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Cellular and Molecular Mechanisms Underlying Long-Term Synaptic Plasticity: mRNA Localization and Synapse to Nuclear Signaling

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Cellular and Molecular Mechanisms Underlying Long-Term Synaptic Plasticity: mRNA Localization and Synapse to Nuclear Signaling

A dissertation submitted in partial satisfaction of the

Requirements for the degree Doctor of Philosophy

in Biological Chemistry

by

Elliott Meer

2014

ABSTRACT OF THE DISSERTATION

Molecular Mechanisms Underlying Long-Term Synaptic Plasticity:

mRNA Localization and Synapse to Nuclear Signaling

by

Elliott Meer

Doctor of Philosophy in Biological Chemistry

University of California, Los Angeles, 2014

Professor Kelsey C. Martin, Chair

Synaptic plasticity, changes in the number and strength of synaptic connections in the brain, represents the best-characterized correlate of learning and memory. Long-term synaptic plasticity is a transcription- and translation-dependent process that can be restricted to subsets of synapses within a single neuron. The model system *Aplysia californica* provides an excellent model system to study the cellular and molecular mechanisms underlying long-term, learning-related synaptic plasticity. In this thesis, I investigate two cell biological questions that emerge from the requirement for gene expression during synapse-specific plasticity. The first question concerns the spatial regulation of gene expression within neurons. Here, we propose that one mechanism for restricting gene expression to individual synapses is through mRNA localization and regulated translation. I have identified a 66 nucleotide sequence in the 5'UTR of sensorin mRNA that when paired with the sensorin 3'UTR is required and sufficient for sensorin mRNA synaptic localization. My experiments further indicate that this localization element is encoded by a stem-loop structure (Meer et al., 2012). The second question concerns the mechanisms by

which synaptically-generated signals are transported from the synapse to the nucleus to initiate transcription. Using the *Aplysia* sensory-motor co-culture system, I found that the transcriptional regulator CREB-regulated transcriptional co-activator (CRTC) is required for activity-dependent long-term facilitation. *Aplysia* CRTC (ApCRTC) accumulates in the nucleus when cultures are treated with stimulation inducing activity-dependent heterosynaptic plasticity, but not when treated with activity-independent heterosynaptic plasticity inducing stimulation. Electrophysiological studies show that ApCRTC is functionally required in the postsynaptic (motor) neuron, as injection of dominant-negative constructs block long-term synaptic plasticity.

The dissertation of Elliott Meer is approved.

Douglas L. Black

James Akira Wohlschlegel

Feng Guo

Kelsey C. Martin, Committee Chair

University of California, Los Angeles

2014

The work presented herein is dedicated to my family and friends.

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To the rest of my committee: Doug Black, James Wohlschlegel and Feng Guo. Thank you for your valuable comments, advice, and taking the time to keep my graduate career on track.

The work presented in chapter 2 of this dissertation is reproduced from: Meer EJ*, Wang DO*, Kim S, Barr I, Guo F, Martin KC, Identification of a cis-acting element that localizes mRNA to synapses, PNAS. 109, 4639-44, 2012. PMCID: PMC3311331. *equal contribution

The work presented in chapter 3 of this dissertation is reproduced from: Meer EJ, Uzgil B, Ch’ng TH, DeSalvo M, Lee P, Martin KC. Synapse to nuclear signaling of CRTC is required postsynaptically in response to activity-dependent forms of long-term synaptic plasticity. In preparation, 2014.

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Meer EJ, Uzgil B, Ch'ng TH, DeSalvo M, Lee P, Martin KC. Synapse to nuclear signaling of CRTC is required postsynaptically in response to activity-dependent forms of long-term synaptic plasticity. In preparation, 2014.

SELECTED PRESENTATIONS\ABSTRACTS

Elliott Meer, Austin Peck, Peter Markiewicz, Les Wilson, Stuart Feinstein. High Resolution Imaging of Microtubules using DIC and AFM. Poster and Abstract presented at UCSB Summer Undergraduate Research Colloquium and Summer Intern Symposium, Santa Barbara, CA, 2005.

(Pro)renin Receptor Activation Causes Acute Production of Macula Densa Prostaglandins Elliott Meer, Jung Julie Kang, Sarah Laurin Vargas, Ildiko Toma, Janos Peti-Peterdi. Poster presented at FASEB Experimental Biology Meeting in 2008.

Identification of a cis-acting synaptic RNA localization element in Aplysia sensory neurons Elliott Meer. Talk given at the Biological Chemistry Departmental Retreat, UCLA, April 2010.

Identification of a cis-acting synaptic RNA localization element in Aplysia sensory neurons Elliott Meer, Dan Ohtan Wang, Sangmok Kim, Kelsey Martin. Poster presented at Society for Neuroscience in 2010.

Chapter 1: Introduction

> …the power of the brain to synthesize sensations makes it also the seat of thought: the storing up of perceptions gives memory and belief and when these are stabilized you get knowledge.
>
> - Alcmaeon of Croton[1]

As early as the 6th century BC Greek philosophers waxed poetic over the function of the human brain. Such was the desire to understand where thought, perception, and cognition arose from within ourselves. Even centuries after the first association of the brain with the concept of the mind[2], a deeper understanding of the basis for our ability to learn, remember, perceive and act eluded scientists.

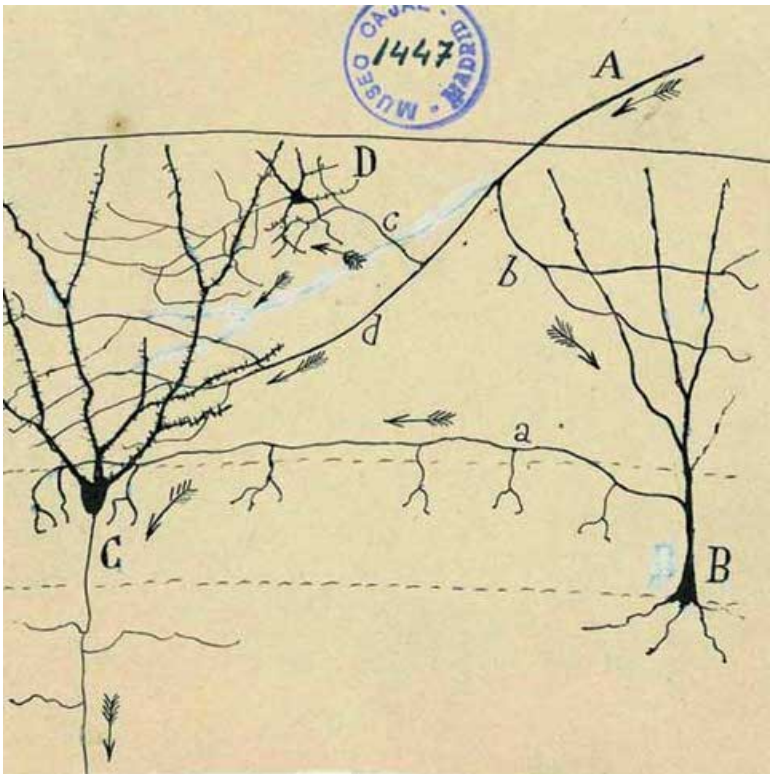

Figure 1-1

The drawings of Santiago Ramón y Cajal. Unprecedented detail and accuracy gave rise to not only neural theory, but also to the idea of information flow (the idea that neurons

receive input through what is now know as dendrites and output information through axonal transmission)[3].

It took until the late 19th - early 20th centuries before scientists were able to formulate theories that would begin to satisfactorily explain the brains ability to learn and form memories. William James in 1890 theorized that the brain displays plasticity, and that it is this plasticity of organic components that allows for living beings to form habits. In addition James stipulated that existing pathways in the brain could be reinforced while activity could drive formation of new associative paths[4]. While James' theories were important contributions to neuroscience, the seminal work of Santiago Ramón y Cajal provided concrete evidence of the microscopic structure of the brain and gave rise to our understanding of brain function as we know it today. Cajal was able to produce highly accurate morphological information through the use of Golgi staining; in contrast to prevailing dogma, Cajal's data clearly showed that the nervous system is contiguous and not continuous[5]. Thus Cajal was able to show that the brain follows Schleiden's 'cell doctrine'; as with the rest of the body, the brain is made up of cells (neurons) that act as the fundamental units. This 'neural doctrine' was highly influential to Cajal's contemporaries. Eugenio Tanzi combined James' idea of brain plasticity with Cajal's demonstration that the brain is composed of individual cells to generate his own theory of synaptic plasticity. Tanzi was the first to describe a connection between associative memories, motor skills and the facilitation of connections between neurons[6]. His theories postulated that under basal conditions "impulses" experience a set amount of resistance when transmitted from neuron to neuron. Repeated activity results in hypertrophy of neural connections and thus reduces the resistance of "impulses" traveling across them. Where along the neuron were these "impulses" being transmitted? Sherrington, in the years after Cajal and Tanzi's initial theories, identified the synapse and described its function for ensuring the unidirectionality of transmission[7]. Taken

together, the work of these neuroscience pioneers began to give us a plausible explanation of how human biology would be able to solve the complex task of learning and memory.

For the most part, the progression of learning and memory theory, and experimental data supporting it, would languish for almost half a century. Finally, in the 1940's, technology such as intracellular microelectrodes would allow for recording directly from neurons[8]. It was during this same period that the ideas of Tanzi and his contemporaries would be resurrected and restated in a manner more amenable to modern scientific investigation. Rather than a reduction in resistance, Donald Hebb described a strengthening of synaptic connections as a biological mechanism for long-term memory. Simplified as "neurons that fire together, wire together," Hebb postulated that repetitive, associative firing of a given pre- and postsynaptic neuron result in the strengthening of synaptic connections at sites of contact between those two cells[9]. To this day this type of homosynaptic plasticity is referred to as 'Hebbian,' although the basic ideas predated Hebb by decades.

The impetus for understanding the neurobiological mechanisms of learning and memory are not driven by scientific curiosity alone. Worldwide, 36 million people are currently diagnosed with Alzheimer's Disease, which is marked by a progressive loss of memory and cognitive abilities. Alzheimer's Disease is characterized by a loss of neurons and synapses, but how exactly this occurs is still unknown[10]. The most common form of dementia, Alzheimer's Disease currently has no treatments to stop or reverse its progression[11]. Unfortunately Alzheimer's Disease is just one of many cognitive neurological diseases, which include Parkinson's, Huntington's and Autism Spectrum Disorders. The numerous, severe neurological disorders affecting learning and memory have motivated scientists to elucidate the mechanisms by which humans learn and form memories so as to better understand how and why things go awry in disease states.

In 1973, Tim Bliss and Terge Lømo discovered a form of homosynaptic plasticity in the mammalian brain. Long-term potentiation (LTP) was elicited by repetitive electrical stimulation of the perforant pathway; this pathway consists of presynaptic cells located in the entorhinal cortex that synapse onto granule cells in the dentate gyrus of the hippocampus (this is the same pathway mostly likely drawn by Cajal, in Fig. 1-1, nearly 100 years earlier). Data from recordings of granule cells showed a massive potentiation that lasted for hours, while a control pathway showed no potentiation[12]. The importance of LTP to neuroscience cannot be understated, with Bliss and Lømo demonstrating for the first time what Donald Hebb postulated 25 years earlier. Another important aspect of the discovery of LTP was that it was discovered in the mammalian hippocampus, a region of the brain already known to be important in learning and memory. The study of patient HM, now known to be Henry Molaison, which predated the discovery of LTP, exemplifies this importance. Patient HM had a bilateral resection of his medial temporal lobe, the region of the brain that contains both the entorhinal cortex and the hippocampus, and showed huge impairments in certain forms of learning and memory[13].

While initially discovered in the hippocampus, LTP has also been found to exist in other brain regions, including the amygdala, a region of the brain involved in fear response. Fear conditioning is a behavioral paradigm in which an unconditioned stimulus (US, foot shock) is paired with a conditioned stimulus (CS, tone). Before conditioning, the animal freezes in response to the US, but not to the CS alone. After conditioning, the animal learns to associate the CS with the US and now the CS (the tone) is able to induce freezing of the animal. Rogan, Stäubli and LeDoux took advantage of this paradigm using awake, freely behaving rats in which electrodes had been implanted into the amygdala. Concurrent with measuring the behavioral fear induction of the rats, electrical recordings showed potentiation of the synapses known to be involved in fear conditioning[14]. Not only does this data provide evidence for LTP outside of the hippocampus, it also gives a more direct link between LTP and memory while showing that LTP

occurs in the free-living animal. While I have only described a few instances, the literature concerning LTP is vast. Combined, the previously mentioned data has given neuroscience a biological correlate of learning and memory, in which changes in the strength of synaptic connections change over time due to experience.

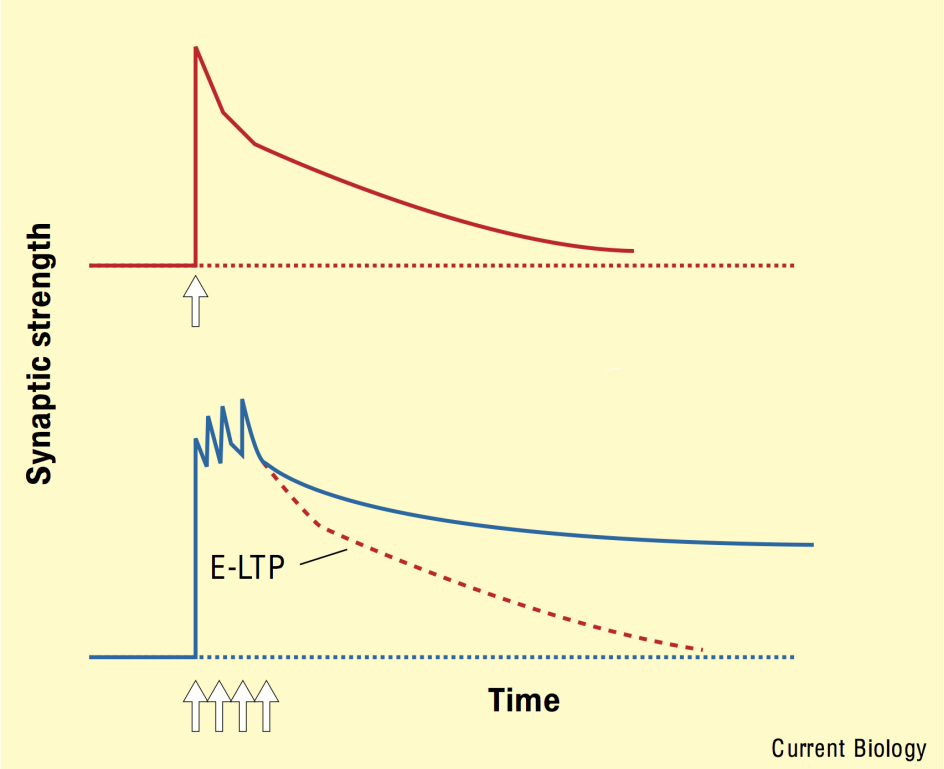

Figure 1-2

Early and late phases of long-term potentiation (LTP). A train of repetitive stimuli (arrows) induces an increase in synaptic strength known as LTP. A single train of stimuli induces E-LTP (red), which decays over the course of a few hours. Multiple trains also induce L-LTP (blue), which remains stable for many hours[15].

As might be implied with the terminology used, in addition to LTP, the brain also exhibits transient forms of potentiation. In mammalian brain, this is referred to as early phase (E-LTP) whereas transcription-dependent LTP is termed late phase (L-LTP). These two phases were

defined as it was found that a single tetanic stimulus would cause potentiation of stimulated synapses that would persist for minutes to a few hours. This is in contrast to L-LTP, which requires multiple spaced tetanic stimuli for physiological expression and can last for hours to days[16,17]. What might the behavioral consequences be for having distinct forms of LTP that are dependent on the strength or repetition of stimuli? In companion papers from Mansuy et al. and Winder et al., data is presented that suggests a correlation between E-LTP and short-term memory, and L-LTP and long-term memory[18,19]. Using a mutant mouse line expressing a modified form of calcineurin (a phosphatase important in LTP), Mansuy and Winder showed that loss of L-LTP correlates to deficits in a hippocampal dependent spatial long-term memory task. Mutant mice do however show normal E-LTP and when tested for novel object recognition, designed to test short-term memory, no loss in ability was seen as compared to wildtype mice. Thus the possibility exists that E-LTP could act as a mechanism for short-term memory storage, and L-LTP could sub-serve long-term memory.

The ability to differentially induce E-LTP and L-LTP suggests the involvement of distinct molecular pathways. Multiple decades of work, trying to generate a compendium of all molecules and processes necessary for LTP, has resulted in the implication of just about every kinase and receptor[20]. During the first phase of LTP at the hippocampal Schaffer Collateral pathway, E-LTP, stimulation of excitatory glutamatergic synapses triggers intracellular calcium increases via N-methyl-D-aspartate (NMDA) receptors that results in activation of Ca2+/calmodulin-dependent protein kinase IIα (CaMKII). Active CaMKIIα phosphorylates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and also initiates signal transduction pathways that increase trafficking of AMPA receptors to the post-synaptic membrane[21]. The increased phosphorylation and number of synaptic AMPA receptors leads to an increase in synaptic efficiency and is thought to be the primary mechanism by which E-LTP is expressed. Rather than invoke distinct pathways, L-LTP requires that additional molecules

be activated in addition to those necessary for E-LTP. Increase in intracellular cyclic AMP (cAMP) are of critical importance to L-LTP, with subsequent activation of protein kinase isoform A (PKA) and mitogen-activated protein kinase (MAPK)[8]. The activation, and translocation to the nucleus, of these kinases culminates in the activation of cAMP response element binding protein (CREB) by phosphorylating it. Phosphorylated CREB, bound to DNA sequence elements containing cAMP response elements (CRE), then recruits CBP and downstream transcriptional machinery.[22]. CREB dependent transcription is one of the hallmarks of L-LTP; in addition L-LTP has been shown to require new protein synthesis[23].

An average neuron in the mammalian brain makes on average 1000 synaptic connections. Is it possible for a neuron to restrict plasticity only to synapses that were specifically activated? Work by Dr. Kelsey Martin in 1997 helped to unequivocally show that it is possible for a neuron to selectively potentiate a subset of synapses in response to stimulation. Dr. Martin took advantage of the *Aplysia* culture system that allows for individual sensory (SN) and motor neurons (MN) to be isolated and placed into culture. By pairing a single SN with two spatially separated target MNs, two sets of distinct synaptic connections are made (Fig 1-3). Synapses made between the SN and lower MN can be stimulated, independently of those between the SN and upper MN, by applying spaced pulses of serotonin via pipette. Measuring the synaptic efficacy before and after stimulation shows that the connection between the SN and lower MN are dramatically potentiated while those between the SN and upper MN remain unchanged[24].

