, and Kupfermann 1970), and body wall (Walters et al. 2004; E. T. Walters et al. 1983). The sensory neurons form excitatory glutamatergic synapses onto siphon, tail, and gill motor neurons (Dale and Kandel 1993; Hening et al. 1979; Hickie and Walters 1995). This direct sensory-to-motor circuit is sufficient, on its own, to produce withdrawal behaviors (Antonov, Kandel, and Hawkins 1999), but motor neuron output is also sculpted by populations of interneurons that receive sensory inputs and which relay both excitation and inhibition on to reflex motor neurons (L. J. Cleary and Byrne 1993; R. D. Hawkins, Castellucci, and Kandel 1981). The reflex circuits in *Aplysia* are also regulated by modulatory inputs. This includes polysynaptic innervation from neurons releasing Phe-Met-Arg-Phe-NH_2 [FMRFa; Mackey et al. (1987)], an inhibitory neuromodulator, as well as diffuse serotonergic inputs (Marinesco et al. 2004c), which serve a key role in the induction of sensitization.

Long-term sensitization memory is encoded at multiple sites in the defensive reflex circuits of *Aplysia*, with notable long-term changes in the input, interneuron, and motor-neuron layers (L. J. Cleary, Lee, and Byrne 1998b; Trudeau and Castellucci 1995). Although encoding is diffuse, modification of the nociceptors at the input layer of the circuits represents a prominent site of storage. These nociceptors are strongly activated by the noxious shocks used for sensitization training [@walters1987]. This barrage of activity coupled with shock-induced serotonin release (Marinesco and Carew 2002) produces long-lasting physiological changes, including enhanced excitability (Scholz and Byrne 1987c), spike narrowing (Antzoulatos and Byrne 2007), and facilitation of synaptic contacts with motor neurons (W. N. Frost et al. 1985). With extensive training protocols, these physiological changes are accompanied by morphological changes, with the nociceptors sprouting new synapses and enlarging synaptic active zones (C. H. Bailey and Chen 1983; Craig H. Bailey and Chen 1989; Wainwright et al. 2002). The summative impact of these physiological and structural changes is the expression of long-term sensitization memory. Consistent with this, blocking transcription during training also prevents long-term changes in synaptic strength (Vincent F. Castellucci et al. 1986) and synaptic outgrowth (Craig H. Bailey et al. 1992).

2.7 Analogs of sensitization memory can be observed in whole ganglia and in cell culture

The study of sensitization in *Aplysia* has been greatly facilitated by the development of analogs that can be studied in whole ganglia (e.g. F. Zhang, Goldsmith, and Byrne 1994) and cell culture (e.g. Montarolo et al. 1986), providing opportunities for both manipulation and

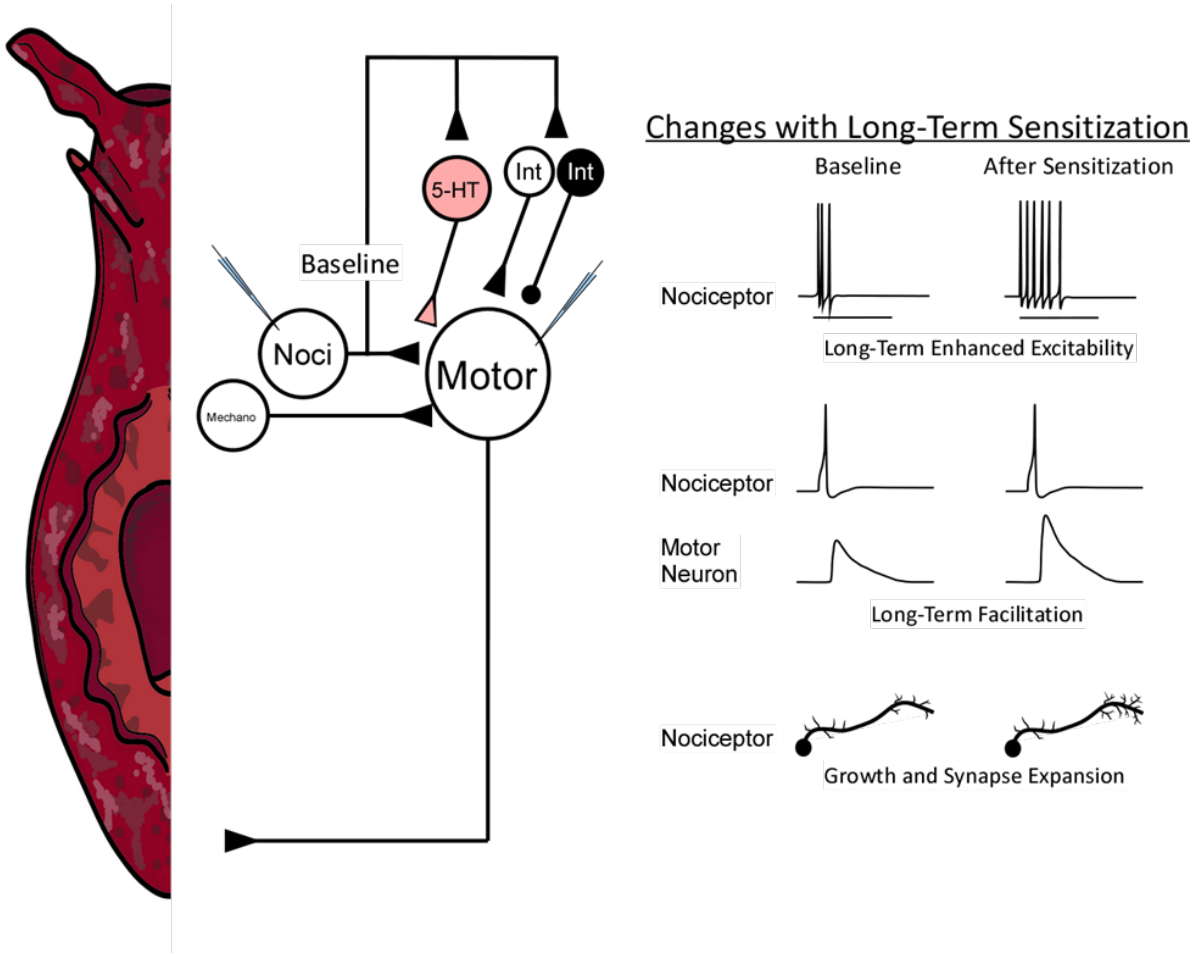

Figure 2.4: **Basic Structure of Defensive Withdrawal Circuits in Aplysia and Selected Changes Induced by Long-Term Sensitization.** At left is a schematic representation of shared features of defensive withdrawal reflex circuits in Aplysia. Sensory input is provided by mechanorecptors (M) with cell bodies in the periphery and several populations of nociceptors (N) with cell bodies in the central nervous system. Both populations of sensory neurons provide direct glutamatergic input to motor neurons (M), producing defensive withdrawal behavior. This circuit is regulated by neuromodulatory inputs, especially from serotonergic neurons (5-HT) and from a population of interneurons (Int) that provide both inhibitory and excitatory feedback. Physiological recordings (electrodes) show that long-term sensitization produces long-lasting changes throughout the defensive withdrawal circuits. Nociceptor populations form an especially prominent site of storage, and the right side of the figure shows 3 distinctive changes observed for at least 1 day after training: long-term enhancement of excitability (top), long-term facilitation (middle) and, with sufficient training, morphological changes (increases in arborization length and synaptic contacts as well as expansion of the active zones and vesicular content of existing synapses). All 3 of these long-term changes are also observed in the cellular model of sensitization in which training is simulated by repeated pulses of serotonin applied to cultures consisting of only nociceptors and their partner motor neurons. Blocking transcription during training prevents the long-term development of these physiological hallmarks of sensitization. The representative changes following sensitization are based on data from L. J. Cleary, Lee, and Byrne (1998a) and (**wainright2002?**).

measurement that would not be feasible when studying intact animals. For cell culture, components of the defensive withdrawal circuits can be reconstituted by co-culturing sensory nociceptors with gill or siphon motor neurons, reproducing the direct glutamatergic pathway that strongly contributes to both normal withdrawal behavior and the encoding of sensitization memory.

In cellular studies, serotonin exposure is used to simulate sensitization training. In both whole ganglia and in culture, serotonin produces the same distinctive patterns of plasticity in *Aplysia* nociceptors that help encode sensitization memory in intact animals, including enhanced excitability of the nociceptor sensory neuron and both short- and long-term synaptic facilitation of this sensory neuron-motor neuron synapse (R.-Y. Liu et al. 2011a; Montarolo et al. 1986). Moreover, cellular models precisely recapitulate the phases of sensitization observed in intact animals: a single pulse of serotonin produces a rapid but transient increase in nociceptor excitability and synaptic strength that is unaffected by treatments that prevent transcription; 5 spaced pulses of serotonin produces a long-term facilitation of synaptic strength and the sprouting of new synapses, and these long-term effects depend on changes in neuronal transcription (Montarolo et al. 1986). Even more striking, cellular models mirror the impact of pattern of training, with massed exposure of serotonin (a single prolonged exposure) ineffective at producing long-term changes (Mauelshagen, Sherff, and Carew 1998), and some intermittently-spaced protocols producing especially strong changes (G. Zhang et al. 2012b).

3 Induction: Why do only some experiences activate transcription to encode long-term sensitization memories?

Limited sensitization training (a single shock) produces only transient neural plasticity and a sensitization memory that rapidly decays. More extensive training (5 shocks) activates changes in neuronal transcription, produces long-lasting neural plasticity, and encodes a long-term sensitization memory. What mechanisms link long-term sensitization training to the activation of transcription, and how do these mechanisms discriminate between different training protocols?

3.1 Long-Term Sensitization Induction Depends on Synchronized Activation of Two Nuclear Signaling Pathways

Intensive study of the cellular models of sensitization has helped provide detailed answers to these questions. The consensus model that has emerged is that long-term sensitization requires activation of two inter-related signal-transduction pathways that regulate transcription, and that the effectiveness of different training protocols is based on the degree to which activation of these pathways is synchronized. Figure 5 provides an overview of this synchronization-based model of the induction of long-term sensitization:

- Noxious shock activates serotonergic neurons. The released serotonin activates metabotropic receptors expressed by *Aplysia* nociceptors, producing a complex local signal-transduction cascade that rapidly but transiently induces the physiological changes that help express short-term sensitization. This includes activation of calmodulin-dependent protein kinase (CamKII), protein kinase C (PKC), and the cyclic adensosine-3-monophasphate (cAMP) to protein kinase A (PKA) pathway (John H. Byrne and Robert 2015).
- The impact of serotonergic modulation is not only local, but also activates signals that can translocate to the nucleus to regulate gene expression. These signaling pathways are complex, but include:

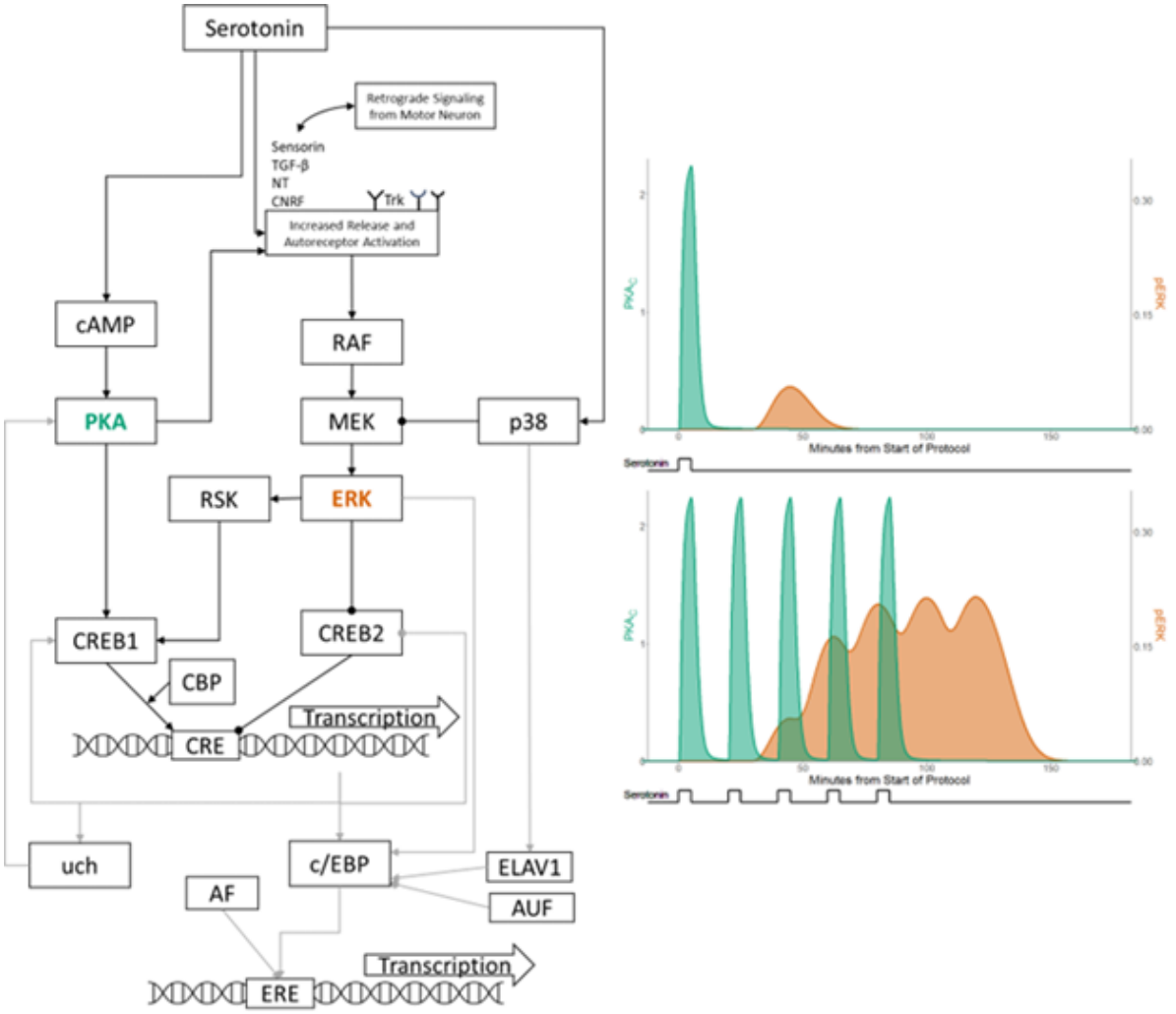

Figure 3.1: **Figure 5 – Simplified Model of the Induction and Stabilization of Long-Term Sensitization**. In this "synchronization based" model, serotonin activates two entangled signaling pathways that regulate transcription, and the degree to which training synchronizes activation of these pathways determines if long-term sensitization is induced and stabilized. The "fast" pathway is serotonergic production of cyclic adenosine 3 monophosphate (cAMP), which promotes the separation of the regulatory and catalytic subunits of protein kinase A (PKA). The catalytic subunit of PKA (green) then can translocate to the nucleus to phosphorylate cAMP response-element binding protien (CREB1), promoting transcription of genes with CRE elements in their promoters through a partnership with creb-binding protein (CBP). The "slow" pathway involves serotonergic increases in neurosecretion of several signaling molecules, activating autoreceptors that promote phophorolytic activation of RAF, MEK, and then ERK. Phosphorylated ERK (red) can then translocate to the nucleus where it phosphorylates CREB2, relieving its constitutive repression of transcription. Activation of the slow pathway is sharply limited by parallel activation of p38, which works against the continued activation of ERK. Successful encoding of long-term training requires PKA activation of CREB1 at the same time that ERK is alleviating repression from CREB2. Thus, a single pulse of serotonin (top right) fails, because ERK activation develops after PKA activation has faded. Spaced training (bottom right) produces overlapping activation of PKA and ERK. This co-activation activates feedback loops that help extend and stabilize the transcriptional response (pathways marked in gray): 1) Rapid production of UCH helps break down the regulatory subunit of PKA, extending its activation, 2) the expression of c/EBP is stabilized by P38 phosphorylation of