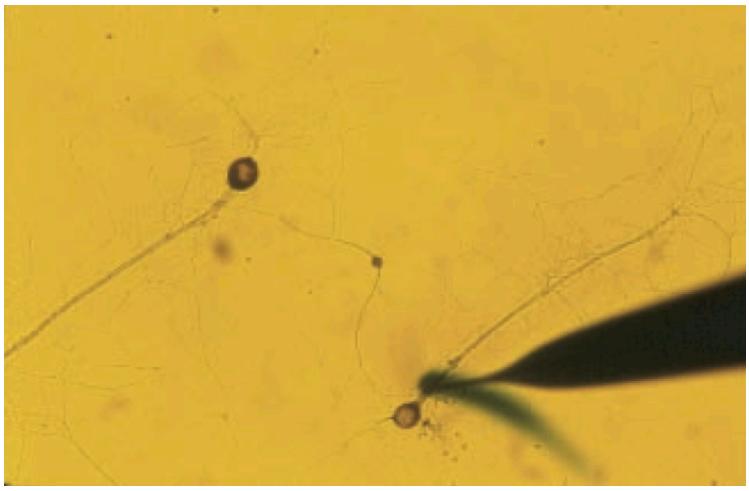

Figure 1-3

Synapse-specific activation using neurons from *Aplysia Californica*. Shown is a SN (center), synapsing onto two spatially separated target MNs. The synapses made between the SN and lower MN are stimulated using a pipette filled with serotonin to induce synaptic plasticity[24].

We now know that synaptic plasticity, the modification of the number and the efficacy of synapses in a neural circuit, provides a cellular substrate for learning and memory. In addition, numerous studies have shown that such modifications can occur at the level of individual synapses. These major findings, along with the knowledge that long-term, learning-related synaptic plasticity requires both transcription and translation, raises an important question: where in the neuron does the production of new proteins, necessary to initiate and sustain synaptic plasticity, take place[24,25]? One possibility is that it takes place in the soma, with proteins then being trafficked to synapses. Another possibility involves translation of localized transcripts at synapses. The latter mechanism provides the cell with an energy-efficient means of spatially and temporally regulating gene expression in response to synaptic stimuli[26].
In the brain, the restriction of protein synthesis to discrete neuronal compartments is critical to the development and experience-driven refinement of neural circuits, playing roles in axon

guidance, synaptogenesis, and synaptic plasticity[26,27]. A large but select population of transcripts has been identified in axons and dendrites[28-33], indicating that local translation subserves diverse cell biological functions, and that messenger RNA (mRNA) localization in neurons is a highly regulated process. Studies of mRNA localization in neurons and other asymmetric cell types have revealed that such cis-acting mRNA localization elements are recognized by specific trans-acting RNA binding proteins (RNABPs), with the resulting messenger ribonucleoproteins (mRNPs) packaged into RNA transport granules that interact with molecular motors to be delivered, in a translationally repressed state, from the soma to their final subcellular destination (See Figure 1-4) [34,35].

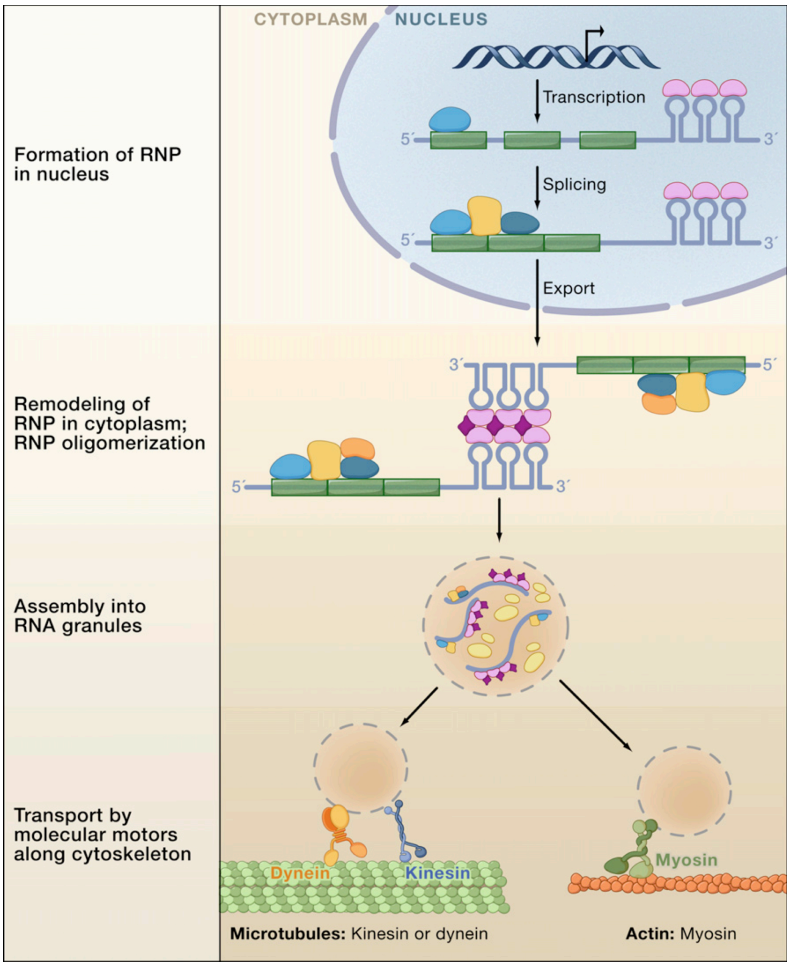

Figure 1-4

A model of mRNA localization[26].

In addition to localizing to neuronal processes, mRNAs exhibit distinct patterns of subcellular localization within processes. For example, β-actin mRNA displays multiple developmentally distinct and activity-regulated patterns of localization. In developing neurons, β-actin mRNA localizes to axonal growth cones in response to Neurotrophin-3 stimulation, while in mature neurons, the transcript undergoes depolarization-dependent localization to distal dendrites [36,37]. Specific BDNF mRNA isoforms have also been shown to localize to distal dendrites in a manner that is regulated by NMDA or TrkB receptor activation [38]. While a number of studies have identified *cis*-acting RNA localization elements (LEs) that target mRNAs to dendrites [39-45], less is known about LEs that mediate regulated localization to more distinct subcellular loci. The importance of determining the fundamental mechanisms underlying mRNA localization and regulated translation in neurons is underscored by the fact that mutations in genes encoding RNABPs are the genetic basis for a number of inherited neurological disorders [46]. A pertinent example is that of fragile-X mental retardation protein (FMRP), an RNABP involved in mRNA transport and translational regulation. Loss of function of FMRP is the cause of Fragile X mental retardation and the most common single gene cause of Autism.

To study the cell biology and function of mRNA localization and local translation at synapses during learning and memory, the Martin Lab utilizes the *Aplysia californica* SN-MN co-culture system. The SN-MN synapse mediates a simple defensive reflex in the animal, the gill withdrawal reflex. Sensitization of this reflex involves release of serotonin from serotonergic interneurons onto the SN-MN connections, leading to increased synaptic strength and enhanced gill withdrawal reflex. This can be reconstituted in culture by pairing a single SN with a single MN, and applying serotonin into the bath with a pipette or perfusion electrode. The large size of *Aplysia* neurons allows for relatively easy microinjection of DNA while the processes themselves can survive without the soma allowing for examination of local events.

Importantly, the molecular and cellular mechanisms underlying long-term synaptic plasticity in *Aplysia* have been found to be conserved in mammalian systems [22].

Studies of LTP in mammalian brain have mainly focused on homosynaptic (Hebbian) plasticity. While this type of LTP has strong evidentiary support, the contribution of neuromodulatory inputs requires further investigation in order to determine the whole story when it comes to physiologically relevant L-LTP. Heterosynaptic plasticity involves the strengthening of a set of synapses via the release of a neuromodulator by a modulatory interneuron onto those synapses. The best-known example of heterosynaptic plasticity comes from studies of the *Aplysia* gill-withdrawal reflex mentioned above. The sensitization of this reflex, achieved by applying a noxious stimulus to the animal, is known to be the result of serotonin being released onto and facilitating the synapses between the siphon (SN) and gill (MN). Importantly the facilitation of the SN-MN synapse can be achieved without homosynaptic activation of these neurons; stimulation of the serotonergic interneuron or application of serotonin directly is sufficient for facilitation. Like homosynaptic plasticity discovered in mammals, heterosynaptic plasticity was found to have phases and that these phases mirror the molecular requirements of homosynaptic plasticity for their induction. Whereas a single pulse of serotonin will result in facilitation of SN-MN synapses for a few minutes, and is due to modification of pre-existing proteins, multiple spaced pulses of serotonin will drive facilitation for hours to days and requires synthesis of new protein and mRNA[22,47]. Because this form of plasticity does not require synaptic activation of either the SN or MN, it is termed activity-independent, in contrast to homosynaptic plasticity that requires associative firing of both neurons and therefore termed activity-dependent.

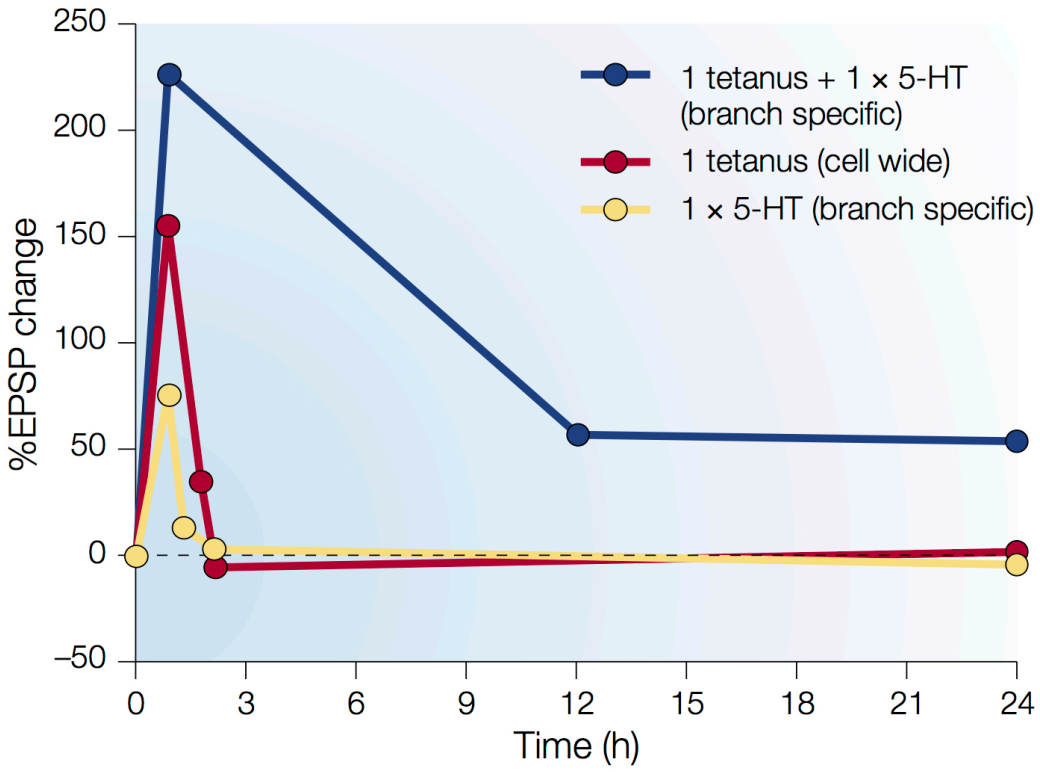

Figure 1-5

The combination of hetero- and homosynaptic stimuli produces a form of synaptic plasticity that is more than just additive in terms of temporal persistence[8].

The existence of these two forms of synaptic plasticity raises the question of whether physiological LTP is predominantly driven by homosynaptic, heterosynaptic or some combination of both? The high degree to which neuromodulatory inputs in the mammalian brain are intertwined with studied neural pathways makes answering this question difficult[8,48]. The *Aplysia* model system has been crucial in that it allows for independent activation of either hetero- or homosynaptic mechanism as well as a combination of both. A purely heterosynaptic form of facilitation in *Aplysia* has been described in the preceding paragraph. Homosynaptic plasticity can be activated in *Aplysia* SN-MN cultures by delivering tetanic stimulation to the SN, but even if multiple trains of stimulation are used the resulting potentiation only lasts for a period

of 1-2 hours[49]. Along with data from mammalian studies of dopamine-mediated receptors, where pharmalogical agents were used to either block or enhance neuromodulatory input, there is strong evidence that homosynaptic plasticity on its own is not capable of activating L-LTP[50,51]. While homosynaptic activation doesn't appear to induced L-LTP and heterosynaptic facilitation on its own is sufficient to do to so, the possibility still exists that *in-vivo* these two mechanisms work together to produce L-LTP. Data to support such an idea come from the observation that pathways shown to exhibit L-LTP, such as the perforant pathway mentioned earlier, contain neuromodulatory interneurons that terminate onto neurons/synapses involved in L-LTP[48]. Work in *Aplysia* has also shown that a single application of tetanic stimulation to the SN in conjunction with branch-specific serotonin treatment results in a form of long-term facilitation. This is interesting because on their own each of these treatments would otherwise result in short-term facilitation, showing that together hetero- and homosynaptic stimuli can result in plasticity that is more multiplicative rather than just additive (Figure 1-5)[49]. The idea of requiring both type of plasticity is attractive, as it would imply that another level of association is required in activating L-LTP. Homosynaptic plasticity ensures synaptic specificity at the post-synaptic neuron's synapses, however activation of the pre-synaptic neuron could still result in neurotransmitter release on all its targets. Heterosynaptic plasticity suffers from the same possible specificity issues, therefore involving both mechanisms to activate L-LTP would produce an additional level of regulation in requiring coincidental activation of both mechanisms [8].

Common to both hetero- and homosynaptic plasticity in activating L-LTP is the absolute necessity for transcription[16,47]. Central to the activation of downstream target genes is the transcription factor CREB. CREB1 is required for L-LTP in both *Aplysia* and mammalian system, and is activated upon phosphorylation at serine 133 by PKA that has translocated to the nucleus[52-55]. While PKA is undoubtedly important in the activation of L-LTP, Martin et al. have shown that MAPK translocation to the nucleus is also critical for long-term synaptic plasticity[56].

In the paper Martin et al. (Neuron, 1997) suggest that MAPK translocation is required, at least in part, in order to drive derepression of CREB2. Furthermore, CREB-dependent transcription is induced in response to a larger number of stimuli that ultimately drive a wide range of cellular processes. These stimuli all result in phosphorylation at serine 133, but each can cause modifications to other sites on CREB or binding of proteins to CREB that can modify its transcriptional activity. Thus the transcriptional profile activated by CREB is specific to the stimulus the cell receives and this activity does not always correlate with phosphorylation at serine 133[57]. It would seem then, and extensive literature shows, that transcriptional activation is thus not simply regulated by a single molecular pathway.

Given that CREB can be activated by any number of stimuli, what are the possible mechanisms by which highly polarized cell like a neuron could convey signals to the nucleus to activate CREB? In a review of retrograde signaling mechanisms in neurons Ch'ng and Martin discuss how signaling at the synapse can be conveyed to the nucleus in order to activate transcription[58]. Rapid signaling can occur through electrochemical signaling via opening of ion channels in distal compartments ultimately resulting in transcription factor activation through somatic calcium increases. Another form of rapid signaling which results in increases in calcium, again activating transcription factors in the nucleus, is through regenerative calcium waves that are initiated in the endoplasmic reticulum (ER) that is present at distal sites and continuous with the nuclear membrane. In addition to these relatively rapid forms of signaling, the neuron can also utilize mechanisms that transport soluble molecular signals from the synapse to the nucleus with the authors mentioning diffusion, signaling endosomes and active transport of soluble proteins. Strong evidence exists for active transport of soluble proteins using the classical importin-mediated nuclear import pathway to initiate transcription, which would provide a direct mechanism for synaptic activation to be conveyed to the nucleus (Figure 1-6)[58].

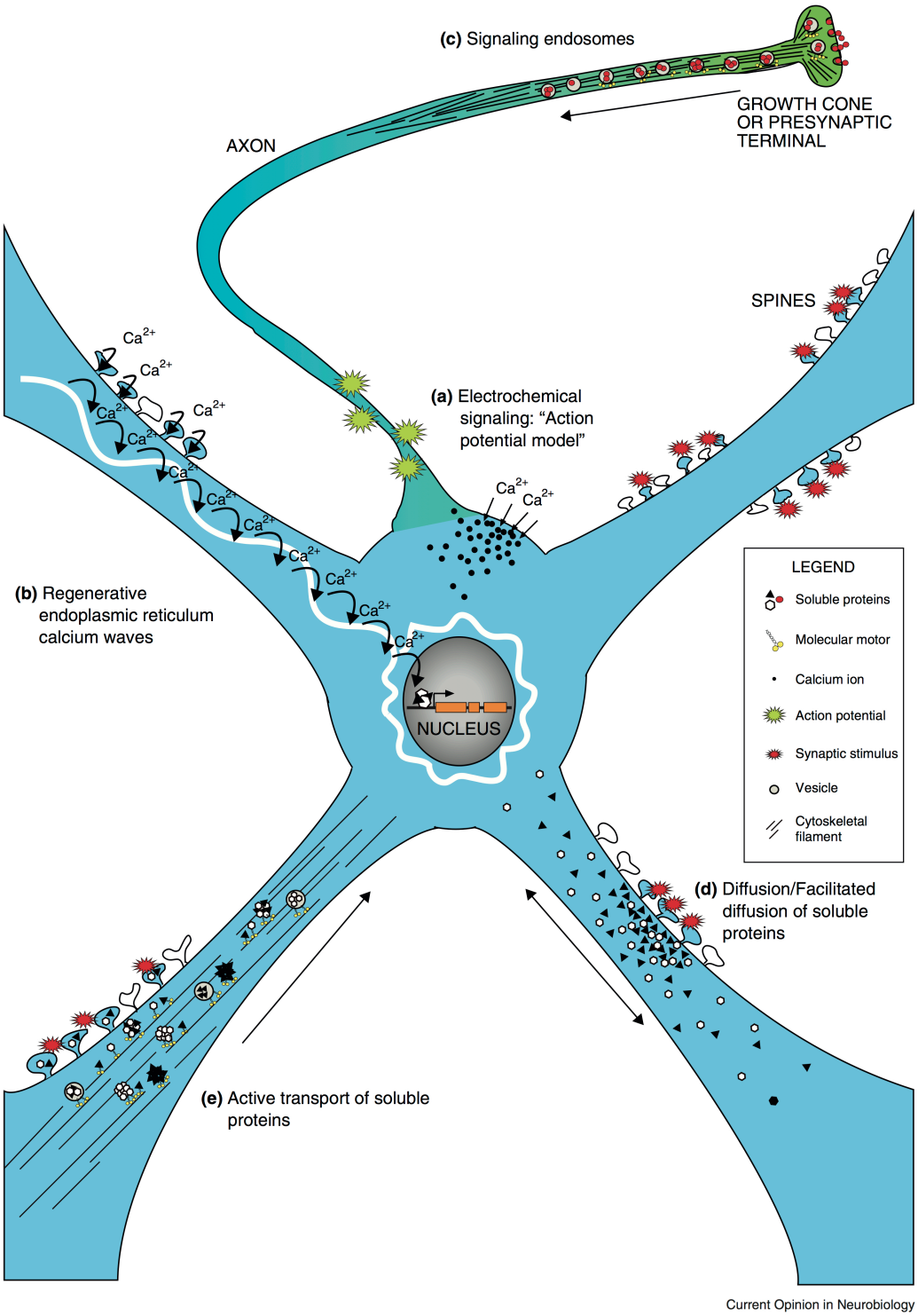
(c) Signaling endosomes
GROWTH CONE
OR PRESYNAPTIC
TERMINAL
AXON
SPINES
(a) Electrochemical
signaling: "Action
potential model"
(b) Regenerative
endoplasmic reticulum
calcium waves
NUCLEUS
LEGEND
Soluble proteins
Molecular motor
Calcium ion
Action potential
Synaptic stimulus
Vesicle
Cytoskeletal
filament
(d) Diffusion/Facilitated
diffusion of soluble
proteins
(e) Active transport of soluble
proteins
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Figure 1-6

Activation of transcription required for L-LTP results from signals transmitted at the site of synaptic contact. These sites are often located very distally to the nucleus. Shown are possible mechanisms by which these signals could be conveyed to the nucleus[58].