  - Activation of the cAMP/PKA pathway, producing a rapid but transient translocation of the catalytic subunits of PKA to the nucleus (Bacskai et al. 1993; Müller and Carew 1998; Y. Zhang et al. 2021)
  - Activation of MEK (MAPK/ERK kinase), which phosphorylates the extracellular signal-related kinase (ERK) isoform of mitogen-activated protein kinase (MAPK); this produces a delayed translocation of ERK to the nucleus (Philips et al. 2013; Y. Zhang et al. 2021)

- The nuclear signals initiated by serotonin exposure converge on the phosphorylation of two key transcription factors: *Aplysia* homologs of the transcriptional activator cAMP response element binding protein [CREB1; Bartsch et al. (1998)] and the transcriptional repressor CREB2 (Bartsch et al. 1995). Both of these basic leucine-zipper transcription factors bind to cAMP-response elements in the promoters of diverse genes but have opposing effects on the expression of their target genes: CREB1 shows low basal activity, and its phosphorylation enhances expression of its target genes; CREB2 is constitutively active as a repressor, and its phosphorylation relieves this repression.
- Long-term impacts only occur with training protocols that activate the cAMP/PKA pathway around the same time that ERK is activated (Philips et al. 2013; Y. Zhang et al. 2021). This stabilizes activation of these pathways, producing sufficient CREB1 activation and CREB2 de-activation to produce an immediate-early wave of transcriptional change. This immediate-early wave of transcription increases the expression of effector genes and recruits additional transcriptional factors, including an *Aplysia* homolog of CCAAT Enhancer Binding Protein [c/EBP; Alberini et al. (1994)] and *Aplysia* Activating Factor [ApAF; Bartsch et al. (2000)], producing a complex transcriptional cascade.
- This transcriptional cascade organizes the expression of the lasting physiological changes that help encode long-term sensitization memory in *Aplysia* nociceptors. This includes global changes, such as long-term enhancement of excitability, as well as selective modification of synaptic properties through signaling cascades that enable recently-activated synapses to capture transported mRNAs for localized protein expression (Casadio et al. 1999; Martin et al. 1997).

According to this model, even a single learning event (shock or serotonin exposure) sets in motion all the mechanisms required to form a long-term memory (including PKA and ERK activation), but does so sequentially, and thus cannot, on its own, unlock the transcriptional changes required for long-term memory. Successful learning thus requires multiple rounds of training timed in a way that produces activation of PKA and ERK at the same time. Although much remains to be discovered, this synchronization model of long-term sensitization provides a biochemical basis for the apparently universal superiority of spaced training over massed training and has proven to have remarkable predictive powers. Given that the transcription factors that play a central role in this model, CREB1 and CREB2, are implicated in long-term memory formation across the animal kingdom (Kandel 2012; Silva et al. 1998), this

detailed understanding of the requirements for long-term sensitization in *Aplysia* may have broad implications for understanding learning in general. In the remainder of this section, the key components of this model are described, along with selective review of supporting evidence.

3.2 Serotonin produces rapid but transient activation of PKA that is stabilized and extended by repeated exposure

Intensive study has shown that the pathways linking serotonergic signaling to the phosphorylation of CREB1 and CREB2 are remarkably complex. What initially seemed like two parallel pathways (PKA -> CREB1 and ERK -> CREB2) has been shown to involve entangled signal-transduction pathways with complex feedback loops and potential functional redundancies.

The "fast" cAMP/PKA pathway is also the most straightforward. Serotonin activates metabotropic receptors positively coupled to the activation of adenylyl cyclase (Cohen et al. 2003; Y.-S. Lee et al. 2009), leading to an increase in the production of cAMP (Bernier et al. 1982). cAMP promotes the separation of the regulatory and catalytic subunits of PKA (Bacskai et al. 1993). Once translocated to the nucleus (Bacskai et al. 1993) the catalytic subunits of PKA phosphorylate CREB1 (Dash and Moore 1996), helping to trigger the transcriptional changes that help encode a long-term sensitization memory. The critical involvement of the cAMP/PKA pathway in long-term sensitization is well established: The long-term effects of serotonin are mimicked by treatment with cAMP (Nazif, Byrne, and Cleary 1991; Schacher, Castellucci, and Kandel 1988; Scholz and Byrne 1988) or the catalytic subunits of PKA (Chain et al. 1999a).

Studies comparing the effects of short- and long-term protocols show that activation of PKA is rapid, with an increase in free catalytic PKA evident almost immediately after serotonin exposure (Müller and Carew 1998; Y. Zhang et al. 2021). With short-term protocols (a single pulse of serotonin), activation of PKA is transient, fading within 15 minutes. Repeated serotonin exposure produces not only repeated PKA activation but also stabilization, extending PKA action to more than 1 hour after induction.

3.3 Serotonin produces a complex and delayed activation of ERK that is terminated by P38 MAPK activity and stabilized by repeated exposure

In addition to activating the cAMP/PKA pathway, serotonin promotes phosphorylation of ERK (Michael et al. 1998), activating its ability to translocate to the nucleus to regulate transcription (Martin et al. 1997). In contrast to the cAMP/PKA pathway, activation of

ERK is highly complex and "slow", features that are probably related. In terms of time-course, a single pulse of serotonin produces ERK phosphorylation that does not develop until 45 minutes after exposure (Philips et al. 2013). This activation then fades very rapidly, by 60 minutes after exposure. This very tight window of activation is produced by parallel activation of the competitive P38 isoform of MAPK (Y. Zhang et al. 2017). With long-term protocols, ERK activation is stabilized and extended for at least 3 hours after training (Sharma et al. 2003; Ye, Marina, and Carew 2012). The activation of ERK has been clearly established as a requirement for long-term sensitization. For example, the long-term effects of serotonin are blocked by manipulations that block ERK activation [Martin et al. (1997); @shobe2016].

The delayed activation of ERK is probably related to the fact that its induction involves complex signaling cascades that integrate extracellular and retrograde signaling. Serotonin stimulates secretion of several different signaling molecules from *Aplysia* nociceptors, including *Aplysia* homologs of neurotrophin [ApNT; Kassabov et al. (2013)], a cysteine-rich neurotrophic factor)(ApCNRF; (Pu et al. 2014)), transforming growth factor [TGF- ; Q. R. Liu et al. (1997a)], and the peptide neurotransmitter sensorin (Hu et al. 2004). These changes in neurosecretion activate autoreceptors, including tyrosine receptor kinases (Trk receptors). This autoreceptor activation is key for then activating the "classic" pathway for ERK activation (RAF -> MEK -> ERK). Thus, serotonergic activation of ERK requires a cascade of extracellular and intracellular events. Blocking TGF- signaling, TrkB-receptor activation, or sensorin secretion during training blocks both ERK activation and long-term plasticity (Hu et al. 2004; Kassabov et al. 2013; Kopec et al. 2015). Adding even more complexity, these neurosecretory signaling pathways are influenced by retrograde signals from synaptic partners, as both long-term facilitation and increased sensorin expression in *Aplysia* nociceptors depends on protein synthesis and calcium signaling in post-synaptic motor neurons (Cai, Chen, and Glanzman 2008). The way serotonin initially triggers neurosecretory signaling is not entirely clear, but likely involves PKA, as blocking serotonergic activation of PKA blocks serotonin-induced ERK phosphorylation (Y. Zhang et al. 2021).

The effects of ERK signaling are also complex, producing phosphorylation of not only CREB2 but also CREB1. The involvement of ERK in CREB2 phosphorylation was discovered first, with a notable increase in phosphorylation produced by co-incubation of *Aplysia* CREB2 with an activated form of ERK (Michael et al. 1998). It has been shown that phosphorylation of ERK is accompanied by activation of the P90 ribosomal S6 kinase (RSK), which then promotes the phosphorylation of CREB1 (R.-Y. Liu et al. 2020). Blocking RSK diminishes CREB1 phosphorylation and the long-term effects of serotonin, suggesting that the ERK pathway makes an important but not necessarily dominant contribution to the activation of CREB1.