With CREB playing a central role in activation of distinct transcriptional profiles in response to different stimuli, molecules that translocate into the nucleus selectively and modulate CREB in an activity-dependent manner would provide a manner in which neurons could differentially respond to a multitude of synaptic inputs. CREB-regulated transcriptional coactivator (CRTC) was identified in a screen for CREB coactivators as a potent transactivator of CREB[59,60]. Data from CRTC2 in ß-islet cells has shown that CRTC undergoes stimulus-induced nuclear/cytoplasmic shuttling with its subcellular localization dependent on its state of phosphorylation. Once inside the nucleus CRTC is able to activate CREB-dependent transcription by binding to its bZIP domain. CRTC is able to active CREB independent of phosphorylation of serine 133 on CREB[61]. Thus translocation of CRTC in response to a subset of stimuli would therefore allow for distinct downstream target genes to be activated, as CRTC would modulate the transcriptional activity of CREB.

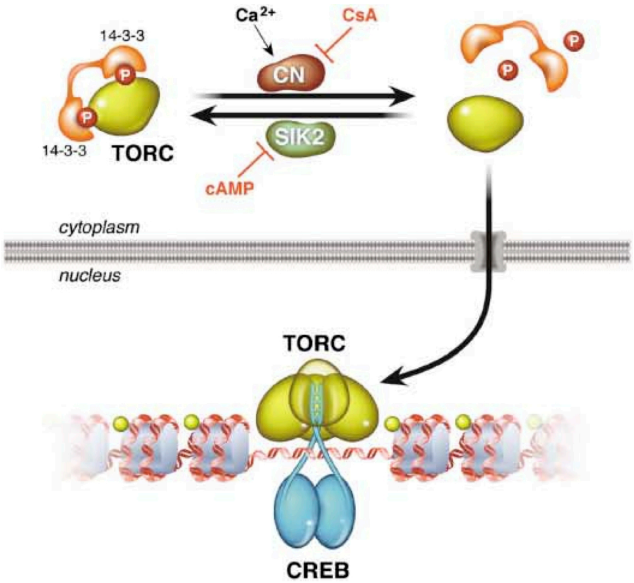

Figure 1-7

CRTC (here referred to as TORC) subcellular localization is dependent on its phosphorylation state. Nuclear translocation is dependent on calcium influx and activation of the phosphatase calcineurin, increased cAMP levels prolong its nuclear presence[61].

The previously mentioned data would point to CRTC as an attractive molecule to study in learning and memory. One could imagine that CRTC is positioned at synapses, poised to respond directly to synaptic stimuli and convey this activity to the nucleus in order to activate target downstream genes required for L-LTP. Indeed CRTC1 has been implicated in long-term memory formation: expressing dominant-negative CRTC1 in mammalian hippocampal neurons has been shown to block L-LTP (but not E-LTP) while overexpression of CRTC1 results in L-LTP when stimulation that normally results in E-LTP only is given[62,63]. Data from the Martin lab has given insight into the mechanism by which CRTC is able to translocate to the nucleus in neurons and thus affect L-LTP induction. Ch'ng et al. showed that CRTC1 is localized to the spines and dendrites of mammalian hippocampal neurons where it is tethered to 14-3-3 proteins[64]. Synaptic activity resulted in a loss of CRTC1 from spines and dendrites, subsequent accumulation in the nucleus corresponded with a dephosphorylation of CRTC1. Consistent with previous studies of CRTC2, CRTC1 nuclear accumulation in neurons was found to be dependent on increases in intracellular calcium and activation of calcineurin. cAMP is able to prolong this nuclear accumulation, but is not required for nuclear import[64]. We now know that CRTC1 is able to respond to synaptic activity by translocating to the nucleus in neurons and the mechanism by which CRTC1 translocates to affect CREB-dependent transcription. Questions still remain however: Is CRTC functionally required in the pre- or postsynaptic neuron? Is the CRTC mechanism conserved outside of mammals? Does CRTC accumulated in the nucleus

differentially in response to hetero- vs homosynaptic stimuli? And in which cases is it functionally required? Questions that would be otherwise difficult to answer in mammalian systems are perfectly suited for study using *Aplysia*. Taking advantage of the ability to dissect out individual pre- and postsynaptic neurons it would be possible to determine in which cell type CRTC is undergoing nuclear import. Studying CRTC in *Aplysia* would also show the importance of the CRTC mechanism in L-LTP as being conserved would show that it was selected for over evolution. As stated previously the technical advantages of the *Aplysia* cultures system allow for inducing synaptic activity in either a homo-, heterosynaptic manner or both. If CRTC responds in a differential manner it would give credence to its ability to help activate specific transcriptional profiles required for long-term memory.

Discussed in the subsequent chapters are two stories; the first aims to elucidate the requirements for mRNA location to synapses while the second looks at how synapse to nuclear signaling could result in activation of specific transcriptional profiles required for long-term memory.

Chapter 2: Identification of a cis-acting element that localizes mRNA to synapses

Abstract

mRNA localization and regulated translation can spatially restrict gene expression to each of the thousands of synaptic compartments formed by a single neuron. While *cis*-acting RNA elements have been shown to direct localization of mRNAs from the soma into neuronal processes, less is known about signals that target transcripts specifically to synapses. In *Aplysia* SN-MN neuronal cultures, synapse formation rapidly redistributes the mRNA encoding the peptide neurotransmitter sensorin from neuritic shafts into synapses. We find that the export of sensorin mRNA from soma to neurite and the localization to synapse are controlled by distinct signals. The 3'UTR is sufficient for export into distal neurites while the 5'UTR is required for concentration of reporter mRNA at synapses. We have identified a 66-nucleotide element in the 5'UTR of sensorin that is necessary and sufficient for synaptic mRNA localization. Mutational and chemical probing analyses are consistent with a role for secondary structure in this process.

Introduction:

mRNA localization and regulated translation provide a means of spatially restricting gene expression within distinct subcellular compartments. In the brain, local protein synthesis is critical to the development and experience-driven refinement of neural circuits, playing roles in axon guidance, synaptogenesis, and synaptic plasticity [26,27].A large but select population of transcripts localizes to axons and dendrites [29-32,65,66], indicating that local translation subserves diverse cell biological functions. Where studied, the localization of mRNAs to axons or dendrites has been shown to depend on *cis*-acting localization elements (LEs) usually found in the 3' UTR, although occasionally present in the coding sequence or 5'UTR [26,27,67]. These *cis*-acting mRNA LEs recruit specific trans-acting RNA binding proteins, and the resulting messenger ribonucleoproteins (mRNPs) are packaged into RNA transport granules that interact with molecular motors to be delivered to their final subcellular destination [68-70].

In situ hybridization studies in neurons indicate that localized mRNAs in neurons are targeted to distinct subcellular compartments and domains within neuronal processes. For example, MAP2 mRNA concentrates within proximal dendrites, while CaMKIIα mRNA extends to distal dendrites [71]. mRNA localization also appears to be dynamically regulated during development and with activity. In mature neurons, β-actin mRNA localizes to dendrites, and its concentration to distal dendrites is stimulated by depolarization [37]. Stimuli that activate NMDA or TrkB receptors drive specific BDNF mRNA isoforms into distal dendrites of hippocampal neurons [38]. High frequency stimulation of perforant path projections to the dentate gyrus has been shown to direct localization of the mRNA encoding the immediate-early gene Arc selectively and specifically to activated dendritic lamina [72], and to drive localization of pre-existing CaMKIIα mRNA into synaptosome fractions [73]. Together, these findings indicate that specific transcripts undergo constitutive as well as developmental- and activity-dependent localization to distinct subcellular compartments.

Although a handful of *cis*-acting RNA localization elements, ranging in size from five to several hundreds nucleotides have been shown to mediate constitutive localization of specific mRNAs into distal processes of neurons [74], little is known about *cis*-acting elements that target mRNAs to more restricted subcellular compartments, such as synapses, or that mediate activity-dependent redistribution of mRNAs within neuronal processes. To identify such *cis*-acting LEs, we generated and expressed chimeric reporters to study the localization of the mRNA encoding the *Aplysia* SN specific peptide transmitter, sensorin [75]. Release of sensorin from the SN is required for both synapse formation and long-term facilitation [75,76]. The localization of sensorin mRNA is regulated by synapse formation, such that it is diffusely localized in neurites of isolated SNs (which do not form synapses), but concentrates at synapses in SNs paired with target motor neurons (MNs, [75]. Synapse formation does not alter the localization of other neuritically localized mRNAs, including those encoding α-tubulin and β-thymosin [30], suggesting that sequences within the sensorin mRNA specify its synaptic localization. Dissection of the mechanisms underlying sensorin mRNA localization thus provides not only a means of identifying *cis*-acting LEs involved in the export of mRNA from the soma to distal neuronal processes and in the localization of mRNA specifically to synapses, but also LEs that dynamically mediate changes in mRNA localization in response to synapse formation.
We previously demonstrated that the full-length 5' and 3'UTRs of sensorin are sufficient for synaptic localization of reporter mRNAs, and for stimulus-induced translational regulation of the reporter at synapses [77]. While the 3'UTR was sufficient for localization of reporter mRNA to distal neurites, the 5'UTR was required for synaptic localization of the reporter [77]. To define the minimal LE for synaptic targeting, we have now characterized a series of deletion and point mutations in the 5'UTR, focusing on stem-loop structures. Our studies identify a 66-nucleotide-long stem-loop cis-element in 5'UTR, just upstream of the translational start site that functions as a synaptic LE (sLE).

Results:

To define the RNA sequences that mediate localization of sensorin mRNA to neurites and to synapses, we fused the 5' UTR, coding sequence and/or the 3' UTR of sensorin to the coding sequence of fluorescent protein dendra2 (Fig 1A). We microinjected each construct into cultured *Aplysia* SNs, fixed the cultures 48 hrs later and processed them for fluorescence *in situ* hybridization (FISH) with antisense dendra2 riboprobes to detect reporter mRNA. To differentiate between sequences mediating neuritic localization and sequences mediating synaptic localization, we performed experiments in isolated SNs, which do not form chemical synapses [75,78], and in SNs forming glutamatergic synapses with target MNs.

As shown in Fig 1B, when no sensorin sequences were included, dendra2 reporter mRNA was restricted to the soma and proximal neurites of isolated SNs. Addition of either the 5'UTR or the coding sequence of sensorin to dendra2 did not alter this pattern of distribution. In contrast, the sensorin 3'UTR distributed dendra2 reporter mRNA into distal neurites, and addition of 5' UTR increased the distal distribution. To quantify the localization, we measured fluorescence pixel intensity along neurite length and measured the percent of FISH signal in the proximal, middle and distal third of the neurites. As shown in Fig 1C, in the absence of any sensorin sequence, or with the sensorin 5'UTR or coding sequence alone, little reporter RNA was present in the distal third (9±1%, 12±1%, and 14±4% respectively). In contrast, addition of the 3' UTR significantly increased the FISH signal in the distal third (to 26±3%), and when both 5' and 3' UTRs were included, the percent of reporter RNA in the distal third rose to 45±3%. By comparison, FISH for endogenous sensorin mRNA in parallel sets of cultures revealed that 34±2% of signal was present in the distal third (Fig 1C and Fig S1). Note that the mean pixel intensity in the cell body did not differ significantly between reporter constructs (Fig S1), indicating that differences in distal localization were not due to changes in expression levels. Collectively, our results show that the sensorin 3'UTR is sufficient to promote mRNA localization

from the soma into distal processes. The sensorin 5'UTR does not promote distal localization on its own, but enhances distal localization of 3'UTR-containing reporters.

We next asked which sequences were required for synaptic localization by expressing the reporters shown in Fig 1A in SNs that were paired with MNs. As shown in Fig 2A, while the reporter with the 3'UTR of sensorin localized to distal sensory neurites, it did not concentrate at synapses. In contrast, the reporter with both the 5' and the 3' UTR of sensorin not only localized to distal neurites, but also concentrated at SN-MN synapses. We quantified this redistribution by comparing the coefficient of variation (CV) of the FISH signal in isolated SNs and in SN-MN cocultures (Fig. 2B). As previously reported for endogenous sensorin mRNA [75], we found that the CV was significantly greater when the 5'3' UTR reporter construct was expressed in SN-MN cocultures than in isolated SNs (0.46±0.04 in SN vs. 1.07±0.09 in SN-MN, $p<0.001$, unpaired Student's t-test), whereas the CV of 3'UTR reporter was not significantly different in cocultures as compared to isolated SNs (0.46±0.04 in SN vs. 0.51±0.04in SN-MN). The CV of cytoplasmic diffusible dendra reporter proteins from the same neurites did not differ between SN and SN-MN, showing that the different CV of reporter mRNA was not due to local volumetric variation (5'3'UTR reporter protein: 0.53±0.05 in SN vs. 0.52±0.03 in SN-MN; 3'UTR-reporter protein: 0.41±0.02 in SN vs. 0.56±0.03 in SN-MN).

To confirm that the sites of reporter mRNA concentration represented synapses, we expressed the pre-synaptic marker **V**esicle-**A**ssociated **M**embrane **P**rotein (VAMP)-mCherry in the SN and labeled the postsynaptic MN with Alexa-fluor 647 (Fig. 2C). We defined synapses as SN varicosities of >2 micron diameter containing a concentration of VAMP-mCherry and contacting the Alexa-fluor (blue) labeled MN. To measure synaptic localization, we quantified the percent of VAMP-mCherry positive varicosities adjacent to MNs that contained clusters of reporter mRNA. We limited our data analysis to neurons in which reporter mRNA was abundant in adjacent neurites (mean fluorescent RNA in situ intensity above 40 in an 8 bit image). As

shown in Fig 2D, 87±3% of synapses colocalized with 5'3'UTR reporter mRNA concentration while only 38 ±7% also contained 3'UTR reporter mRNA concentration (see also [77], Fig S2). Together, these data indicate that the dynamic redistribution of sensorin mRNA from neuritic shafts to synapses upon synapse formation is mediated by signals in the 5'UTR.

Studies of mRNA localization in other cell types have revealed that LEs often consist of stem-loop structures [26,74,79]. To define the minimal sequences within the sensorin 5'UTR that mediate localization to synapses, we focused on stem-loop secondary structures predicted by structural motif discovery programs. Twenty stem-loop structures were predicted, present within four distinct regions of the 5'UTR (Fig 3A and S6 [79-81]. We deleted each region from the 5'3'UTR dendra reporter to generate reporters with intact 3'UTR and 5'UTRs lacking one of the four regions predicted to contain local stem-loop structures (ΔR1-R4, Fig 3B).

We expressed these reporters together with VAMP-mCherry in SNs paired with MNs and quantified synaptic localization. As shown in Fig 3C and D, ΔR1 and ΔR3 retained synaptic localization comparable to the intact 5'3'UTR reporter (81±2% for ΔR1 and 80±9% for ΔR3, compared to 87±3% for intact 5'UTR). The synaptic localization of ΔR2 was not as high as that of the intact 5'3'UTR reporter or endogenous sensorin mRNA, but was significantly greater than a 3'UTR-dendra reporter lacking the 5'UTR (68±7% compared to 38±7%). In contrast, the synaptic localization of ΔR4 mRNA was completely abolished, and did not differ significantly from that of the 3'UTR reporter (33±6% compared to 38±7%). RNA FISH intensity in the soma for each mutant was used as an indicator for overall RNA expression level. There was no correlation between RNA FISH intensity and synaptic localization, indicating that synaptic localization of mRNA did not depend on mRNA expression level (Fig S2).

To test whether R4 was sufficient for localizing distally localized reporter mRNA to synapses, we inserted R4 (iR4, 66 nts) into the dendra reporter, downstream of the RSV promoter and upstream of the translation initiation site (iR4, Fig 3E). This reporter showed

synaptic localization that was not significantly different from endogenous sensorin mRNA (Fig 3F, 70±7% and 83±3% respectively), indicating that the 66 nt R4 sequence is sufficient for localizing RNA to synapses (Fig 3F). By comparison, insertion of a sequence including R1, 2 and part of 3 (iR1-3) or R2 (iR2) was not sufficient to mediate synaptic mRNA localization (iR1-3: 37±9% and iR2: 35±10%, Fig 3E and F). Since R4 was necessary and sufficient for synaptic RNA localization, we term it a synaptic LE (sLE).

We next set out to further define the sLE. Closer inspection of the primary sequence of this structure revealed a 24 nt-long sequence that contains two repeated 7mers (5'-CAGTCTTGAAACAGAAACAGTCTT-3', with two "CAGTCTT"s at the ends, and two "GAAACAG"s in the center, Fig 4A). Repeated hexamers or heptamers have been identified in LEs in various RNAs, often sites of recognition by RNA binding proteins. We thus set out to test whether this 24 nt tandem repeat element constituted the minimal synaptic LE. When we deleted either the entire 24 nt double-7mer-repeating sequence from 5'3'UTR-dendra reporter (ΔR5), just one set of the two heptamers (ΔR6), or 10-nt of the center repeat (ΔR7) the synaptic concentration of the reporter mRNA was strongly reduced (Fig 4A and B, 34±6%, 52±9% and 53±11%, respectively). The fact that loss of a single repeat reduced localization suggested to us that synaptic RNA targeting depended on the secondary structure rather than the primary sequence of the sLE.