3.4 Synchronization of PKA and ERK activation is critical for the induction of long-term sensitization.

Research comparing short- and long-term training protocols suggests that the key factor for producing long-term sensitization is the degree of synchronization between serotonin-induced PKA and ERK activation. This hypothesis was developed, in part, by the observation in intact animals that long-term sensitization could be produced by just 2 shocks spaced 45 minutes apart, but not by slightly shorter (15 minutes) or longer (60 minutes) training intervals (Philips, Tzvetkova, and Carew 2007). This narrow temporal window was found to be reflected in the delayed but transient phosphorylation of ERK produced by the first training stimulus. This led to hypothesis that the two-shock protocol succeeds in producing long-term sensitization only if the second shock is timed to re-activate PKA in the narrow time window of ERK activation from the first shock. Consistent with this hypothesis, preventing the first shock from producing a late-developing activation of ERK prevented a second shock from producing long-term sensitization (Philips et al. 2013). These findings strongly suggest that the key switch from short- to long-term sensitization is training that synchronizes the nuclear signaling induced by serotonin, producing PKA activation around the same time as ERK activation.

The synchronization model of the induction of long-term sensitization has also been validated by computationally modeling parts of the PKA and ERK pathways and then testing predictions derived from these models. In one study, an abstract model of the PKA and ERK pathways was developed and fit to empirical measurements of PKA and ERK activation from single pulses of serotonin (Y. Zhang et al. 2012). The model was then used to explore a broad parameter space of possible stimulation protocols. This exploration identified an idiosyncratic training protocol (5 pulses with inter-stimulus intervals of of 10, 10, 5, and 30 minutes) predicted to produce more temporal overlap of PKA and ERK activation than the standard long-term training protocol (5 pulses of serotonin at even 20-minute intervals). Remarkably, real-world testing of this idiosyncratic protocol in the cellular model of sensitization produced stronger phosphorylation of CREB1 and longer-lasting synaptic facilitation at *Aplysia* nociceptors; in intact animals shocks applied at the same uneven intervals produced longer-lasting long-term sensitization of the siphon withdrawal reflex.

Subsequent computational studies have incorporated more of the complex signaling pathways involved in serotonergic activation of PKA and ERK and have continued to generate novel *in silico* predictions that have then been validated *in vivo*. In one study (R.-Y. Liu et al. 2013b), the model was extended to represent PKA- and ERK-based phosphorylation of CREB1 and CREB2, and their interaction with CREB-binding protein (CBP) to regulate expression of the immediate-early gene c/EBP. The extended model correctly mimicked the *in vivo* finding that knocking down CBP expression limits the effectiveness of the standard long-term protocol, producing lower levels of long-term facilitation than under control conditions. The model was then used to search for training protocols that could overcome the reduction of CBP, identifying a predicted rescue protocol with intermittent timing (5 pulses at intervals of 10, 10, 20, and 10 minutes). This *in silico* prediction was confirmed in culture, with the rescue protocol restoring

the expression of long-term sensitization during CBP knock down. Similarly, the impact of reducing CREB1 expression has been modelled and successfully rescued via a computationally-derived intervention (Zhou et al. 2015).

Additional elaborations of the computational model of long-term sensitization include incorporation of serotonin-induced activation of a P38 isoform of MAPK that reduces ERK activation (Y. Zhang et al. 2017) and the addition of both PKA and ERK-mediated RSK activation (Y. Zhang et al. 2021). At this point, the model incorporates many of the complex signaling pathways known to regulate PKA and ERK activation. It has generated multiple validated predictions, offered new insight into the failure of massed training to produce long-term memory, and provided a tool for dissecting the role of different feedback pathways in stabilizing and extending the induction of long-term sensitization. While much remains to be learned, it seems clear that the temporal dynamics by which serotonin activates nuclear signaling plays a key role in determining which experiences succeed in activating transcription to generate long-term memories.

3.5 Activation of CREB1 and de-activation of CREB2 initiate an immediate-early transcriptional response that includes up-regulation of C/EBP

Synchronized PKA and ERK activity converge on the regulation of the transcription factors CREB1 and CREB2. Together, these transcription factors serve as a two-factor key for unlocking neuronal transcription to induce lasting plasticity in *Aplysia* nociceptors. Successful long-term training protocols achieve sufficient activation of the ERK to relieve the constitutive repression of CREB2 while also activating the cAMP/PKA pathway and RSK to phosphorylate CREB1; this combined action produces an immediate-early transcriptional response, a designation for transcriptional changes triggered rapidly by constitutively expressed transcription factors without the need for intervening protein synthesis.

The immediate-early wave of transcription increases the expression of multiple proteins (Kuhl et al. 1992a). One of the clearest readouts is a dramatic up-regulation of C/EBP, a transcription factor which targets genes with an CCAAT enhancer-response element (Alberini et al. 1994). C/EBP is not constitutively expressed in *Aplysia* nociceptors, but is induced rapidly (within 15 minutes) but transiently by serotonin exposure in cell culture and by noxious shock in intact animals (Lyons et al. 2006). This increase in expression requires translocation of CREB-binding protein (CBP) to the nucleus, where it binds with CREB1 to the promoter of the *c/ebp* gene, displacing constitutive repression by CREB2 (Guan et al. 2002). Serotonergic activation of C/EBP requires histone acetylation at the *c/ebp* promoter (Guan et al. 2002) and can be fostered by the nucleolar protein known as activity-induced activation LAPS18-like protein (ApLLP' Kim et al. 2003, 2006).

The dual roles of CREB1 and CREB2 in initiating transcription are very well-established. Long-term protocols produce phosphorylation of CREB1 (Bartsch et al. 1998; Chain et al. 1999a) and CREB2 (Bartsch et al. 1995), displace CREB2 binding from the promoters of genes it represses while increasing CREB1 binding (Mohamed et al. 2005), and increases the expression of CREB1's target genes (Kaang, Kandel, and Grant 1993). Manipulations that disrupt CREB1 function completely block serotonin from producing long-term increases in synaptic strength and excitability in *Aplysia* nociceptors (Bartsch et al. 1998; R.-Y. Liu et al. 2011b) while direct injection of phosphorylated CREB1 into *Aplysia* nociceptors mimics the effects of repeated serotonin exposure (Bartsch et al. 2000). The repressive influence of CREB2 is highlighted by studies which have disrupted CREB2 function; this makes it easier to induce long-term plasticity, enabling short-term training protocols (a single pulse of serotonin) to produce lasting effects (Bartsch et al. 1995; J. a. Lee et al. 2001).

4 Encoding: What are the transcriptional changes induced by long-term sensitization training?

Successful long-term sensitization training protocols activate CREB1 and C/EBP while deactivating CREB2, triggering a transcriptional response that produces the cellular and synaptic changes required to encode a long-term sensitization memory. What, exactly, is this transcriptional response and how is it organized?

A framework for answering this question was developed from tracking not transcription but translation. Specifically, measurement of the rate of protein production in *Aplysia* nociceptors showed that serotonin produces two waves of increased translation: a small early wave evident within 1 hour that rapidly fades, and a larger late wave that arises more than 3 hours after the end of induction (Barzilai et al. 1989). The early wave is part of the immediate-early response, as it persists through treatments that block transcription. Subsequent studies have found that transcriptional changes are also organized into early and late waves, identifying transcripts that show rapid but transient changes in expression (Zwartjes et al. 1998), transcripts that show delayed changes in expression (Kuhl et al. 1992b), and some that bridge these phases of regulation (Hart et al. 2011; R.-Y. Liu et al. 2011c).

The early and late waves have been further characterized via microarray studies conducted at different time points after long-term sensitization training (Figure 6). These studies estimate that the early wave is focused, involving only about 50 transcripts (Herdegen, Holmes, et al. 2014), that the late wave is complex, involving over 1,000 transcripts, and that very few (about 25) transcripts participate in both waves (Conte et al. 2017). As sensitization memory is forgotten, the late wave also decays, but a handful of transcripts have been identified that remain regulated even beyond forgetting (Perez et al. 2018).

This section summarizes what is known about the transcriptional changes forming the early and late waves of regulation after the induction of long-term sensitization and its *in vitro* analogs. Special focus is given to two interesting classes of transcriptional change: those that participate in both early and late waves of regulation, and those that persist even after sensitization is forgotten.