To specifically test the role of secondary structure in mediating synaptic RNA localization, we generated and analyzed two additional reporters in which we introduced 1) eight mutations to "collapse" the two internal loops into a long stem (Fig 4C and D, "Zipper"); and 2) nine mutations to maintain the predicted secondary structure while disrupting the primary sequence of the tandem repeats (Fig 4C and D, "RTR" for Remove Tandem Repeats"). The synaptic localization of the Zipper mutant was completely abolished (Fig 4E, 34±7% of VAMP-mCherry sites containing reporter RNA). In contrast, the synaptic localization of the RTR mutant

was intact (Fig 4E, 84±8% of VAMP-mCherry sites containing reporter RNA). Moreover, insertion of the RTR 66 nt sequence into the dendra reporter, downstream of the RSV promoter and upstream of the translation initiation site (iR4-RTR) was sufficient to mediate synaptic localization of reporter RNA, while insertion of the Zipper 66 nt sequence (iR4-Zipper) was not (iR4-RTR: 70±4%, iR4-Zipper: 20±3%, Fig 4F and G). Together, these data are consistent with a critical role for the secondary structure of the sLE in mediating synaptic RNA localization. Since the half-life of a RNA could affect the ability of that transcript to concentrate at synapses, we measured the stability of a subset of constructs by expressing them in isolated SNs, severing the soma and then fixing the remaining neurites at 0 or 48hrs after soma removal. FISH was performed and mean pixel intensity measured. Our data show that all reporter RNAs were stable over a 48hr period (Fig S4).

To further analyze the secondary structure of the sLE, we employed selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE, [82,83]. SHAPE takes advantage of the difference in reactivity between base-paired and unpaired nucleotides to the electrophile N-methylisatoic anhydride (NMIA). Modification of unpaired RNA at the 2' hydroxyl group with NMIA blocks primer extension by reverse transcriptase, which can be detected by sequencing gels with single nucleotide resolution.

We used SHAPE to test the structure of two sequences that localize to the synapse, R4 and RTR, and one sequence that does not, Zipper (Fig. 5). Consistent with algorithm-based prediction, Zipper collapses the two loops into a long stem while maintaining the tandem repeat elements. In contrast, the reactivity of RTR is remarkably similar to the wildtype sequence R4. Incorporation of the SHAPE data (ΔG_s) into a secondary structure prediction program [84] decreased the minimal free energy (ΔG) of R4 from -7.2 to -23.1, of RTR from -7.7 to -21.3, and of Zipper from -20.5 to -74.1 kcal/mol (Fig 5). Chemical analysis by SHAPE thus supports the predicted *in silico* secondary structure of the identified synaptic LE.

Discussion:

Our studies identify the first, to our knowledge, *cis*-acting LE that mediates localization of mRNA to neuronal synapses. Our data support a multi-step mRNA localization mechanism within neurons, in which specific cis-acting LEs mediate localization from the soma to the neuronal process, while other cis-acting LEs mediate further targeting to synapses [85]. This type of multi-step mechanism for localization to distinct subcellular compartments has previously been reported for myelin basic protein mRNA localization in oligodendrocytes [85] and protein kinase Mzeta in neurons [86]. While our data demonstrate that the 5'UTR is necessary for synaptic localization, they do not, however, rule out the possibility that synaptic localization involves combined actions of the 5' and 3' UTRs.

Bioinformatic analyses of the 5' UTR of sensorin does not reveal homology at the primary sequence level with any known localization elements. Although it is possible that the identified 66 nt sequence is unique, we believe that it is more likely that the conservation is at the level of secondary structure or three dimensional structure since mutations that maintain secondary structure localize reporter RNA to synapses despite disrupting the primary sequence (Fig 4A-C). As RNA secondary structure alignment programs are developed and optimized [87,88], it will be interesting to use the R4 stem-loop structure to search for similar structures in other mRNAs, and to then determine whether these mRNAs are synaptically localized.

The large size of cultured Aplysia SN-MNs facilitates the study of mRNA subcellular localization in neurons. The ability to compare mRNA localization in neurons that do and do not form chemical synapses provides a means of detecting dynamic changes in mRNA localization that occur upon synapse formation. Together, this level of spatial resolution and control over stimulation (synapse formation) permits determination of where and when mRNAs localize in neurons. As such, our studies extend studies in mammalian neurons that have described cis-acting LEs for mRNA localization and/or dynamic changes in mRNA localization, where analysis

has been restricted to the level of localization to proximal and distal dendrites, rather than to specific compartments within the dendrite (e.g., [72,89].

Our data show that mRNAs are remarkably stable within isolated neuronal processes. As shown in Fig S4 we do not detect any decrease in FISH signal for endogenous sensorin or for any of the reporter RNAs in neurites that have been severed from their cell bodies after 48 hrs. Although it has been proposed that localized mRNAs are transported in structures in which they are protected from degradation [68], to our knowledge these are the first studies to specifically monitor RNA stability in isolated neuronal processes,

Our discovery of a cis-acting RNA sLE is a first step towards dissecting the mechanisms whereby RNAs localize to distinct subcellular compartments within neurons. It opens the door to identifying the specific RNA binding proteins, cytoskeletal elements, molecular motors and transport structures that function to regulate gene expression with exquisite spatial and temporal control within neural synapses and circuits.

Materials and Methods:

Reporter Constructs:

pNEX-dendra2, sensorin 3'UTR pNEX-dendra2, sensorin 5'UTR pNEX dendra2, sensorin 5'3'UTR-dendra2 and mCherry-VAMP constructs were generated as described in [77].

***Aplysia* neuronal cultures, microinjection, electrophysiology, stimulations:**

Aplysia SN-MN cultures were prepared as described in [90]. Reporter plasmids were microinjected into SNs 24-36 hours after plating. Synaptic connectivity was assayed by measuring EPSP amplitude between SN and target MN, as described in [77].

Live cell imaging:

Confocal images were acquired on a Zeiss Pascal scanning laser microscope (Zeiss, Germany). Green dendra2 protein was excited with a 488nm Argon laser (at 2.5mW). To detect VAMP-mCherry positive SN varicosities in contact with Alexa-fluor 647 labeled MNs, pNEX vectors encoding VAMP-mCherry were microinjected into SNs 24-36 hrs after plating and the MN was microinjected with Dextran, Alexa Fluor 647 (Invitrogen, Eugene, OR) 60 minutes prior to imaging on DIV 4. Neuronal morphology was traced from DIC images using Neurolucida trace software (MBF Bioscience, Vermont).

Fluorescence *in situ* hybridization (FISH):

Cells were fixed with 4%PFA/30% sucrose in PBS and processed for FISH as previously described [77]. Sense riboprobe did not produce any background signal; antisense riboprobe did not produce signal in MNs (which do not express sensorin, [77]). Since dendra2 protein fluorescence does not persist following processing of samples for FISH, we manually aligned RNA images to live cell images based on the morphology of SN and MN. We limited our data analysis to neurons in which reporter mRNA was abundant (e.g. mean fluorescent RNA in situ intensity above 40 in an 8 bit image) in adjacent shafts.

Statistical analysis

GraphPad Prism software (La Jolla, CA) was used for all statistical analysis as specified in figure legends. Kruskal-Wallis test followed by Dunnett's multiple comparison tests were used when data distribution did not follow Gaussian distribution.

SHAPE analysis

SHAPE was used to determine paired and unpaired regions within predicted secondary structures [82,83]. Details are provided in Supplemental information.

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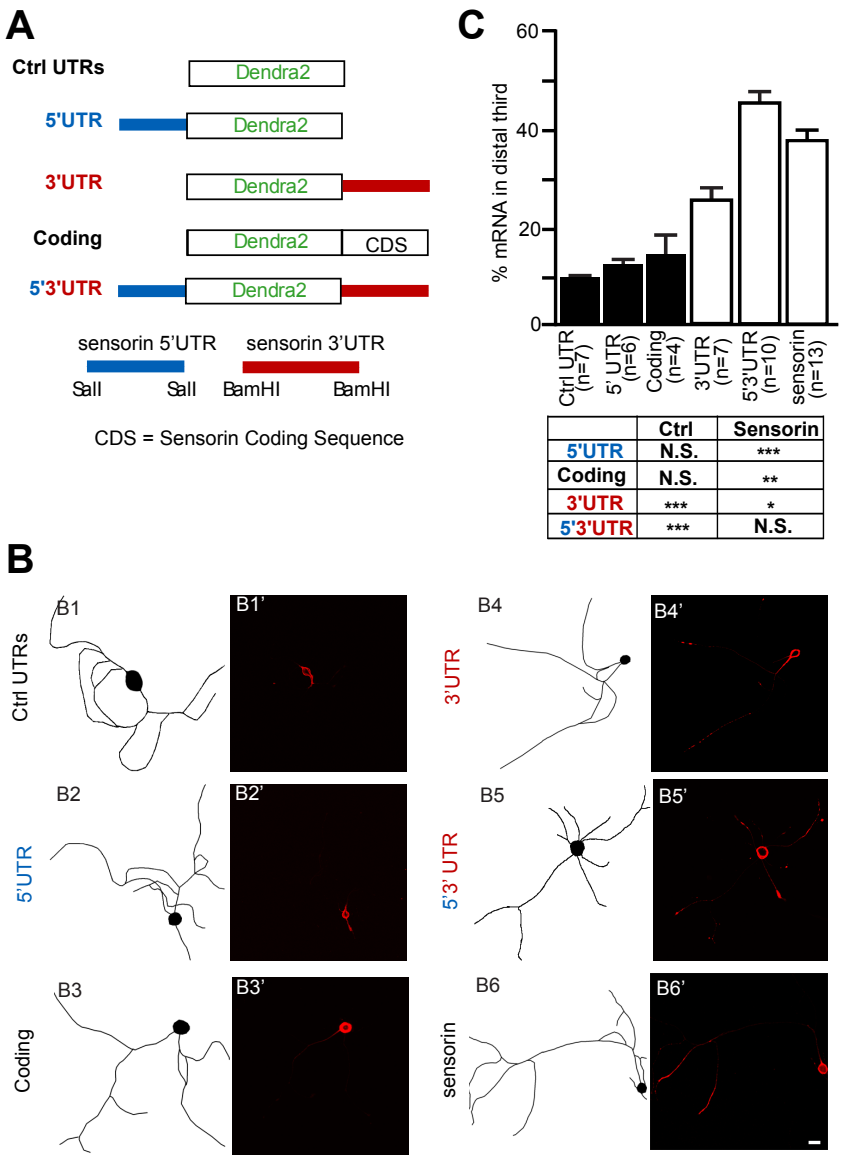


Figure 2-1

Fig. 1. The 3'UTR of sensorin is sufficient to target reporter mRNA into distal neurites. pNEX vectors encoding translational reporters were microinjected into isolated *Aplysia* sensory neurons (SNs) in culture (DIV2). Neurons were fixed (DIV4) and processed for fluorescence *in situ* hybridization (FISH) with dendra antisense riboprobes. *A*, Cartoons of reporter constructs in pNEX™3 expression vector. *B,* Representative images of reporter (dendra2) mRNA FISH in isolated SNs. B1-6, neurolucida tracing of each SN; B1'-6', FISH (detected with dendra

antisense riboprobes for B1'-5', and with sensorin antisense riboprobes for B6'). The FISH signal only extends to distal neurites when the 3'UTR of sensorin is present; distal localization is enhanced by 5'UTR. Scale bar, 100μm; *C,* Quantification of the distribution of reporter mRNA within sensory neurites. Neurites were linearized and divided into proximal, middle and distal segments. The percent of total FISH signal in distal segments is shown (see also Figure S1. ***p<0.0001, Kruskal-Wallis one-way analysis of variance followed by Dunnett's multiple comparison test.

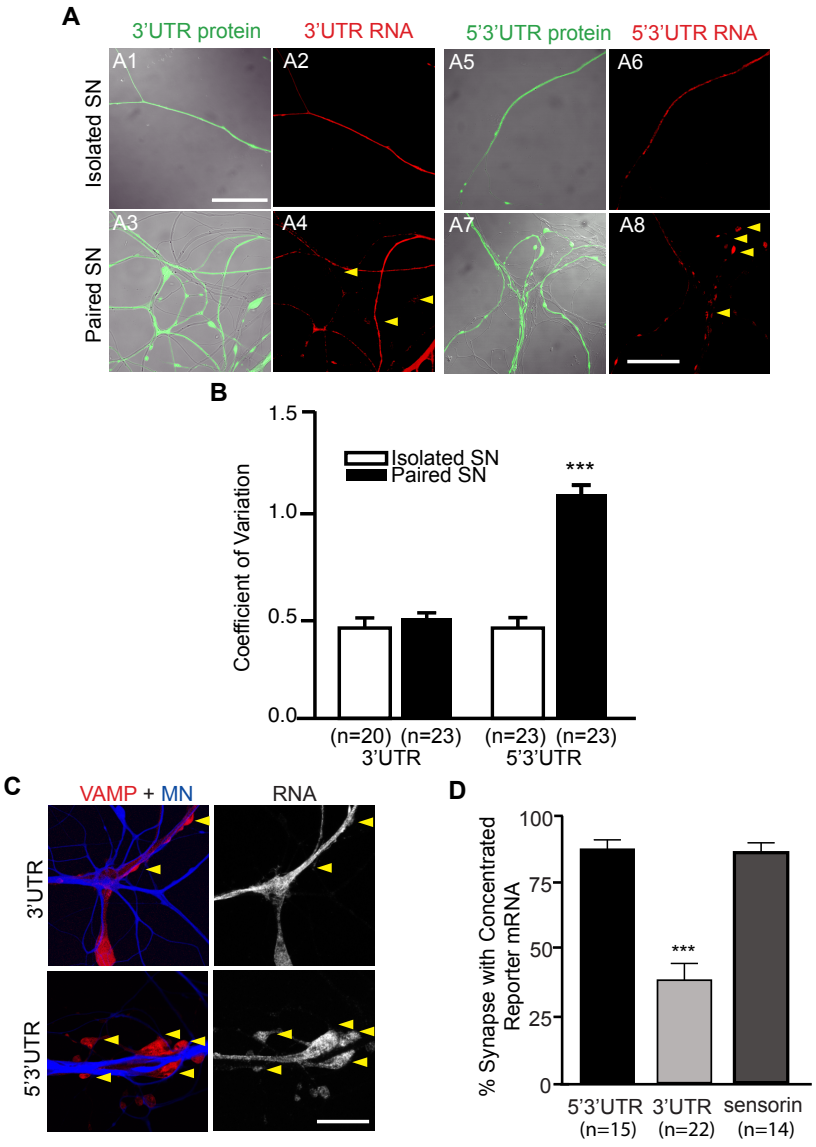


Figure 2-2

Fig. 2. 5'UTR of sensorin is required for localizing reporter mRNA to synapse.

Expression vectors encoding dendra2 reporters with either the 3'UTR or both the 5' and 3' UTRs (5'3'UTR) of sensorin were microinjected into *Aplysia* sensory neurons (SN, DIV2) cultured in isolation (isolated SN) or with motor neurons (MNs). Neurons were fixed (DIV4) and processed for fluorescence *in situ* hybridization (FISH) using dendra2 antisense riboprobes. *A,* Representative photomicrographs of dendra reporter protein (green, A1, 3, 5, 7) and mRNA (red, A2, 4, 6, 8) distribution in isolated and paired SNs. Scale bar, 50μm. *B,* Quantification of the change in distribution of reporter mRNA by measuring the coefficient of variation (CV, standard deviation/mean) of the FISH signal. ***p<0.0001, unpaired Student's t-test. *C,* The 5'3'UTR reporter or the 3'UTR reporter was co-expressed with the presynaptic marker VAMP-mCherry in *Aplysia* SNs paired with target MNs on DIV2. On DIV4, the MN was injected with the volume filling dye Alexa-fluor 647 (blue), and images of VAMP-mCherry and blue Alexa-fluor were acquired, followed by fixation and FISH with dendra antisense riboprobes. Left panels, merged VAMP/MN images of a co-culture with SN over-expressing 5'3'UTR or 3'UTR reporter and VAMP-mCherry (VAMP-mCherry in red, MN in blue); right panels, FISH signal for reporter mRNA. Scale bar, 100μm. *D,* Quantification of the percent of synapses (VAMP-mCherry clusters adjacent to MN) containing reporter mRNA (error bars = SEM). ***p< 0.0001, One-way ANOVA followed by Dunnett's multiple comparison test. *(*See also [77], Figure S2).

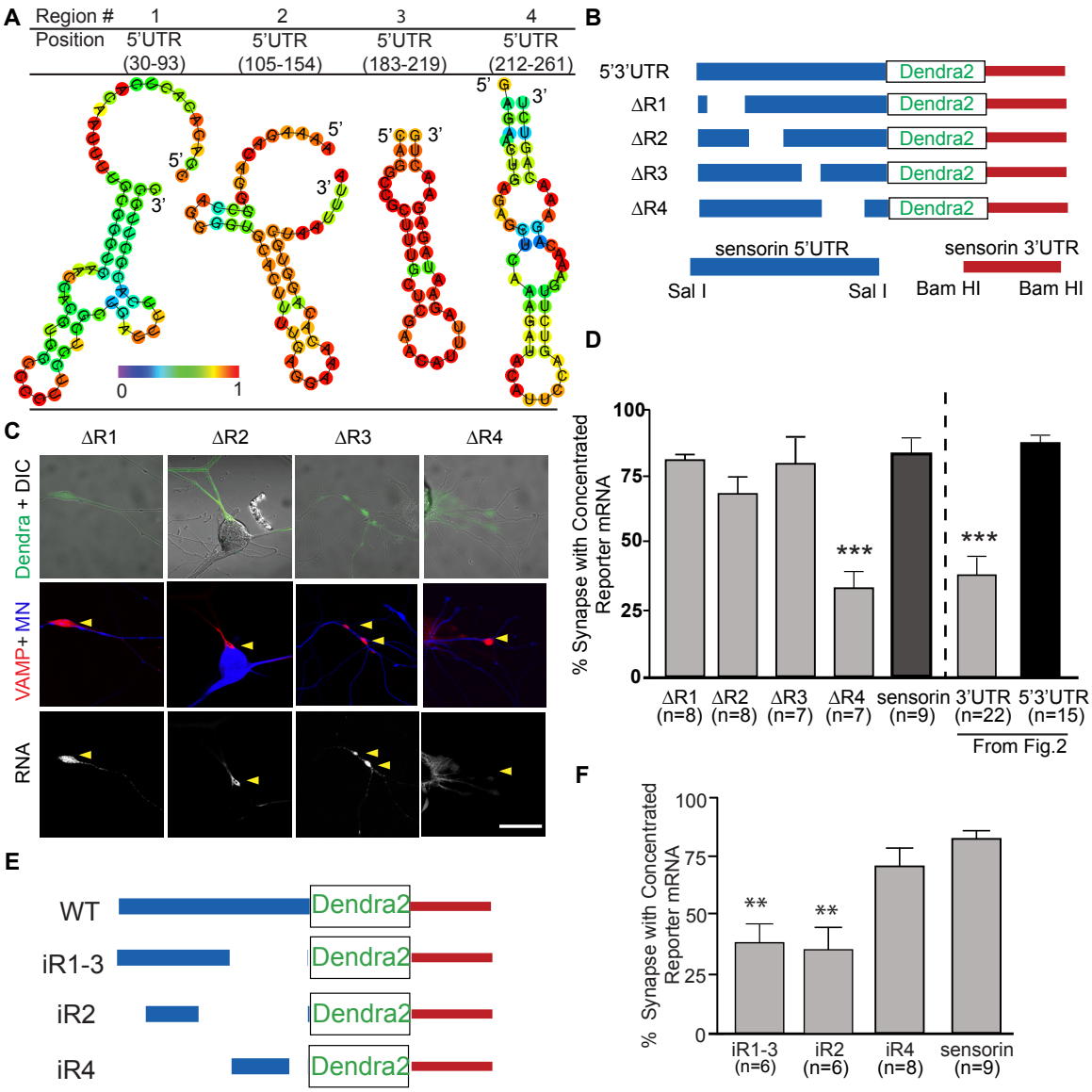


Figure 2-3

Fig. 3. A region directly upstream of the sensorin translation start site, in the 5'UTR, is necessary and sufficient for synaptic localization of the reporter mRNA. *A,* Representative graphic depictions of potential stem loop structures in 5'UTR of sensorin predicted by RNAfold [91], color scale denotes base pair probabilities; *B,* Cartoon representation of deletions made to the reporter construct. *C,* Representative images of deletion constructs ΔR1-R4 expressed in SNs synaptically connected with MNs. Top panel, photomicrograph of Dendra protein (SN, green) merged with DIC image; middle panel, synapses marked as VAMP-mCherry clusters

(red) contacting the MN (blue, Alexa 647); bottom panel, FISH images showing clustering of reporter mRNA. *D,* Quantification of synaptic localization of reporter mRNAs as the percent of synapses (VAMP-mCherry clusters adjacent to MN) containing reporter mRNA. *E,* Cartoon of insertion constructs (iR1-4) in which the designated regions of the sensorin 5'UTR were inserted into a control pNEX 5'UTR to test their ability to localize the reporter mRNA to synapses. The mutants were co-expressed in SNs paired with target MNs at DIV2 and neurons were fixed on DIV4 and processed for FISH. *F,* Percent of synapses (VAMP-mCherry clusters adjacent to MN) containing reporter mRNA.

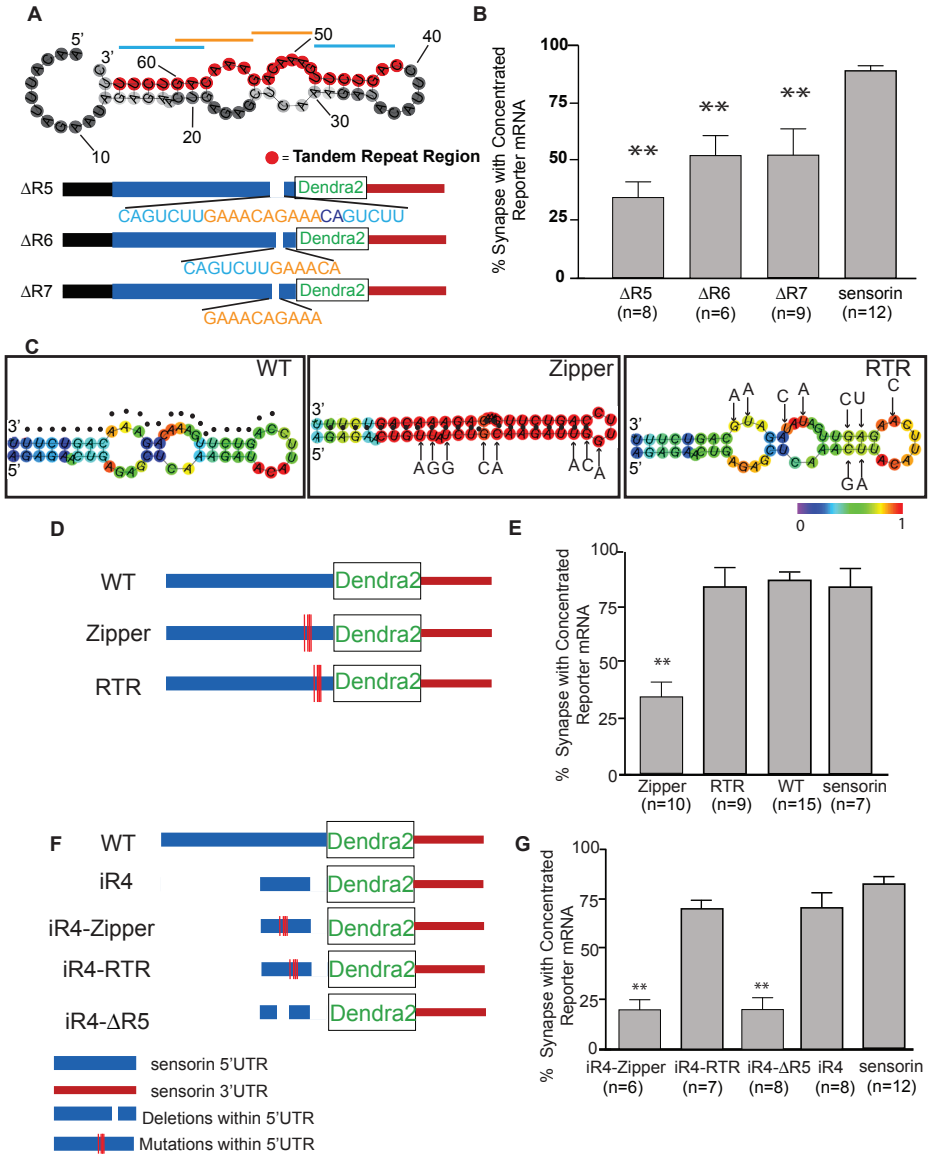


Figure 2-4

Fig. 4. A 66 nt stem-loop structure localizes reporter mRNA to synapses. *A,* Predicted secondary structure of the region corresponding to R4 (RNAfold, [91]; highlighted in red is the 24-nt primary sequence containing a double-tandem 7mer-repeat (indicated with blue/orange lines). Mutants were generated in which the entire 24-nt (ΔR5), the first 13-nt set of repeat elements (ΔR6) or 10-nt of the center repeat (ΔR7) were deleted from the 5'3'UTR reporter construct. *B,* Mutants were co-expressed with VAMP-mCherry in SNs paired with target MNs and the percent of synapses containing reporter RNA was measured. ** p<0.01, one-way ANOVA followed by Dunnett's multiple comparison test compared to 3'UTR reporter or endogenous sensorin. See also Figures S2, S3 and S6. *C,* Predicted secondary structures (RNAfold, [91]) of WT (dots denote tandem repeat region described in Fig 3); zipper construct, 8 point mutations were introduced to collapse the predicted secondary structure without disrupting the tandem repeat sequence; RTR construct, 9 mutations were introduced to disrupt the primary sequence of the tandem repeat region, while retaining predicted secondary structure. *D,* Cartoon showing the location within the sensorin 5'UTR of the mutations in the zipper and RTR constructs. The mutants were co-expressed in SNs paired with target MNs at DIV2 and neurons were fixed on DIV4 and processed for FISH. *E,* Percent of synapses (VAMP-mCherry clusters adjacent to MN) containing reporter mRNA. *F,* Cartoon of mutant insertion reporter constructs, including WT iR4 (66 nt), iR4-RTR (iR4 with mutations shown in RTR in C), iR4-Zipper (iR4 with mutations shown in zipper in C), or iR4-ΔR5 (iR4 lacking the 24 nt repeat element). The mutants were co-expressed in SNs paired with target MNs at DIV2 and neurons were fixed on DIV4 and processed for FISH. G, Percent of synapses (VAMP-mCherry clusters adjacent to MN) containing reporter mRNA. ** p<0.001, One way ANOVA followed by Dunnett's multiple comparison test.

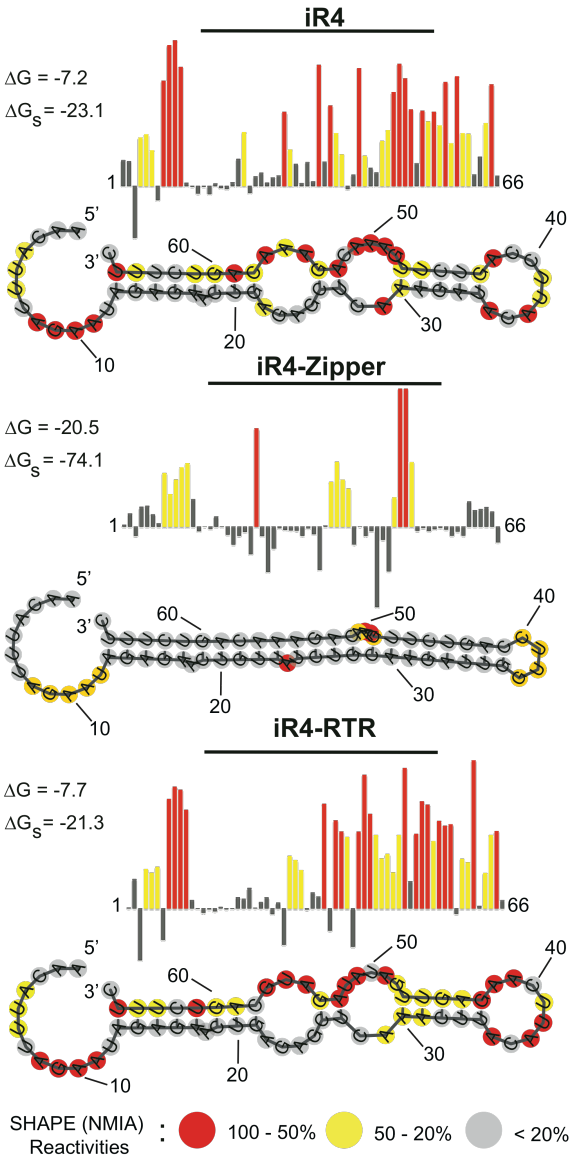


Figure 2-5

Fig. 5. SHAPE analysis of the 66-nt element and mutants. Above, SHAPE reactivities, determined from the sequencing gels in Fig S5. Residues with intensities between 0% and 20%, 20% and 50% and 50% and 100% are labeled gray, yellow and red, respectively. Bars show the amounts of modification at each position relative to the most highly modified nucleotide. Numbers denote nucleotide position. Below, SHAPE modification intensities mapped onto the predicted secondary structures; structures were generated using RNAfold [91]. Residues with intensities between 0% and 20%, 20% and 50% and 50% and 100% are

shown in gray, yellow and red, respectively. Both ΔG and ΔG_s (predicted with SHAPE Data) were generated using RNAstructure [84]. Numbers denote nucleotide position.

Supplemental Materials and Methods

SHAPE

Constructs were generated by inserting PCR generated fragments containing: T7 promoter – 5' linker – iR4 or iR4-Zipper or iR4-RTR – 3' linker with a RT primer binding site into the pUC19 vector between HindIII and XbaI restriction sites. Cloned plasmids were linearized with XbaI and used as templates for *in vitro* transcription (Ambion, Austin, TX). Transcriptions were purified on an 8% denaturing polyacrylamide gel (29:1 acrylamide:bis-acrylamide, 7M Urea). The RNA was resuspended in 0.5X TE buffer. RT primer oligos (Operon) were labeled using γ^{32}P-ATP and polynucleotide kinase and purified using G-25 MicroSpin columns (GE Healthcare, Piscataway, NJ). NMIA modification of RNA followed the method of Wilkinson et al. [83] except that we used 12 pmol of RNA. RNA was treated with 13mM NMIA in DMSO or DMSO alone for 45 min at 37°C, then ethanol precipitated and reverse transcribed. Sequencing reactions were analyzed using a 12% denaturing polyacrylamide gel (7M Urea). The gel images were produced using a Typhoon phosphorimager (GE Healthcare). Analysis and quantification were carried out using the SAFA program [92]. Band intensities in the NMIA (-) lane were subtracted from the (+) lane in order to determine the SHAPE (NMIA) reactivites, and the highest degree of NMIA modification was set to 100%.

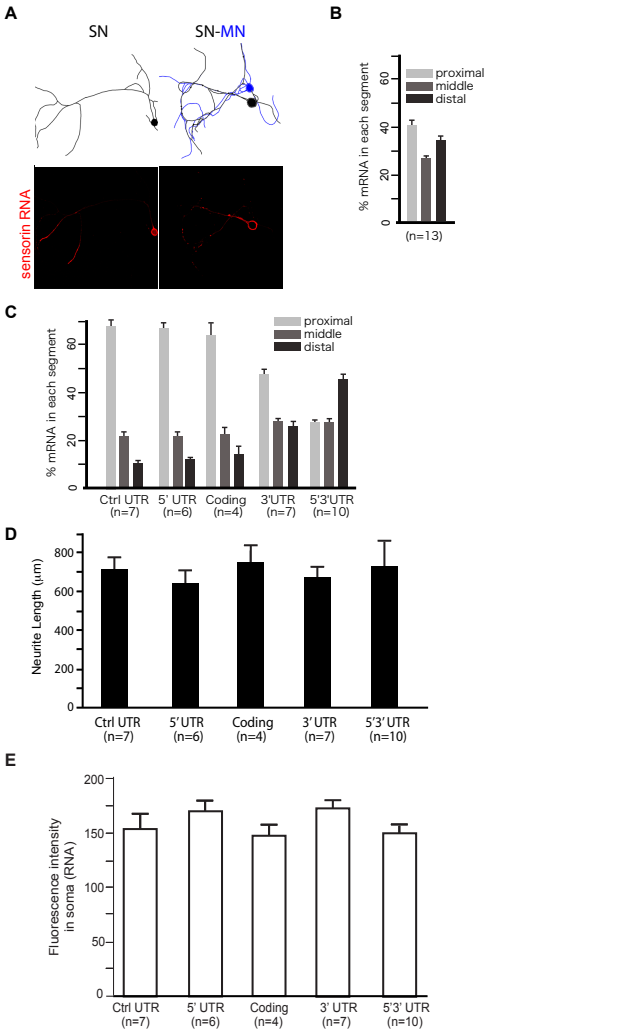


Figure S1

Figure 2-6

Fig. S1, related to figures 1 and 2. A, Localization of endogenous sensorin mRNA in isolated sensory neurons (SNs) and SNs co-cultured with target motor neuron (MN). Top panel, neuronal morphology traced from DIC images of cultured SNs and MNs (DIV 4). Black, SN, Blue, MN; bottom panel, fluorescent in situ hybridization (FISH) of endogenous sensorin mRNA. Note that sensorin mRNA is diffusely localized in distal neurites of isolated SNs but concentrated at varicosities in SNs co-cultured with target (LFS) MNs. Relevant to figs. 1 and 2. B, Quantification of sensorin mRNA localization in isolated SNs quantified as percent of mRNA

FISH intensity in proximal, middle, and distal neurites. C, Quantification of reporter mRNAs FISH intensity in the proximal, middle and distal neurites in isolated sensory neurons (DIV 4, this data completes the data presented in Fig 1; see Fig 2D for quantification of synaptic localization of sensorin mRNA). D, The average length of sensory neurites is similar across groups analyzed. This indicates that expression of the various reporters does not alter neuritic growth. E, The mean intensity of FISH signal in the soma is similar across groups analyzed. This indicates that the various reporters are expressed at relatively similar levels.

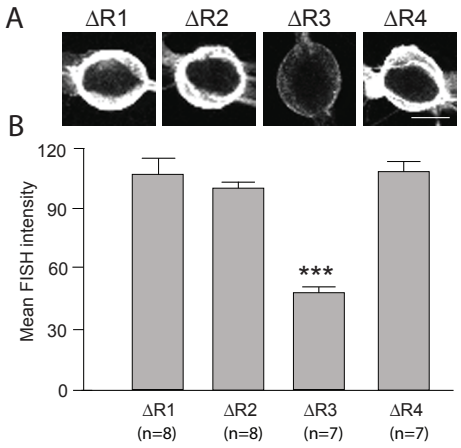


Figure S2

Figure 2-7

Fig. S2, Expression level of deletion mutants, related to fig 3. A, Confocal images of somatic FISH signals for ΔR1-4 in SN-MN cultures; B, quantification of fluorescence intensity of dendra2 FISH intensity. Only ΔR3 shows decreased RNA concentration, yet this construct retained synaptic localization.

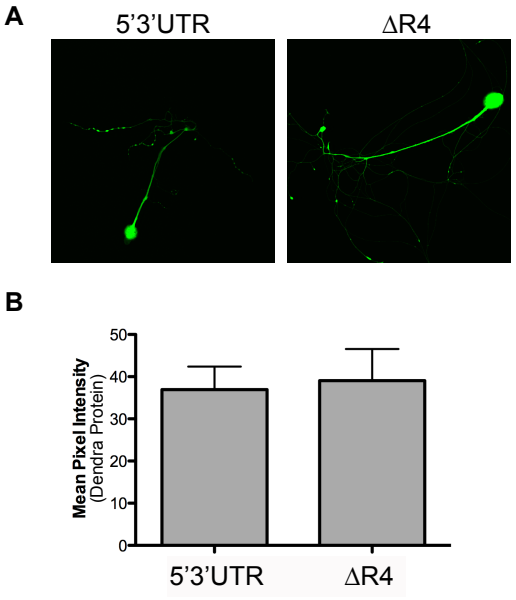


Figure S3

Figure 2-8

Figure S3. Deletion of the synaptic localization element does not alter dendra2 protein concentration, related to fig 3. A, Representative confocal images of dendra2 protein (green) in

sensory neurons (paired with motor neurons) expressing the 5'3'UTR reporter or the ΔR4 reporter. B, Quantification of mean pixel fluorescence intensity of dendra2 protein; student's t-test, ns.

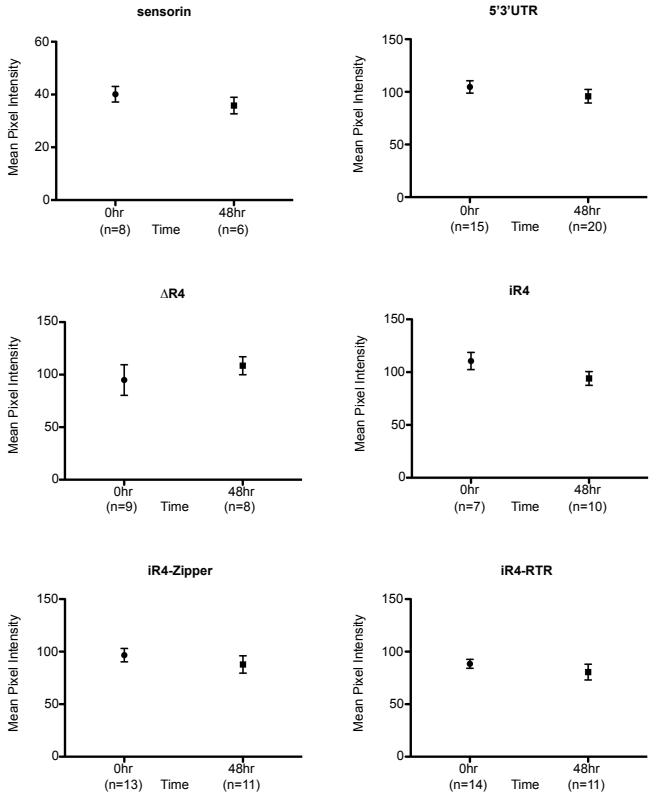


Figure S4

Figure 2-9

Fig. S4. Endogenous sensorin and reporter RNAs are stable in isolated neurites.