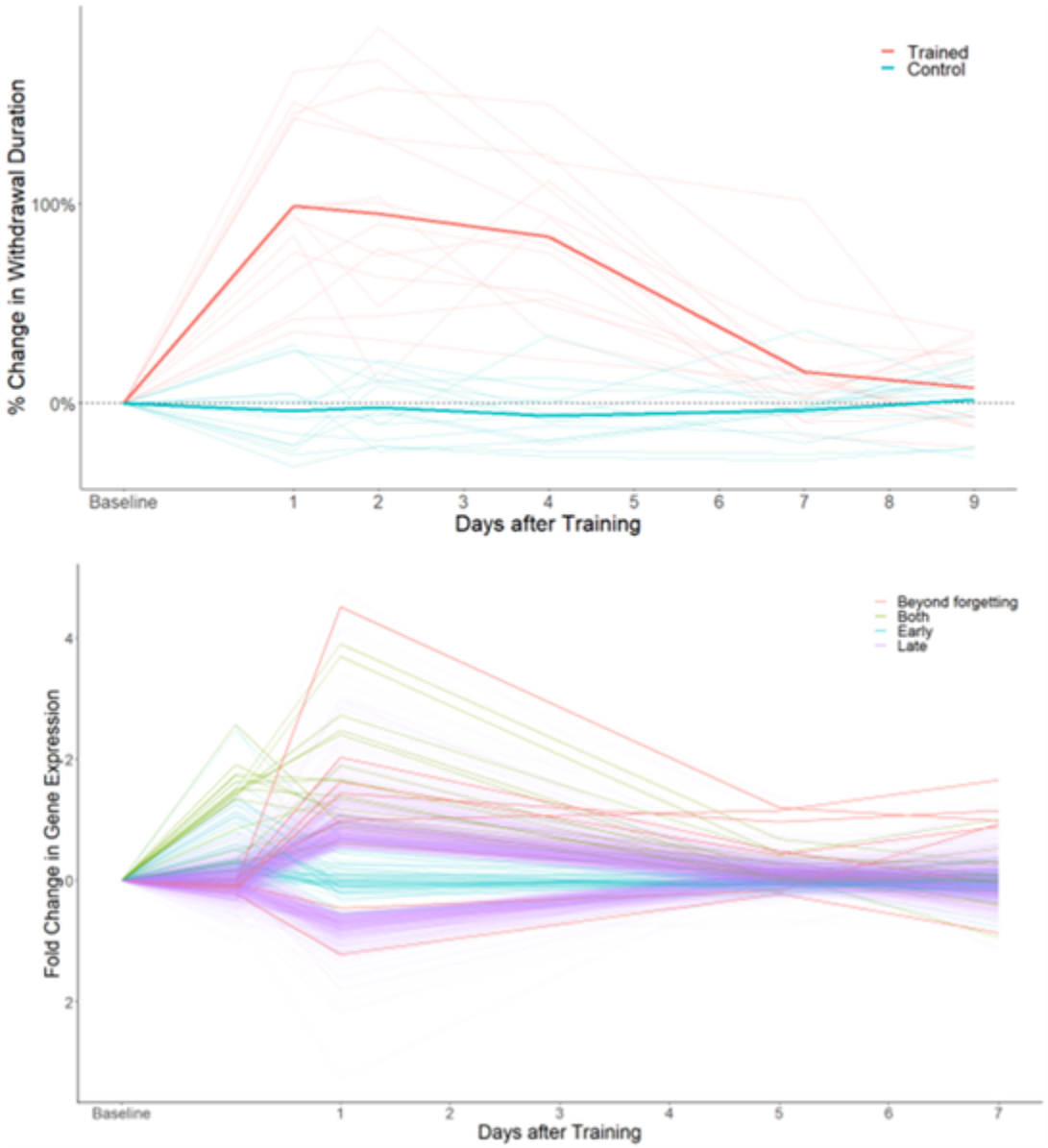

Figure 4.1: **Figure 6 – Time Course of Behavioral and Transcriptional Changes Following Long-Term Sensitization.** At top is the time-course of sensitization memory, showing both mean (solid lines) and individual (transparent lines) changes in defensive withdrawal durations on the trained (red) and untrained (sides) of Aplysia after long-term sensitization training (4 shocks). Sensitization memory is strongly expressed at 1 and 5 days after training, but fades back to baseline within 9 days. At bottom are changes in gene expression measured from microarray at 1 hour, 1 day, 5 days, and 7 days after the same long-term training protocol. Color-coding helps reveal the distinct patterns of regulation: There is an early-wave of transcripts up-regulated at 1 hour that then decays (blue), a large delayed wave of transcripts that show delayed up- or down-regulation at 1 day after training (purple with high transparency due to the large number of transcripts in this category), and a few transcripts that participate in both waves (green). Notably, most transcriptional changes resolve within 5 days of training, a time-point at which the expression of sensitization memory is still strong. A few transcripts, though, remain regulated even beyond forgetting (red).

4.1 The early wave of transcriptional change includes up-regulation of transcription factors, effector genes, and specific isoforms of CAM

Long-term sensitization training produces an immediate-early response that includes up-regulation of c/EBP. Successful training stabilizes and extends this rapid phase of transcriptional change, but it remains transient, with c/EBP mRNA levels returning to baseline within 5 hours of induction. The early wave of transcription and translation detected after sensitization training consists of this immediate-early response and its subsequent stabilization and elaboration.

In addition to *c/EBP*, several specific transcripts in the early wave have been identified. Some are transcription factors, with sensitization training producing rapid increases in the expression of transcripts that encode CREB1 (Bartsch et al. 1998) and a putative *Aplysia* homolog of C/EBP [aka Ig/EBP; Herdegen, Conte, et al. (2014)]. In addition, several effector genes are regulated in the early wave of transcription. This includes transcripts encoding *Aplysia* homologs of ubiquitin hydrolase [uch; Chain et al. (1999b); Hegde et al. (1997)], calmodulin (Zwartjes et al. 1998), the peptide neurotransmitter sensorin (Sun, Wu, and Schacher 2001), and an *Aplysia* Tolloid/BMP-1-like protein (Q. R. Liu et al. 1997b).

There are probably additional transcriptional changes in the early wave, with a microarray analysis identifying over 50 additional transcripts showing rapid but transient regulation after sensitization training (Herdegen, Holmes, et al. 2014). Most of these additional transcripts have not yet been mapped to well-annotated gene models. In addition, a microarray analysis conducted immediately after *in vivo* serotonin exposure in *Aplysia kurodai* identified regulation of 4 novel transcripts that were validated via qPCR: up-regulation of transcripts predicted to encode homologs of matrilin, antistasin, and eukaryotic translation initiation factor 3 (EIF3) and down-regulation of a BAT1 (Lee et al. 2008). Time-courses were not evaluated, but EIF3 is likely one of the rare transcripts regulated both rapidly and persistently; it is discussed in (**encoding_both_waves?**).

One intriguing aspect of the early wave of transcription is that it can also produce changes in the expression of specific isoforms of the same gene. Specifically, work in cell culture shows that serotonin treatment shifts the expression of different isoforms of mRNA for an *Aplysia* homolog of NCAM immunoglobulin-related cell adhesion molecule (ApCAM), changing the balance of isoform expression in *Aplysia* nociceptors and motor neurons without changing overall mRNA levels across all ApCAM isoforms (Schacher et al., 2000). These changes in isoform expression require trans-synaptic interactions, as they only occur when sensory and motor neurons are co-cultured together. Changes in both overall and synaptic ApCAM expression play critical roles in producing *in vitro* long-term sensitization, contributing to both transcription factor activation (Lee et al., 2007) and synapse formation (Zhu et al., 1995). The changes in ApCAM isoform expression produced by serotonin suggests that the trans-isoform measurement of gene expression that has so far been common in *Aplysia* research may miss

important forms of transcriptional regulation, pointing to an important priority for future research.

4.2 The early wave of transcriptional response produces feedback loops that help stabilize and extend induction mechanisms

The immediate-early transcriptional response is initially fragile; it is extended and stabilized by complex interactions triggered by long-term training protocols. This can be observed in the dynamics of the key immediate-early expression of c/EBP. Even a short-term protocol (a single shock) is sufficient to produce a rapid increase in c/EBP transcription, but this fades in less than 1 hour, producing no lasting effects (Kopec et al., 2015). With repeated shock, c/EBP becomes upregulated for several hours (Yamamoto et al., 1999). Treatments that prevent the stabilization of c/EBP expression impair the induction of long-term plasticity (Lee et al., 2012; Mirisis et al., 2021; Yim et al., 2006); treatments that extend its activation enable short-term protocols to produce lasting effects (Kim et al., 2006; Lee et al., 2001).

Stabilization of the immediate-early response occurs, at least in part, through regulation of c/EBP activity and stability by MAPK isoforms. First, c/EBP is phosphorylated by ERK, and this enables the binding of C/EBP to ERE motifs in its target genes (Yamamoto et al., 1999). In addition, the p38 isoform of MAPK phosphorylates an *Aplysia* homolog of the RNA-binding protein embryonic lethal visual system (ELAV), protecting c/EBP mRNA from rapid degradation (Mirisis et al., 2021; Yim et al., 2006) that would otherwise occur from ARE/poly(U)-binding/degradation factor (AUF1; Lee et al., 2012). These interactions integrate the extracellular signaling pathways contributing to ERK activation into the regulation of c/EBP: TrkB-receptor-activation is required for the initial increase in c/EBP transcription and NGF-B activation of P38 is required for the stabilization and extension of c/EBP transcription with long-term protocols (Kopec et al., 2015; Mirisis et al., 2021).

Stabilization of the early wave of transcription is also mediated by feedback between expressed genes and induction mechanisms. For example, the rapid increase in the expression of uch degrades the regulatory subunit of PKA, extending the time during which the catalytic domain is active (Hegde et al., 1997). In addition, CREB1's promotion of its own expression extends CREB1 activity (Mohamed et al., 2005); calmodulin mediates serotonergic activation of adenylyl cyclase (Lin et al., 2010); sensorin secretion activates autoreceptors that help activate ERK (Hu et al., 2004), and tolloid regulates TGF- signaling that is also important for ERK activation (Chin et al., 1999). Functional studies have confirmed that importance of these feedback loops, showing that the long-term persistence of serotonin-induced plasticity depends on upregulation of CREB1 (Liu et al., 2011) and uch (Hegde et al., 1997) as well as signaling from TGF- , which is activated by tolloid (Chin et al., 1999; Zhang et al., 1997). For sensorin, serotonin produces not only a rapid but transient increase in its mRNA expression, but also export of sensorin mRNA to the neurites of nociceptors co-cultured with motor neurons, producing in increase in sensorin secretion that is essential for strengthening ERK activation

and enabling serotonin to produce long-lasting plasticity (Hu et al., 2006). Some negative-feedback may also possible, as C/EBP lacks a transcriptional activation domain and in other animals has been shown to act as a negative inhibitor C/EBP function (Cooper et al., 1995); functional studies have not yet investigated this possibility.

4.3 The late wave is complex and involves changes potentially related to neural plasticity, protein production, mRNA transport, and meta-plasticity

Early proteomic screens showed that sensitization training and its *in vitro* analogs produce not only rapid changes in translation but also late-developing regulation, with several changes in expression becoming detectable only several hours after induction (Barzilai et al., 1989; Castellucci et al., 1988). Intense work subsequently identified two of these late-regulated genes: *Aplysia* homologs of the calreticulin (Kennedy et al., 1992) and binding immunoglobin protein (BiP; Kuhl et al., 1992), proteins involved in protein production and trafficking in the endoplasmic reticulum.

The late phase of transcription may be surprisingly complex, with microarray identifying 1,172 transcripts that show clear regulation that does not develop until 1 day after long-term sensitization training (Conte et al., 2017). In contrast to the early wave consisting almost exclusively of up-regulation, late-regulated transcripts were both up-regulated (722, 62%) and down-regulated (450, 38%). Given that the current draft of the *Aplysia* genome contains around 19,000 protein-coding gene models, the formation of a long-term sensitization memory is thus accompanied by regulation of an appreciable fraction of the genome. One caution to this conclusion is that proteomic screens have not uncovered the same degree of complexity, identifying just over 30 proteins showing clear changes in expression 1 or 2 days after treatment with serotonin (Monje et al., 2012, 2013). The reason for this discrepancy is not yet clear. It could be that not all transcriptional changes lead to translational changes, or it may be that changes in protein expression may be too subtle to easily detect, perhaps due to localization via transport and synapse-specific translation.

Although the late wave of transcriptional change is complex, some overall regulatory themes seem apparent. As might be expected, many late-regulated transcripts seem related to changes in neuronal signaling that could help express long-term sensitization memory. For example, there is a sharp up-regulation in mRNA and protein levels of an *Aplysia* homolog of synaptotagmin (Conte et al., 2017; Monje et al., 2012), a protein which plays a conserved role in regulating neurotransmitter release (Fernández-Chacón et al., 2001). There is also an increase in transcription of an putative glutamate receptor (Conte et al., 2017). It is feasible that these changes contribute to increases in synaptic connectivity that help express sensitization. Indeed, two of the hallmarks of *in vitro* sensitization are long-term increases in nociceptor transmitter release and post-synaptic glutamate sensitivity (Trudeau & Castellucci, 1995; Zhu

et al., 1997). Other potential changes relevant to neural signaling include transcripts annotated to encode proteins involved in synaptic targeting (e.g. semaphorin), growth-factor signaling (e.g. TGF- receptor-associated protein), ion channels (e.g. volage-gated potassium channel subunit), and synaptic plasticity (e.g. menin-like, insulin-like growth factor 2, and fibroblast growth factor receptor 4 like). At the protein level, serotonin produces an increase in the expression of an *Aplysia* homolog of flotillin-1, which plays an important role in synapse formation and hippocampal-based memory formation in mammals (Monje et al., 2013)

Beyond specific changes in neuronal signaling, many late transcriptional changes seem related to the infrastructure required to produce neuronal plasticity (Conte et al., 2017). That is, many of the transcriptional changes would be expected to foster the production, maturation, and trafficking of proteins, potentially providing the resources necessary to produce the signaling changes that encode sensitization. This includes up-regulation of putative *Aplysia* homologs of proteins related to transcription (e.g. RNA polymerase II elongation factor ELL-like), RNA processing (e.g. CUGBP Elav-like family member 3-B), translation (e.g. Translational activator GCN1) and post-translational processing (e.g. BiP). Especially notable is a widespread increase in the expression of translation initiation factors (eIF1, eIF2, eIF3, eIF4, and eIF5). Consistent with a global increase in protein production, studies in culture show a late-developing down-regulation of CREB2 mRNA produced through serotonergic activation of small non-coding RNAs that interact with the protein Piwi to repress CREB2 transcription (Rajasethupathy et al., 2012).

Although there seems to be a global increase in the infrastructure for protein production, this does not rule out the possibility of synapse-specific changes should mRNAs be transported and targeted for local translation. Consistent with this possibility, long-term sensitization also produces delayed up-regulation of several transcripts annotated to encode heterogeneous nuclear ribonucleoproteins and one annotated to encode a KIF protein (GenBank: EB225867.1); some of these changes are also detectable in proteomic screens (Monje et al., 2012). As these proteins are all implicated in neuronal mRNA transport (Hirokawa, 2006), regulation of their expression could relate to targeting translational changes to specific synapses (Wang et al., 2010).

A fourth theme of the late phase of transcription is meta-plasticity: transcriptional changes that could feasibly be involved in resetting thresholds for additional learning and plasticity. Specifically, microarray analysis indicates delayed down-regulation of several transcripts associated with the induction of long-term sensitization (Conte et al., 2017). This includes decreases in the expression of 3 of the 4 known *Aplysia* homologs of adenylate cyclase, a type-4 cAMP-specific phosphodiesterase (PDE4) critical to the activation of cAMP (Park, 2005), and a putative serotonin receptor transcript. This set of transcriptional changes suggests the possibility that long-term sensitization makes it harder to induce additional sensitization. If sensitization produces a new learning context, this could help explain reconsolidation effects that have been observed after training (Cai et al., 2012).