The stability of endogenous sensorin and select reporter RNAs was measured by injecting isolated sensory neurons (DIV 1) with pNEX vectors encoding sensorin reporters. Cell bodies were mechanical removed 48 hrs later, and neuritis were fixed at either 0 or 48hr after soma

removal and processed for FISH. Images were analyzed to determine the mean pixel intensity for each cell. The concentration of endogenous sensorin and of all reporter RNAs did not decrease significantly between 0 and 48 hrs. (student's t-test). n = number of cells analyzed.

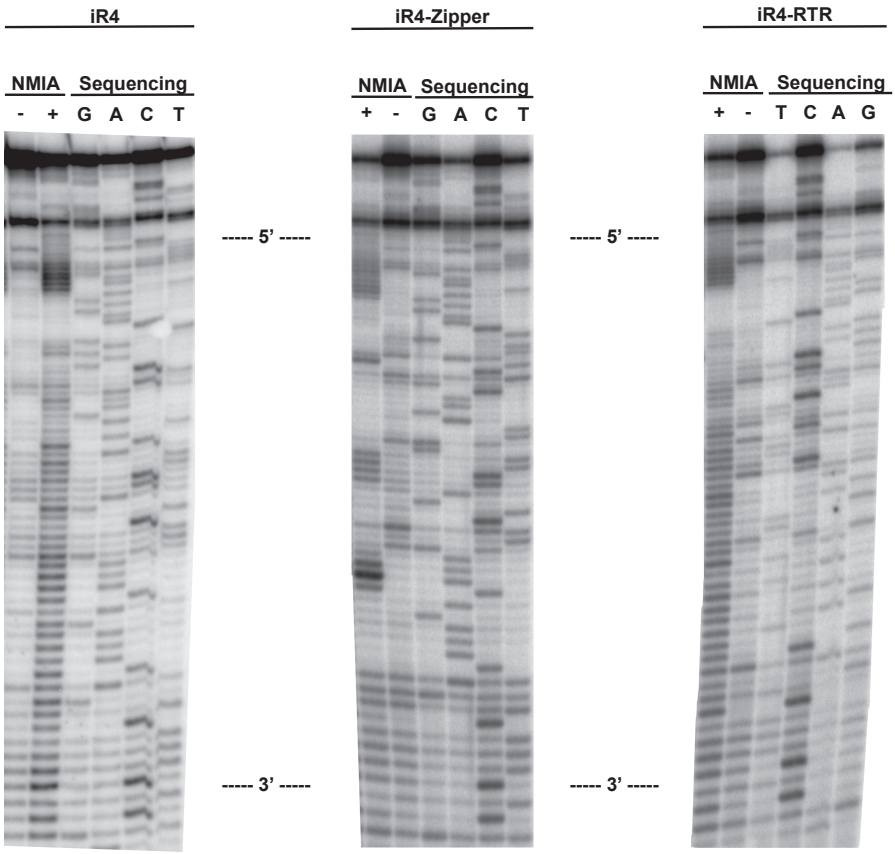


Figure S5

Figure 2-10

Fig. S5. SHAPE Polyacrylamide gels

Sequencing polyacrylamide gels for SHAPE [83] analysis of the RNA (from 5' to 3', as denoted) The lanes marked sequencing are lanes in which ddNTPs were added to the reverse transcription to cause chain termination and are exactly 1 nucleotide longer than the corresponding NMIA lanes. NMIA lanes (+) and (-) correspond to incubation with 130mM NMIA

for 45 min at 37°C and a control with NMIA omitted. Un-based paired segments are visible as regions of increased NMIA modification in the (+) lane as compared to the (-) lane.

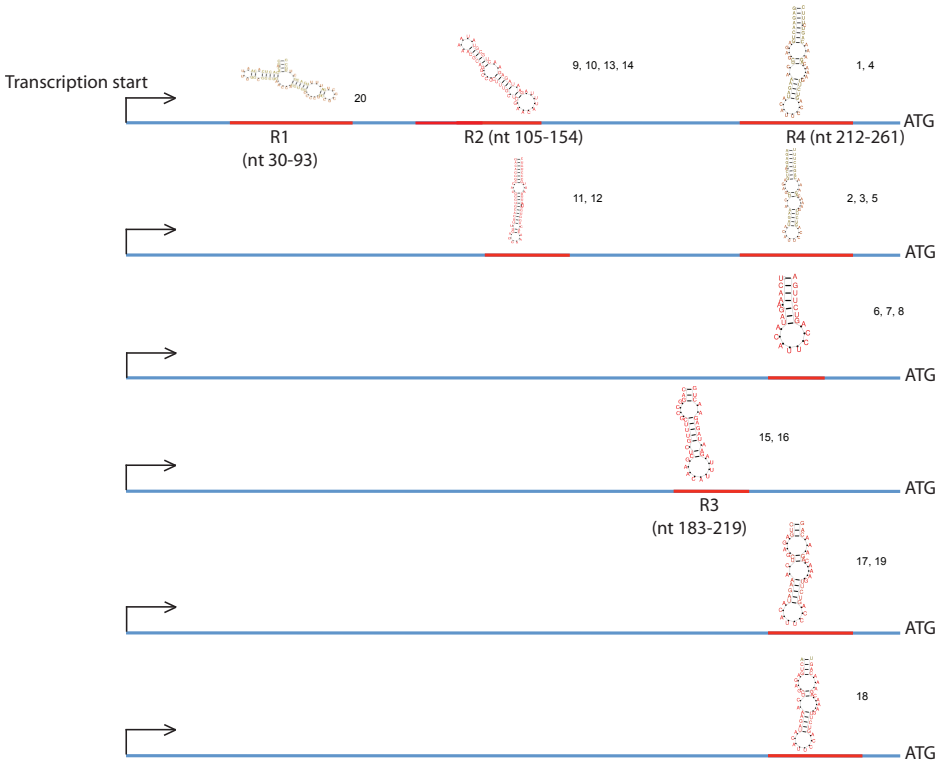


Figure S6

Figure 2-11

Figure S6: Secondary structures predicted using RNApromo [79] and their corresponding regions within the sensorin 5'UTR. RNApromo was used in the initial screen for secondary structure(s) within the 5'UTR. Relevant to figs. 3 and 4. A total of 20 candidate secondary structures were predicted using RNApromo software, numbered 1 through 20 in Figure S6. The structures that were located in the same region (red line) and that shared similar secondary structures were clustered together and a representative structure is displayed (e.g. structures with probability color code variation). After further secondary structure analysis with RNAfold [91], R1-4 were

chosen for the initial deletion screening analysis; the numbers in parentheses denote the specific positions of R1-4 within the 5'UTR of sensorin.

Chapter 3: Synapse to nuclear transport of ApCRTC is required postsynaptically during activity-dependent long-term facilitation of Aplysia sensory-motor synapses

Abstract

Neurons must be able to respond to a number of different synaptic stimuli, each generating a distinct transcriptional response. While much is know about gene expression in response to synaptic stimulation, the regulation of synapse to nuclear signals that trigger changes in transcription is less well understood. CREB-regulated transcriptional coactivator (CRTC) has been shown to be necessary for long-term forms of synaptic plasticity and to translocate from the synapse to the nucleus in response to synaptic stimulation. Our objectives were to determine; if CRTC signaling was present in *Aplysia Californica*, does CRTC respond differentially to multiple forms of long-term synaptic facilitation, and ask if CRTC was required in the pre- and/or postsynaptic cell. *Aplysia California* sensory-motor neuronal cultures can undergo multiple forms of synaptic facilitation and offer a high degree of molecular tractability. Our findings show that *Aplysia* express CRTC (ApCRTC), present in both the pre- and postsynaptic cell. ApCRTC accumulates in the nucleus in response to activity-dependent, but not activity-independent long-term facilitation. And finally, ApCRTC is required in the post-synaptic neuron during activity-dependent long-term facilitation. While both forms of long-term facilitation require transcription, CRTC is activated and required in response to only a subset of synaptic stimuli. This differential response would allow for activation of distinct downstream genes.

Introduction

Synaptic plasticity can be induced using a number of different stimulation paradigms, with long-lasting forms of synaptic plasticity requiring new gene expression[22,93]. The transcription factor cAMP Response Element Binding Protein (CREB) has been show to be central regulator of gene expression in response to a number of stimuli, including cell injury[94], development[95], and synaptic plasticity in learning and memory[52,54]. The requirement for CREB to induce synaptic plasticity in response to electrical, chemical or a combination of these stimuli implies common, core pathways for activating transcription are being recruited[23]. Mammalian studies of synaptic plasticity have focused on homosynaptic (Hebbian) plasticity, the contribution of neuromodulatory inputs difficult to dissect out due to the stimuli used to induce synaptic plasticity. Are distinct transcriptional signaling pathways involved in response to the specific stimuli? And what is the role for neuromodulation in driving transcription-dependent, long-term, synaptic plasticity?

Synapse to nuclear trafficking of transcriptionally active molecules would provide neurons the ability to integrate locally generated stimuli at distal sites into signals that activate and modify gene expression[57,64,96]. CREB-regulated transcriptional coactivator 1 (CRTC1), is a coactivator of CREB that is capable of activating CREB independently of phosphorylation of CREB at Ser 133[59,60]. CRTC1 has also been shown to be required for Hebbian forms of synaptic plasticity in rodents[63]. Our group recently demonstrated activity-dependent synapse to nuclear translocation of CRTC1 in hippocampal neurons[64].

In the present study we investigate the requirement for signaling pathways elicited in response to different forms of transcription-dependent, learning-related plasticity, specifically the involvement of CRTC. The invertebrate *Aplysia* californica was chosen as a model system because of the ability to induce both activity-dependent and –independent forms of synaptic plasticity[8]. Activity-independent long-term facilitation results from spaced neuromodulatory

stimulation, whereas activity-dependent long-term facilitation combines pre-synaptic depolarization with neuromodulatory input[97].

We cloned an *Aplysia* CRTC homolog and characterized its protein localization in sensory-motor neuronal co-cultures. Cultures were treated to induce activity-dependent or –independent synaptic plasticity and nuclear accumulation of CRTC assessed. Our findings reveal that only when activity-dependent synaptic plasticity is induced, does CRTC accumulate in the nucleus of both sensory and motor neurons. Consistent with these findings we found that blocking post-synaptic CRTC function through the use of a dominant negative construct completely abrogates increases in synaptic efficacy normally seen while inducing activity-dependent synaptic plasticity. Taken together our results show that synapse to nuclear trafficking of transcriptional regulators is a conserved modality for converting synaptic input into required gene expression changes. Using multiple forms of synaptic plasticity we show that CRTC is capable of responding to a subset of synaptic signals and is functionally required post-, but not presynapticaly. As such, CRTC is critically positioned to integrate synaptic information and in turn modulate the required transcriptional response.

Materials and Methods

Cloning ApCRTC. By BLASTing the Aplysia EST database with highly conserved domains found in mammalian CRTC1 protein, we isolated a strong region of homology in the carboxy-terminal of ApCRTC. Using an Aplysia cDNA library reverse transcribed from (pleural/pedal) ganglia, we designed several primer pairs to amplify the entire coding region of ApCRTC from the cDNA library. Subsequent sequence comparisons indicate that ApCRTC is closely related to the CRTC1 paralog.

Dominant negative ApCRTC (1-42). We used primer pairs (aagctagcgtcgacatggcgaaccccggaag and ttggatccttggcggcggcggcggcgtatcttggaggctccct) to amplify the coding region of ApCRTC that spans amino acids 1 to 42. This fragment was cloned into the pEGFP-N1 vector using the NheI and BamHI restriction site. The resulting fusion protein contains a 12 amino acid linker. The fusion construct was verified via sequencing and expression.

Full length ApTORC-Dendra (pNEX vector). We used primer pairs (aagtcgacccatggcgaaccctcggaagttt and aagtcgaccaatctgtccaacttgaaagt) to amplify full length ApCRTC (including a 20 amino acid linker) and fusing it to the amino-terminus of Dendra-N cloned within a pNEX vector at the SalI restriction site.

Custom ApCRTC antibody. A custom polyclonal antibody was raised against the last 22 amino acids of ApCRTC, sequence: MLTDASLVADPATEDTFKLDRL (Proteintech Chicago, IL). The peptide was n-terminally conjugated to ovalbumin and the resulting protein injected into

rabbits. Serum containing antibodies against ApCRTC was collected, and the antibody affinity purified.

Injection Constructs and Dyes. Experiments referring to the injection of plasmids encoding either GFP or DN-ApCRTC-GFP were expressed using the pNEX vector. Plasmids were generated by cloning PCR generated constructs into the multiple cloning site under the transcriptional control of the RSV-LTR minimal promoter. DN-ApCRTC-GFP was generated by directly fusing the first 54 amino acids of ApCRTC in frame to the N-terminus of the GFP coding sequence. VAMP-mcherry was generated as previously described (Wang 2009). Where dyes were injected, Alexa labeled dextran (10,000 MW, Invitrogen) were diluted to 10 mg/mL in water and Fast-Green (for visibility when injecting, 0.2% final concentration).

Aplysia Neuronal Cultures, Microinjection, Electrophysiology, and Stimulations. Aplysia SN–MN cultures were prepared as previously described (Zhao, 2009), for detailed protocols please visit: http://www.kelseymartinlab.com/protocols. Injection constructs and dyes were microinjected into the stated cell type on DIV 3 with expression/fluorescence confirmed on DIV4 via fluorescence microscopy. Synaptic connectivity was assessed by measuring EPSP amplitude between SN and target MN. SN and MN were impaled with sharp glass electrodes (10-20 mΩ resistance) filled with 2 M K-acetate. MNs were held at -80 mV, and SNs were held at -50mV. EPSPs were evoked by intracellular stimulation of the sensory neuron (2–8 nA for 5 ms) with a Grass (West Warwick, RI) S88 stimulator. EPSPs were recorded and measured with Axoscope 8.2 and pCLAMP 8 software (Axon Instruments, Union City, CA). To elicit activity-independent long-term facilitation (AI-LTF) of SN-MN synapses, cultures received five spaced applications of 5HT according the following protocol: five 5 min applications of 5HT (10 μM) with four intervening 20 min washes of L-15. Activity-Dependent long-term facilitation (AD-LTF) was

induced using a single 5 min application of high-potassium artificial seawater (ASW) plus 5HT (10 μM). All stimulations were performed at least 1hr after eliciting and EPSP.

Immunocytochemistry. DIV5 cells were fixed at room temperature with paraformaldehyde (4%) plus sucrose (30%) for 15 min, incubated with 0.3% Triton X-100 (Calbiochem) for 10 min followed by 50mM NH_4Cl (Fisher) for 15 min and finally blocked in 10% goat-serum for 1 hr. Following fixation and permeabilization, neurons were incubated in primary antibodies at various concentrations overnight at 4°C. Secondary antibodies (diluted to 1:500), and Hoechst nuclear dye (1:1000, Invitrogen) were incubated at room temperature for 1 hr. All antibodies were diluted in 10% goat serum.

Microscopy and Image Analysis. All images were taken on a scanning confocal LSM 700 (Zeiss, Thornwood, NY, USA) using the same acquisition settings. For determining nuclear-cytoplasmic ratios, Hoechst nuclear dye was used to find the imaging plane (corresponds to the plane with the largest Hoechst area and is a single confocal section). Image analysis was performed using ImageJ (Ref XX).

Statistical Analysis. GraphPad Prism software (La Jolla, CA, USA) was used for all statistical analysis. The specific statistical test performed for a given experiment are stated either in the figure legends or results section.

Results

CRTC is conserved in *Aplysia californica* and expressed in both sensory and motor neurons

To study CRTC function in Aplysia neurons, we first identified the *Aplysia* CRTC homolog. Using a combination of degenerate primers and 5' and 3' rapid amplification of cDNA ends (RACE) we cloned a cDNA that showed conservation across the protein coding region with murine CRTC1, which we call ApCRTC At the amino acid level, ApCRTC showed 30.4 % identity and 41.2% percent similarity with mouse CRTC1, and 23.7 and 23.4% identity and 35.1 and 35% similarity with mouse CRTC2 and 3 respectively. ApCRTC showed 31.3% identity and 41.4% similarity with human CRTC1, and only 19.6 and 19.9% identity and 27.9 and 27.2% similarity with *Drosophila melanogaster* and *Caenorhabitis elegans* CRTC, respectively. These results suggest that ApCRTC is most closely related to mammalian CRTC1. The highest levels of conservation were observed in the N-terminal 60 amino acids, which have been shown to constitute the dimerization and bZIP binding domain [59], in which ApCRTC shared 54.1% identity with mouse CRTC1.

A custom antibody was generated against the 15 C-terminal amino acids of ApCRTC, a sequence that is within the transactivation domain and that is also well conserved across species (86% identity with mouse CRTC1, and 63 and 62% identity with mouse CRTC2 and 3 respectively). To confirm the specificity of the antibody, we expressed ApCRTC C-terminally tagged with Dendra2 in HEK cells. As shown in Figure 1A the ApCRTC antibody detected Dendra2 tagged ApCRTC by immunoblotting. Bands of the appropriate molecular weight of tagged ApCRTC were recognized in ApCRTC-Dendra transfected but not in Dendra-transfected HEK cell lysates. Immunoblotting for ApCRTC using *Aplysia* pleural-pedal and abdominal ganglion lysates revealed bands of the expected molecular weight, which collapsed to the lowest molecular weight band after phosphatase treatment of the lysate (Fig. 1A), consistent

with what has been shown for mammalian CRTC1 [64]. While specific bands were detected by immunoblotting, other, non-specific bands were also noted. To ensure that our custom antibody specifically recognized ApCRTC *in-vivo* we expressed Dendra2 tagged ApCRTC in HEK cells and performed immunocytochemistry (Fig 1B). Staining for ApCRTC reveals strong signal in ApCRTC transfected HEK cells, whereas HEK cells transfected with Dendra alone show no signal. HEK cells were also transfected with mouse CRTC1 and 2, using the ApCRTC antibody revealed little or no signal respectively. We performed immunocytochemistry of sensory-motor co-cultures to determine the subcellular localization of ApCRTC (Figure 1C). We injected Alexa Fluors into both sensory and motor neurons so that we could distinguish between sensory and motor neurites that fasciculate together. Low magnification images revealed that ApCRTC is expressed highly in the soma and neurites of both sensory and motor neurons (Fig 1C-1), and higher magnification revealed ApCRTC immunoreactivity in the Alexa-fluor labeled distal neurites of both sensory and motor neurons (Fig 1C-2). To determine whether ApCRTC localized to synapses, as we have recently reported in rodent hippocampal neurons [64], we performed ApCRTC immunocytochemistry in sensory-motor cocultures in which we expressed VAMP-mCherry in the sensory neuron to label presynaptic varicosities. High magnification images of *Aplysia* synapses, defined as concentrations of VAMP clusters, reveals that ApCRTC is present at synapses as well as in the adjacent neurite (Fig 1D1-4). Initial qualitative characterization of ApCRTC cellular localization indicated that little to no protein was present within the nucleus. To quantify the amount of ApCRTC present in the nucleus, mock stimulated co-cultures treated with artificial sea-water (ASW) were used and the mean pixel intensity of nuclear ApCRTC immunoreactivity measured. Hoechst dye was used to find the imaging plane and also to generate a nuclear mask. The mean pixel intensity of nuclear ApCRTC was 7.16±0.44, compared to 27.68±1.09 for somatic signal, confirming that in unstimulated co-cultures very little ApCRTC protein is present in the nucleus (Fig 1E). Together, these results

reveal that CRTC is conserved in *Aplysia*, that it is expressed in both sensory and motor neurons, that it localizes throughout the somatic and neuritic cytoplasm of sensory and motor neurons, and that it is present within the presynaptic varicosity.

Activity-independent long-term facilitation induced by spaced applications of serotonin (5HT) does not trigger nuclear accumulation of ApCRTC.

Previous studies have indicated that long-term facilitation (LTF) of Aplysia sensory-motor neurons induced by five spaced applications of 5HT requires CREB-mediated gene expression in the sensory neuron [24,52,55]. To determine whether this CREB-dependent program of gene expression also requires the activity-induced nuclear accumulation of ApCRTC we performed ApCRTC immunocytochemistry in cultures immediately following induction of LTF by spaced applications of 5HT (1 hr 45 min after the beginning of the stimulation protocol); control cultures received spaced applications of L15 medium. The mean pixel intensity of ApCRTC immunoreactivity was calculated in both the nucleus and somatic cytoplasm of sensory and motor neurons, and nuclear accumulation was expressed as the ratio of nuclear to cytoplasmic mean pixel intensity. As shown in Figure 2A, no statistically significant increase in nuclear accumulation of ApCRTC was observed in either sensory or motor neurons (Fig. 2B). These results indicate that the program of gene expression induced by CREB in the sensory neuron during 5HT-induced LTF does not require ApCRTC.

ApCRTC accumulates in the nuclei of both sensory and motor neurons during activity-dependent long-term facilitation.

We previously reported that the synapse to nuclear translocation of CRTC1 during long-term potentiation of rodent hippocampal synapses depended on calcium influx through glutamate receptors[64]. Unlike hippocampal LTP, 5HT-induced LTF of *Aplysia* sensory-motor

synapses does not involve stimulus-induced elevations in intracellular calcium in either sensory or motor neuron, but rather involves elevations in cAMP in the sensory neuron[98,99]. This type of plasticity has been called heterosynaptic, as opposed to homosynaptic plasticity[97]. Another form of LTF, termed activity-dependent LTF of *Aplysia* sensory-motor synapses (AD-LTF, [49]) has been described that is induced by pairing of depolarization with 5HT application. Given the requirement for glutamatergic stimulation and calcium influx for CRTC1 nuclear import in hippocampal neurons, we thus asked whether AD-LTF triggered nuclear accumulation of ApCRTC. We induced AD-LTF of sensory-motor synapses by incubating cultures for five minutes in Artificial Seawater (ASW) containing high-potassium (100 mM) and 5-HT (10 μM). As this protocol, which we refer to K+5-HT, has been used to study intermediate-term facilitation (ITF) but not LTF in cultured Aplysia neurons[100], we first confirmed that it would in fact induce LTF of sensory-motor co-cultures. As shown in Figure 3A and 3B, a five min incubation K+5-HT resulted in a significant (187.5±42.04%) increase in the amplitude of excitatory post-synaptic potential (EPSP) between the sensory and motor neurons at 24 hrs. In control experiments, we found that a 5 min application of high potassium (Hi-K) did not produced LTF of the EPSP at 24 hrs (Fig 3A). A single 5 min pulse of 5-HT has been previously shown to be unable to result in LTF at 24 hrs[24,47]. There was no statistically significant difference in the baseline EPSPs between control and experimental groups.

The nuclear accumulation of ApCRTC was assayed by immunocytochemistry with ApCRTC antibodies. Co-cultures were fixed immediately after incubation with K+5-HT (or after incubation with ASW in controls). The mean pixel intensity of ApCRTC immunoreactivity was calculated in the nucleus and somatic cytoplasm of both sensory and motor neurons and nuclear accumulation was expressed as the ratio of nuclear to cytoplasmic mean pixel intensity. As shown in Figure 3, we detected increased nuclear-cytoplasmic ratios of ApCRTC in both sensory and motor neurons in cultures receiving K+5-HT as compared to ASW (Fig 3C and D).

Thus, in contrast to activity-independent LTF induced by spaced applications of 5HT, AD-LTF induced by KCl-induced depolarization in combination with 5HT induces nuclear translocation of ApCRTC into the nuclei of both sensory and motor neurons.

ApCRTC signaling is only required in the motor neuron during AD-LTF.

To investigate the functional requirement of ApCRTC during AD-LTF, we generated an expression construct in which the bZIP (CREB) binding domain of ApCRTC was fused to GFP. This construct was designed to act as a dominant-negative, as the construct lacks the trans-activation domain necessary for activating transcription of downstream target genes thus inhibiting endogenous ApCRTC signaling; homologous constructs have been used as dominant negative CRTC1 in rodent hippocampal neurons[59,62,63].

To test the requirement for ApCRTC signaling during AD-LTF, we expressed the dominant-negative construct (DN-ApCRTC) in either the sensory or motor neuron of COCXs by injecting the construct on DIV3. Twenty-four hours later, DIV4, we confirmed expression by imaging GFP signal in the appropriate cell type. Baseline EPSP amplitude was measured on DIV4, and neurons were stimulated with K+5-HT, or with ASW for controls. On DIV5, twenty-four hours after stimulation, EPSPs were again recorded and compared with baseline. The results show that expression of DN-ApCRTC in the motor neuron, but not in the sensory neuron completely blocked AD-LTF elicited by K+5-HT (Fig 4A and 4B).

To ensure that the dominant-negative construct did not disrupt LTF by sequestering CREB, and thereby disrupting CREB-dependent transcription, we expressed DN-ApCRTC in both sensory and motor neurons and stimulated COCXs with 5x5-HT to induced activity-independent LTF. This form of LTF requires CREB-dependent transcription in the sensory neuron[55], and thus DN-ApCRTC in the SN should disrupt 5HT-induced LTF if it functions by

sequestering CREB. As shown in figure 4C and 4D, we found that injection of DN-ApCRTC into either sensory or motor neuron had not effect on LTF induced by 5x5-HT.

Taken together, our functional experiments using DN-ApCRTC indicate that ApCRTC is required in the motor but not the sensory neuron during AD-LTF, and further that it is not required for activity-independent, heterosynaptic LTF induced by spaced applications of 5-HT.

Discussion

The present investigation demonstrates that CRTC is conserved in *Aplysia Californica* and that ApCRTC signaling is required in the motor neuron in response to a subset of stimuli that activate long-term facilitation of sensory-motor synapses. In support of this conclusion we have shown that ApCRTC accumulates in the nucleus of both sensory and motor neurons after stimulation of COCXs using a protocol designed to produce a activity-dependent form of long-term synaptic facilitation, combining neuronal activity with neuromodulatory input. In contrast, using the classical application of five spaced pulses of serotonin to produce an activity-independent form of long-term facilitation did not result in the accumulation of ApCRTC in the nuclei of either sensory of motor neurons. Moreover, ApCRTC signaling appears to be selectively required in the motor neuron for forms of long-term facilitation, specifically in an activity-dependent form of long-term facilitation, but not in a form of heterosynaptic facilitation that is independent activity. Thus demonstrating that independent transcriptional programs, those involving ApCRTC and those that do not, can be activated in response to specific synaptic stimuli.

Interestingly while we see nuclear accumulation of ApCRTC in both the sensory and motor neuron, in response to activity-dependent LTF, ApCRTC signaling is only required in the motor neuron for induction of LTF. AD-LTF involves depolarization of the sensory neuron resulting in neurotransmitter release onto the motor neuron as well as activation of g-protein coupled receptors (GPCRs) by 5-HT[101]. Increased intracellular calcium, required for CRTC translocation[64], would result from activation of NMDA receptors and/or Gq type GPCRs in the motor neuron. In contrast, increased intracellular calcium in the sensory neuron would only result from Gq GPCRs being activated. Intracellular calcium increases via Gq GPCR could be sufficient for translocation of ApCRTC, but not to produce ApCRTC competent for signaling required for AD-LTF induction. It is possible this difference accounts for differential signaling

requirement of ApCRTC in the sensory and motor neurons, implying the source of calcium is critical for ApCRTC signaling. Another possibility is that ApCRTC has a yet unknown function in the senosory, which is not required for AD-LTF.

Both activity-dependent and activity-independent forms of long-lasting synaptic facilitation have been described in Aplysia[8] and been shown to require CREB. Overlapping mechanisms to induce long-term facilitation at sensory-motor synapses exist, however not all pathways are shared. For example, LTF observed after 5x5-HT is not abrogated by NMDAR blockade, whereas calcium entry through NMDAR is required for activity-dependent forms of LTF[49,102]. Activation of cellular pathways, in AD-LTF, stimulation of postsynaptic NMDARs and elevation in postsynaptic calcium likely converge to act on and dephosphorylate ApCRTC, allowing for it accumulate in the nucleus. With 5x5-HT, presynaptic calcium levels do not elevate to the same degree and thus may not be enough to trigger ApCRTC nuclear accumulation[98]. Nuclear accumulation of ApCRTC could provide a mechanism to regulate downstream transcriptional output in response to a subset of stimuli.

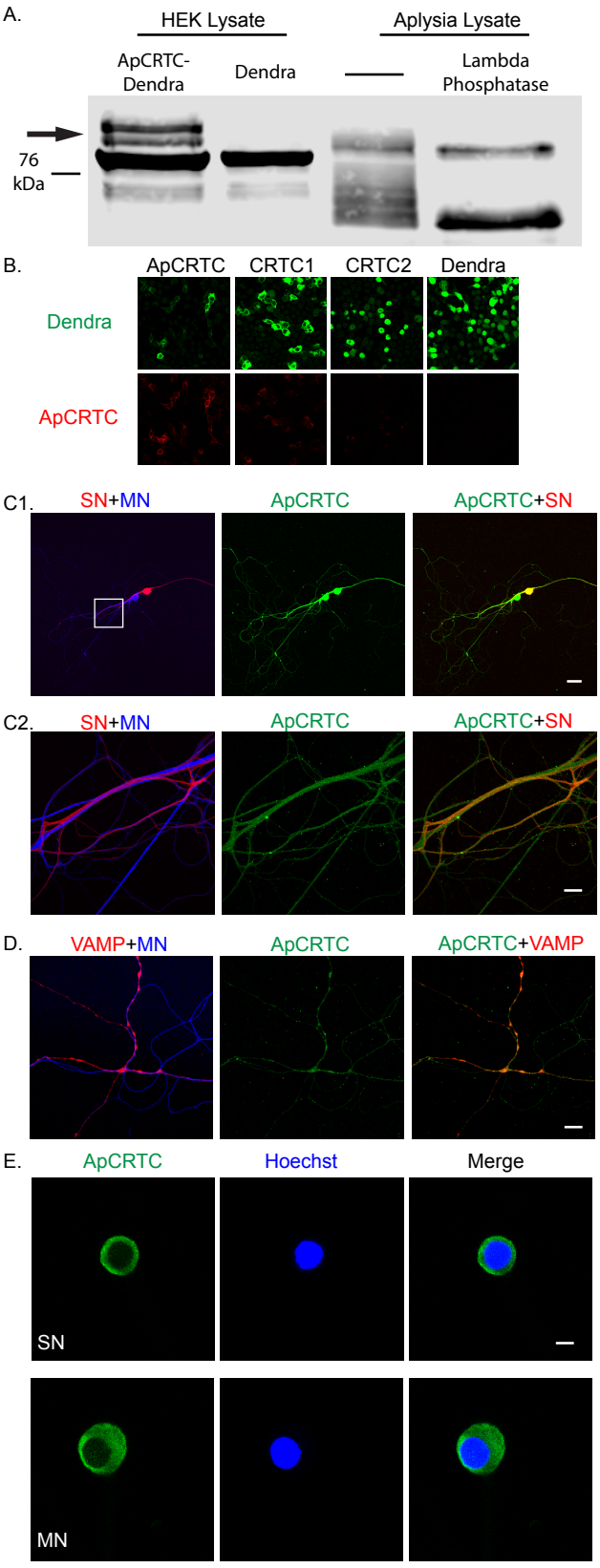
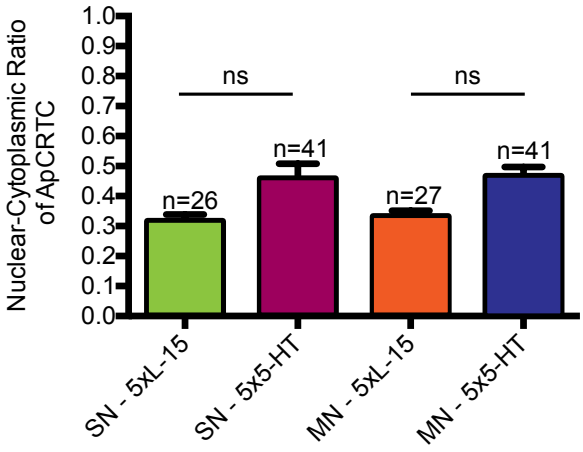

A.
HEK Lysate
Aplysia Lysate
ApCRTC-Dendra
Dendra
Lambda Phosphatase
76 kDa
B.
ApCRTC
CRTC1
CRTC2
Dendra
Dendra
ApCRTC
C1.
SN+MN
ApCRTC
ApCRTC+SN
C2.
SN+MN
ApCRTC
ApCRTC+SN
D.
VAMP+MN
ApCRTC
ApCRTC+VAMP
E.
ApCRTC
Hoechst
Merge
SN
MN

Figure 3-1

Fig. 1. CRTC is conserved in Aplysia and expressed in both Sensory and Motor Neurons. A. Immunoblot using a custom generated antibody against the c-terminal end of ApCRTC: Lane 1, lysate from HEK cells transfected with ApCRTC-Dendra shows the antibody recognizes bands corresponding to the increased molecular weight of ApCRTC tagged with Dendra. Lane 2, lysate from HEK cells transfected with Dendra only. Lane 3, *Aplysia* neuronal lysate (pleural-pedal and abdominal ganglia). Lane 4, *Aplysia* neuronal lysate treated with lamda phosphatase. The custom antibody recognizes a series of bands, consistent with multiple phosphorylated forms of CRTC1 (Ch'ng 2012) that collapses to a single band when lysate is treated with phosphatase. B, HEK cells were transfected with Dendra tagged ApCRTC, mouse CRTC1, mouse CRTC2 or Dendra alone. Top, images show the expression of the Dendra constructs, bottom, immunocytochemistry staining for CRTC using the custom *Aplysia* ApCRTC antibody. C, Images showing Aplysia SN-MN COCXs processed for ICC, ApCRTC (Green), SN injected with Alexa 555 dye (Red) and MN injected with Alexa 647 dye (Blue). C1, 10X images of a representative SN-MN COCX, scale bar 100μm, yellow indicates ApCRTC in the SN. C2, 63X image of boxed region of COCX in C1, colors are the same as in C1, scale bar 20μm. D, 63X image of Aplysia SN-MN COCX processed for ICC showing ApCRTC (Green), SN expressing VAMP-mCherry (Red) and MN injected with Alexa 647 dye. E, 63X Images of SN and MN stained for ApCRTC, green, and Hoechst dye labeling the nucleus, blue, scale bar 20μm.

A.


Nuclear-Cytoplasmic Ratio of ApCRTC
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ns
ns
n=26
n=41
n=27
n=41
SN - 5xL-15
SN - 5x5-HT
MN - 5xL-15
MN - 5x5-HT

B.

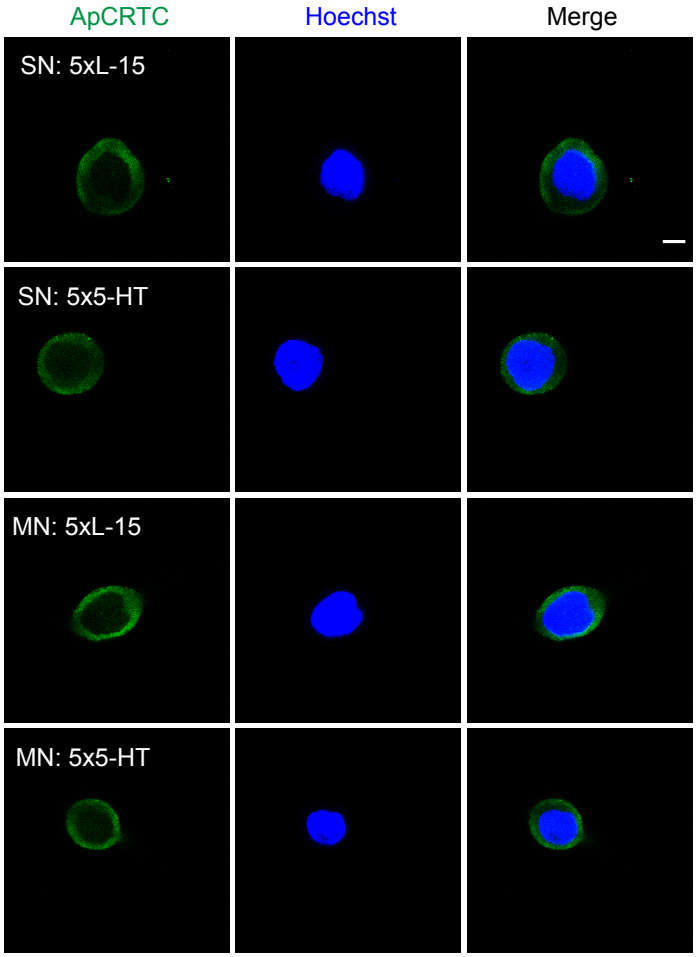

ApCRTC
Hoechst
Merge
SN: 5xL-15
SN: 5x5-HT
MN: 5xL-15
MN: 5x5-HT

Figure 3-2

Fig. 2. Five spaced pulses of serotonin do not cause ApCRTC to accumulate in the nucleus. A, DIV4 Aplysia SN-MN COCXs were treated with either 5xL-15 or 5x5-HT. Immediately after treatment the cultures were fixed using 4%PFA/30% Sucrose then proceed for ICC, staining for ApCRTC. Using a single confocal image the nuclear-cytoplasmic ratio of ApCRTC was calculated. Neither the sensory or motor neuron shows an increase in nuclear-cytoplasmic ratio after 5x5-HT treatment, as compared to 5xL-15 controls. B, representative 63X images of neurons used to calculate the nuclear-cytoplasmic ratio in A, scale bar 20μm.