4.4 A small and diverse set of transcripts participate in both the early and late waves of transcription

Although long-term sensitization produces distinct early and late waves of transcription, some transcripts participate in both phases of regulation, showing changes in expression that begin rapidly but also persist for at least 1 day after training. This is likely to be a rare form of regulation, with microarray analysis after sensitization training identifying only 25 potential "early-but-persistent" transcripts (Conte et al., 2017). Those that are well characterized are diverse, including transcription factors (CREB1 and Egr), synaptic proteins (synapsin), regulators of gene expression (eIF3 and TOB1), and modulatory peptide transmitters (FMRFa).

The early-but-persistent transcripts are of special interest, as their continued regulation suggests they may play some essential role in long-term sensitization. In particular, transcriptional changes involved in maintaining sensitization memory would be expected to show this type of stable regulation. Functional analysis of several "early-but-persistent" bears out this interest, demonstrating essential roles in *in vitro* analogs of sensitization.

One of the first "early-but-persistent" transcripts to be identified was synapsin (Hart et al., 2011), a protein that regulates synaptic vesicles. In culture, repeated pulses of serotonin produce an immediate increase in synapsin mRNA that decays to a moderate down-regulation at 12 hours and then rises back to strong up-regulation within 1 day, indicating that synapsin is part of both the early and late waves of transcription. These changes are likely mediated by activation of CREB1, as serotonin promotes binding of CREB1 to a CRE-like site in the *synapsin* promoter. Transcriptional changes in synapsin are accompanied by a rapid rise in synapsin protein that within 24 hours is targeted specifically to synaptic varicosities in *Aplysia* nociceptors. Synapsin likely plays a critical role in long-term sensitization, as RNA interference timed to prevent changes in synapsin expression prevent serotonin from producing lasting synaptic facilitation.

eIF3 also shows early-but-persistent transcriptional activation. It was initially identified for showing a rapid increase in expression following *in vivo* serotonin exposure in intact animals (Lee et al., 2008a), and subsequent study showed it is also persistently up-regulated after long-term sensitization training (Conte et al., 2017). Consistent with the importance of this class of transcripts, work in culture shows that knockdown of eIF3 expression via RNA interference prevents the long-term synaptic effects of serotonin, while over-expression enables short-term protocols to produce lasting effects (Lee et al., 2008a).

A third well-characterized early-but-persistent transcript is an *Aplysia* homolog of early-growth response protein (Egr; Cyriac et al., 2013), a transcription factor that plays a key in long-term plasticity and memory in mammals (Poirier et al., 2008). Egr mRNA is constitutively expressed throughout the nervous system and is bi-directionally regulated by changes in neural activity. Long-term sensitization produces a rapid and sustained increase in Egr mRNA in *Aplysia* nociceptors (Herdegen et al., 2014a). Although the function of Egr regulation has

not yet been explored, the degree of up-regulation 1 day after training is correlated with the strength of memory expression at that time point (Cyriac et al., 2013).

Although already discussed as an early-wave transcript, CREB1 may be another transcript that participates in both early and late waves. CREB1 binds to its own promoter (Mohamed et al., 2005). In cell culture, repeated pulses of serotonin produce both a rapid increase in CREB1 mRNA that decays quickly and a delayed increase that lasts at least 1 day (Liu et al., 2008). This is accompanied by a long-lasting increase in the expression of CRE-containing promoters and is essential for serotonin to produce long-lasting increases in synaptic strength and excitability in *Aplysia* nociceptors (Liu et al., 2011). As discussed in the Maintenance section, the positive-feedback loop of CREB1 may serve not only to help stabilize the induction of long-term sensitization, but also to help maintain the persistence of sensitization memory. One caveat to this conclusion, however, is that in intact animals, CREB1 seems participate only in the first wave, with multiple samples failing to observe persistent changes in CREB1 mRNA after long-term sensitization training (Bonnick et al., 2012; Conte et al., 2017; Herdegen et al., 2014a).

A surprising member of the early-but-persistent category is a transcript encoding the peptide neurotransmitter Phe-Met-Arg-Phe NH2 (FMRFa, Schaefer et al., 1985). This transcript is strongly up-regulated 1 hour and 1 day after long-term sensitization training in the pleural ganglia, which contain FMRF-amidergic neurons that provide inhibitory neuromodulation to defensive withdrawal circuits. This is accompanied by a late up-regulation of a FMRFa receptor in the pleural ganglia, suggesting an overall up-regulation of signaling from this transmitter system after sensitization. These changes are surprising because FRMFa can suppress memory (Fioravante et al., 2006), producing antagonistic effects on the expression of long-terms sensitization memory (e.g. Abrams et al., 1984). This suggests that in addition to activating transcriptional changes to encode sensitization memory, learning also activates signaling pathways that could eventually erode the expression of sensitization, a form of active forgetting (Berry & Davis, 2014).

Microarray analysis has identified 21 additional transcripts which show clear regulation at both 1 hour and 1 day after long-term sensitization (Conte et al., 2017). Some of these map to gene models that have not yet been well annotated. Those with annotations include ApGlyT2 (sodium- and chloride-dependent glycine-dependent transport 2), ApVPS36 (Vacuolar protein-sorting-associated protein 36-like), and a putative *Aplysia* homolog of TOB1 (transducer of ErbB-2). TOB1 is necessary for some forms of memory and is a key regulator of CPEB, a prion-like protein that plays a key role in the maintenance of the forms oflong-term plasticity that help encode sensitization memory (seeMaintenance section:).

4.5 The late wave of transcription fades before forgetting is complete, but some transcripts remain regulated long-after sensitization is forgotten

Unless training is extensive, long-term sensitization memory is forgotten, becoming progressively less likely to be recalled. The late wave of transcription also fades, but seems to collapse before forgetting is completed. Microarray analysis shows that less 1% of the transcripts identified in the late wave remain regulated just 5 days after training, a time point at which sensitization memory has partly decayed but remains quite strong (Rosiles et al., 2019). This held true even in a subset of animals selected for especially strong maintenance of sensitization memory.

Although the vast majority of transcriptional changes induced by sensitization training decay, a handful of transcripts show very persistent regulation that extends well beyond the behavioral expression of sensitization memory (Patel et al., 2018; Perez et al., 2018). While initially identified via exploratory microarray analysis, beyond-forgetting regulation has now been confirmed via qPCR in multiple independent samples. This includes upregulation of FMRFa, BiP, and 3 un-annotated transcripts and down-regulation of a putative homolog of spectrin and an un-annotated transcript. All of these are late-wave transcripts except FMRFa, which, as discussed above, is a an early-but-persistent transcript expected to work *against* the expression of sensitization memory.

It is not yet clear why some transcriptional changes might persist after sensitization memory is forgotten. They could be involved in producing forgetting, in maintaining a limited engram to enable rapid re-learning, or could even have no functional consequences at all.

5 Maintenance: Does transcription contribute to the long-term maintenance of sensitization memory?

Long-term sensitization training produces a robust transcriptional response, and these transcriptional changes are essential to the long-term neuronal changes that encode a sensitization memory. From that point, the sensitization memory can persist for days to weeks, though even when sensitization memory seems to have fully faded it can be rapidly re-learned (Menges et al., 2015; Philips et al., 2006). How is the sensitization memory maintained and what happens to maintenance memory during forgetting and re-learning?

Given the constant molecular flux that characterizes life, researchers have presumed that the long-lasting expression of memory must involve one or more maintenance mechanisms: molecular interactions that are a) inducible by learning, b) self-sustaining throughout the life of the memory, and c) responsible for the continued expression of a sensitization memory. According to this conceptualization, disrupting a maintenance mechanism would halt the expression of a long-term memory, even long after initial training. There are not clear predictions of how maintenance mechanisms would relate to forgetting: it could be that forgetting represents a disruption of maintenance, or it could be that maintenance mechanisms persist but that additional processes develop that inhibit the continued expression of the memory. Although the notion of a maintenance mechanism makes sense, it has proven difficult to develop definitive accounts of memory maintenance for long-term sensitization in *Aplysia*. In this section, potential maintenance mechanisms are reviewed, touching briefly on some non-transcriptional mechanisms and then considering in more depth the possibility of transcriptional loops that help sustain long-term memory (For an in-depth review, see Smolen et al., 2019).

5.1 Self-sustaining protein interactions could serve as maintenance mechanisms for long-term sensitization memory

At least some aspects of sensitization maintenance occur through self-sustaining protein interactions. For example, PKC, which helps mediate short-term sensitization, can become cleaved by calpain into a truncated, constitutively active form, termed PKM, which can then persistently regulate synaptic strength. PKMs play a role in memory maintenance, as treatments

that disrupt PKMs stop the maintenance of long-term sensitization in intact animals (Cai et al., 2011) and ofits cellular analogs in cell culture (Farah et al., 2019; Hu et al., 2017).

In addition, serotonin produces a self-perpetuating conformational change in synaptically-expressed CPEB, a prion-like protein that regulates local translation (Miniaci et al., 2008; Si et al., 2003, 2010). This enables activated synapses to up-regulate local translation. Synapse-specific depletion of CPEB shows that it is essential to initial maintenance (1 to 2 days after induction) of both synaptic facilitation and synaptic outgrowth, but that maintenance eventually becomes independent of CPEB function (3 days post induction).

5.2 Transcriptional loops could also support memory maintenance, including the inducible auto-activation of CREB1

Self-sustaining protein interactions may be especially important for maintaining synapse-specific encoding of sensitization. Sensitization is also encoded by global changes in neuronal function, including a long-term enhancement of excitability in *Aplysia* nociceptors (Scholz & Byrne, 1987). This suggests the possible operation of neuron-wide maintenance mechanisms. Given that the induction of sensitization is stabilized and extended by transcriptional feedback loops, this raises the possibility that these loops continue to operate after induction to produce sustained neuron-wide changes in function.

The most well-explored possibility relates to the inducible auto-activation of CREB1. As described above, studies in culture show that CREB1 produces a self-sustaining transcriptional loop: induction produces CREB1 phosphorylation and increases binding of CREB1 to its own promoter, producing a long-term increase in CREB1 mRNA, CREB1 protein, and the transcription of CREB1 (Liu et al., 2008, 2011; Mohamed et al., 2005). Moreover, RNA interference designed to block the late up-regulation of CREB1 mRNA stops the maintenance of increased synaptic strength and excitability. Thus, there is strong evidence that CREB1-self-promotion plays an essential role in at least the early phases of sensitization maintenance. There may be additional transcriptional feed-back loops that contribute to sensitization maintenance (e.g. PKA induction of uch transcription, which extends the activity of PKA).

There are several challenges to the notion of transcriptional feedback loops for sensitization maintenance. First, while CREB1 mRNA is persistently up-regulated by serotonin treatment in culture, multiple attempts have failed to detect persistent up-regulation in intact animals following long-term sensitization training (Bonnick et al., 2012; Conte et al., 2017; Herdegen et al., 2014a). A more significant challenge is that it is not yet clear if persistent transcription is always required for the expression of long-term sensitization memory. To date, few analyses have extended more than 1 day after induction, a timepoint that may be more associated with memory stabilization than memory maintenance (Miniaci et al., 2008). Moreover, microarray analysis suggests that the late wave of transcription almost fully collapses before forgetting

(Rosiles et al., 2019), and that reminders can produce a long-term reinstatement of sensitization without re-activating the late wave of transcription (Rosiles et al., 2020). These analyses might lack sensitivity, but they raise the intriguing possibility that sensitization memory can be expressed in the absence of ongoing transcriptional changes.

These challenges are not definitive. One possibility is that persistent transcriptional changes become refined as sensitization memory matures, and can no longer be detected in whole ganglia via low-sensitivity screening techniques. This could be especially true if persistent transcriptional states develop only in sparse sets of memory-encoding neurons, and could even be obscured if non-encoding neurons undergo offsetting homeostatic changes. If this is correct, persistent transcriptional regulation of CREB1 or other feedback-loop transcripts should be detectable via single-cell transcriptional analysis.

Another possible explanation is that long-term sensitization has multiple forms that differentially depend on transcriptional maintenance mechanism. At the behavioral level, very intense training (4 spaced shocks each day for 4 days) produces sensitization memory that lasts for weeks and that is always accompanied by synaptic outgrowth (Bailey & Chen, 1989). Less intense training (4 spaced shocks on 1 day only) produce sensitization that decays within a week and does not produce detectable levels of synaptic outgrowth (Wainwright et al., 2002). This suggests the possibility of a very-long-lasting phase of sensitization memory produced only with extended training. It may be that only this very long-lasting phase requires persistent transcriptional changes, as only this phase involves maintained growth. The discrepancy in CREB1 dynamics between culture and intact animals could reflect differential recruitment of these phases on long-term sensitization: *in vitro* models always produce synaptic growth and therefore may reflect primarily a very-long-lasting form of sensitization, whereas transcriptional analyses from intact animals have so far utilized only a 1-day training protocol, and may therefore have missed persistent up-regulation of transcription in general and CREB1 specifically. If so, transcriptional analysis following more intense training should support the transcriptional-loop model.

5.3 Sensitization memory may be maintained via multiple interacting maintenance mechanisms involving changes in protein function, transcription, and epigenetic regulation

In addition to feedback loops, persistent changes in transcription could be maintained by epigenetic modifications. Indeed, *in vitro* sensitization produces an increase in methylation of a region of the CREB2 promoter, leading to a persistent decrease in CREB2 expression (Rajasethupathy et al., 2012). In addition, pharmacological inhibition of DNA methylation stops maintenance of *in vitro* sensitization, though what transcriptional changes this treatment targets have not been identified.

At this time, there is evidence for self-sustaining maintenance mechanisms operating at the protein level (PKMs and possibly also CPEB), via transcriptional loops (CREB1), and through epigenetic regulation. Rather than considering these mutually exclusive possibilities, it has been proposed that the sustained expression of sensitization memory reflects multiple, interlinked maintenance mechanisms, and that it is their interoperation that sustains the complex patterns of plasticity that express long-term memory (Smolen et al., 2019). In grappling with this complexity, it remains unclear how maintenance mechanisms might change (if at all) during forgetting, and/or how rapid re-learning after forgetting is possible.

6 Conclusions and Future Directions

Long-term sensitization in *Aplysia* is produced by learning experiences that synchronize fast-developing cAMP/PKA activation with slow-developing ERK activation, enabling transcriptional activation via CREB1 to occur at the same time as transcriptional de-repression via CREB2. Successful induction of long-term sensitization triggers an immediate-early transcriptional response that helps stabilize and extend the complex signaling pathways activated during learning, organizing a late-developing wave of transcription that helps encode long-term sensitization memory via lasting changes in excitability, synaptic strength, and (with extended training) growth. The feedback loops that help stabilize the induction of long-term sensitization may also help maintain sensitization memory, but how transcriptional changes relate to forgetting and the potential re-learning of sensitization memory remains unclear.

While our understanding of the transcriptional mechanism of sensitization is detailed [A1] enough to enable computational predictions of new learning phenomena, much still remains to be learned:

· The encoding of long-term sensitization involves multiple cell types, and transcriptional changes are evident beyond the nociceptors that have been the primary focus of study. It is not yet clear, though, how transcriptional mechanisms of memory vary across neurons. One especially intriguing question is if transcriptional changes must be "zero-sum" across a circuit in order to limit the energy costs incurred by information storage.

· Many of the key mechanisms of long-term sensitization in *Aplysia* play important roles in learning and plasticity in other organisms. To date, however, potential conservation of learning mechanisms has not been systematically investigated. Measuring transcriptional states across species and learning paradigms may provide a way to begin elucidating a phylogeny of long-term memory mechanisms.

Long-term sensitization mechanisms are remarkably complex, both in the cross-talk, feedback, and extracellular signaling loops that characterize induction pathways, and in the surprisingly vast array of accompanying transcriptional changes. Why is the encoding of such a simple form of learning so complex? Does it help individual elements in a circuit encode information in concert with the networks they take part in, or is it just an accidental product of the complex evolutionary history of learning?

These and many other fascinating aspects of long-term sensitization in *Aplysia* await exploration.

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