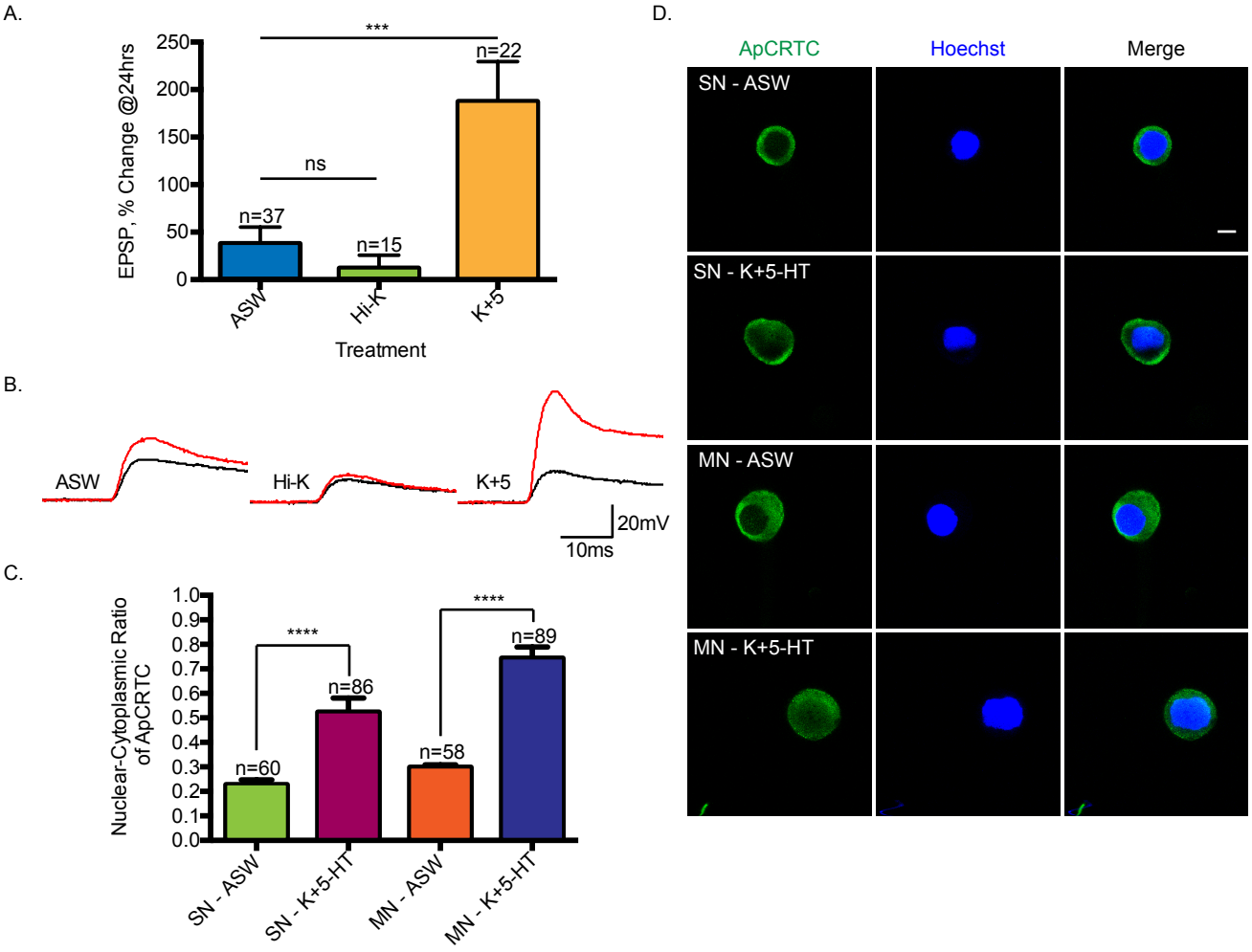

A.
EPSP, % Change @24hrs
250
200
150
100
50
0
***
ns
n=22
n=37
n=15
ASW
Hi-K
K+5
Treatment
B.
ASW
Hi-K
K+5
20mV
10ms
C.
Nuclear-Cytoplasmic Ratio of ApCRTC
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
****
****
n=60
n=86
n=58
n=89
SN - ASW
SN - K+5-HT
MN - ASW
MN - K+5-HT
D.
ApCRTC
Hoechst
Merge
SN - ASW
SN - K+5-HT
MN - ASW
MN - K+5-HT

Figure 3-3

Fig. 3. A single 5-minute treatment of High Potassium plus serotonin (K+5) is able to induce Long-Term Facilitation (LTF) and cause ApCRTC to accumulate in the nucleus.
A, to test the ability of K+5 to induce LTF in Aplysia SN-MN COCXs baseline EPSP values were taken from DIV4 cultures. Cultures were then treated with either ASW or K+5 for 5min after removing the culture media, washed with ASW and then replacing the culture media. 24-hours later EPSP values were taken. Graphed is the percent EPSP change after treatment, calculated by $((EPSP_{Treatment} - EPSP_{Baseline})/EPSP_{Baseline})*100$. B, Representative traces of COCXs stimulated in A. C, DIV4 Aplysia SN-MN COCXs were treated with either ASW or K+5 for 5min. Immediately after treatment the cultures were fixed using 4%PFA/30% Sucrose then proceed for ICC, staining for ApCRTC. Using a single confocal image the nuclear-cytoplasmic ratio of ApCRTC was calculated. Both the sensory and motor neuron shows an increase in nuclear-cytoplasmic ratio after K+5 treatment, as compared to ASW controls. D, representative 63X images of neurons used to calculate the nuclear-cytoplasmic ratio in C, scale bar 20μm.

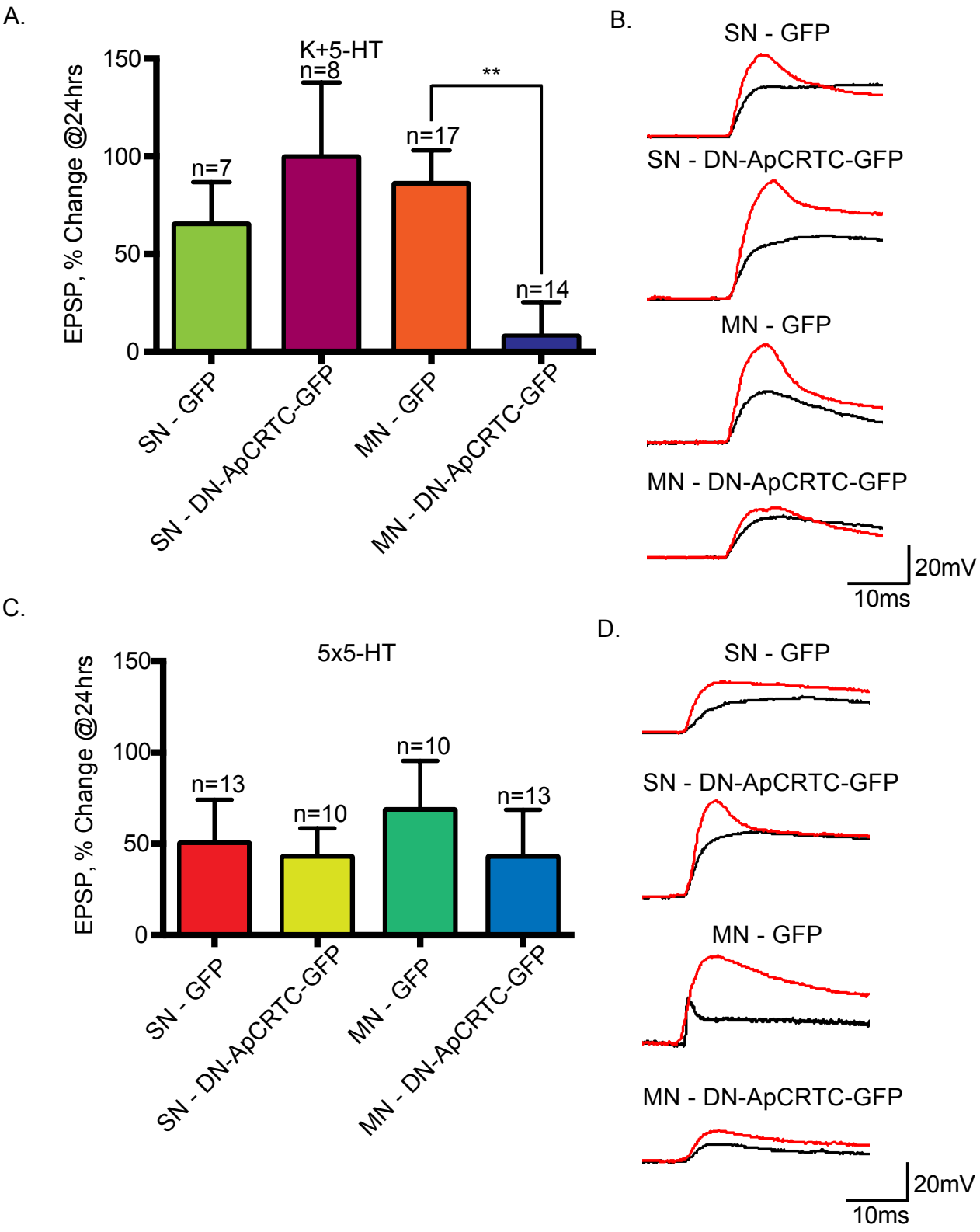

A.
EPSP, % Change @24hrs
150
100
50
0
K+5-HT
n=8
n=7
n=17
n=14
**
SN - GFP
SN - DN-ApCRTC-GFP
MN - GFP
MN - DN-ApCRTC-GFP
B.
SN - GFP
SN - DN-ApCRTC-GFP
MN - GFP
MN - DN-ApCRTC-GFP
20mV
10ms
C.
EPSP, % Change @24hrs
150
100
50
0
5x5-HT
n=13
n=10
n=10
n=13
SN - GFP
SN - DN-ApCRTC-GFP
MN - GFP
MN - DN-ApCRTC-GFP
D.
SN - GFP
SN - DN-ApCRTC-GFP
MN - GFP
MN - DN-ApCRTC-GFP
20mV
10ms

Figure 3-4

Fig. 4. Inhibition of ApCRTC results in the attenuation of EPSP amplitude after K+5 but not 5x5-HT induced LTF.

In order to test if ApCRTC is functionally required for LTF a dominant negative ApCRTC construct, fused to GFP, was expressed in either Aplysia sensory or motor neurons. DIV3 cultures were injected with either DN-ApCRTC-GFP or a GFP control plasmid into the SN or MN. After 24hrs baseline EPSP values were taken. A, Shows group data of SN-MN COCXs treated with K+5 for 5min, after removing the culture media, washed with ASW and then replacing the culture media. 24-hours later EPSP values were taken. Graphed is the percent EPSP change after treatment, calculated by $((EPSP_{Treatment} – EPSP_{Baseline})/EPSP_{Baseline})*100$. Each bar graph is labeled using the cell type and construct that was injected. Only when the DN-ApCRTC-GFP is injected into the MN is the increase in EPSP amplitude blocked after K+5 treatment, as compared to MNs injected with GFP and treated with K+5. B, Representative traces of COCXs stimulated in A. C, same as A, except that SN-MN COCXs are treated with 5x5-HT and washed with L-15. Injecting DN-ApCRTC-GFP into either the SN or MN failed to block an increase in EPSP amplitude after 5x5-HT treatment, as compared to their respective GFP injected controls. D, Representative traces of COCXs stimulated in C.

Chapter 4: Discussion and Concluding Remarks

One of the most important findings to the study of learning and memory, and to the field of LTP, was the discovery that LTP requires new transcription and translation in order to persist (L-LTP). Since its initial discovery nearly 30 years ago, a vast number of studies have aimed to understand the pathways and molecular mechanism that underlie this requirement. In the work presented within this dissertation, we have sought to provide insight into these molecular mechanisms by experimentally determining where in the neuron the new gene products (mRNA and proteins) are necessary and how synaptic input activates transcription of these genes.

The major findings of the second chapter are that reporters containing the 5' and 3' untranslated regions (UTRs) of sensorin, a protein required for long-term memory formation in *Aplysia*, recapitulate the endogenous sensorin mRNA localization pattern. More specifically, a 66-nt region of the 5'UTR is both necessary and sufficient to drive synaptic localization of reporter mRNA. The synaptic localization of the reporter mRNA is dependent on the secondary structure of the RNA, confirmed by selective 2'- hydroxyl acylation analyzed by primer extension (SHAPE). In the third chapter, we identified the *Aplysia* ortholog of CRTC (ApCRTC) and showed that it was expressed in both sensory and motor neurons. We characterized a stimulation paradigm in which depolarization was paired with 5HT to generate activity-dependent in long-term synaptic facilitation of *Aplysia* SN-MN co-cultures. This same protocol was able to drive nuclear accumulation of ApCRTC in both the SN and MN. When co-cultures were treated with five-spaced pulses of serotonin, resulting in purely heterosynaptic, activity-independent long-term facilitation, ApCRTC did not accumulate in the nucleus of either the SN or MN. Finally, when a dominant-negative ApCRTC construct was expressed in the MN synaptic plasticity induced with the combined protocol was blocked. However, when the dominant-negative was expressed in the SN, long-term activity-dependent facilitation was intact. Expresion of the dominant-negative in either the SN or MN did not block long-term activity-

independent synaptic plasticity induced by spaced applications of 5HT. This was expected since spaced 5HT stimulation did not result in nuclear accumulation of ApCRTC.

mRNA localization

The identification of the synaptic localization elements of sensorin, presented in chapter two, was initiated in order to determine how mRNAs could be spatially restricted to synapses. While other studies of mRNA localization in neurons had existed prior to our study, to our knowledge, none had shown examples of mRNAs localizing at the level of individual synapses[44]. The *Aplysia* SN-MN co-culture system provided experimental paradigm that, unlike in mammalian neuronal cultures, provided the ability to observe synapses between a single pair of pre- and postsynaptic neurons. Sensorin was chosen to study mRNA localization in neurons based on previous work in the Martin lab. It had been shown that sensorin mRNA was part of set of distally localized transcripts, and that sensorin mRNA would concentrate at synapses in response to synapse formation. Furthermore de novo translation of sensorin mRNA was found to be required for synapse formation/stabilization[30,75]. From an experimental point of view, an advantage of characterizing sensorin mRNA localization is that the transcript is only expressed in sensory neurons, and thus one can use FISH to study its localization without any interfering signal from the motor neuron. In 2009, work from the Martin Lab showed that a translational reporter, containing the 5' and 3'UTRs of sensorin fused to the coding sequence of the photoconvertable fluorescent protein dendra2, had an identical localization pattern to that of endogenous sensorin[77]. This work allowed us to focus on the UTRs as the regions that could contain the localization elements necessary to drive concentration of the mRNA at synapses. The specific approach we took to identify possible localization elements resulted from previous work in mRNA localization, which showed identified sequences predicting to form stem-loop secondary structures[103]. After discovering that the 5'UTR was required for synaptic localization of sensorin, secondary structure prediction was used to identify step-loop structures.

Identification of a 66-nt element in the 5'UTR of sensorin showed that mRNA localization to synapses is a possible mechanism for a neuron to restrict gene expression[104].

As stated in chapter one, work from other asymmetric cell types had given a basic framework for how the localization elements present in mRNAs could be then bound by trans-acting factors (RNA binding proteins, RNABPs)[26]. It had been our intention to follow up the sensorin synaptic localization element (LE) findings by then determining the RNABPs that recognized this element and trafficked it to the synapse. Preliminary data from electromobility shift assays (EMSA) showed that a protein(s) present in Aplysia neuronal lysate recognized a radiolabeled probe corresponding to the 66-nt sensorin synaptic LE. The mutant LEs which had been used to assay synaptic localization requirements served as positive and negative controls, depending on their ability to localize to synapses (Meer et al. 2012). Mutant LEs that failed to localize reporter mRNA to synapses failed to show similar recognition of protein(s) present in *Aplysia* neuronal lysate, suggesting that the sensorin synaptic LE was being specifically recognized (Figure 4-1).

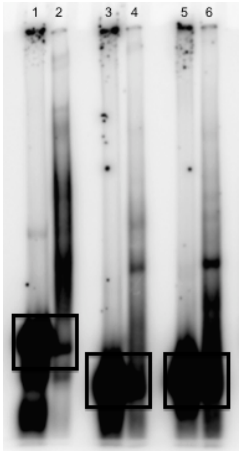


Figure 4-1

Protein(s) present in *Aplysia* neuronal lysate specifically recognize the sensorin synaptic LE. Lanes 1,2: radiolabeled synaptic LE only, and incubated with lysate. Lanes 3,4:

radiolabeled mutant LE only, and incubated with lysate. Lanes 5,6: radiolabeled scrambled probe only, and incubated with lysate. Free probe is boxed.

Subsequent EMSAs corroborated the specificity of the sensorin LE interaction; incubation of unlabeled probe in the reaction was able to compete away the specific decreased mobility band. These initial results had provided enough evidence that further effort into identifying the specific protein(s) recognizing the sensorin LE was warranted (unpublished data). Using an RNA-aptamer based system, which would allow the sensorin synaptic LE to be used as bait, our goal was to pull-down proteins present in *Aplysia* neuronal lysate. This approach had been used previously, with success, in finding the proteins that specifically recognized the *Drosophila* LEs of the transcripts K10, hairy, gurken and I factor[105]. After optimization of the protocol, we showed using silver-stained gels that proteins from *Aplysia* neuronal lysate could indeed be pulled-down. Our goal had been to find specific RNABPs by subtracting out proteins that also came down with mutant sensorin LEs (those that failed to synaptically localize). Eluted samples were to be subjected to Multidimensional Protein Identification Technology (MudPIT) mass spectrometry for identification of all proteins present in affinity-purified lysates. Unfortunately at this point in the project a major roadblock was reached. Mass spectrometry produces a set of MS/MS spectra that act as signatures of particular peptides; these spectra are normally compared against *in-silica* trypsin digested peptides corresponding to entire proteome of your species of interest. In this way MudPIT can identify proteins present in a complex sample. While work on sequencing the *Aplysia* genome had been ongoing for several years at the time this project was started, there is still yet to exist a complete, annotate *Aplysia* genome. This would have been necessary in order to continue so as to identify all known protein coding regions in *Aplysia,* so they could have been subjected to *in silica* trypsin digestion for comparison against the MS/MS spectra generated from affinity-purified samples. Although

determining the proteins present in *Aplysia* lysate that bound the sensorin synaptic LE was not possible, we did explore the possibility that RNABPs that recognize the LE are conserved. Using the aptamer-based system described above, we tested the ability of the sensorin synaptic LE to pull-down proteins present in mammalian (mouse) brain lysate. Even though initial experiments were unsuccessful in elucidating proteins of interest, it is possible that with further troubleshooting positive results could be obtained.

To date most, if not all, mRNA localization studies have used fixed samples and looked at one transcript at a time. The information provided has been valuable in forming an understanding of how spatial gene regulation can serve to restrict activation of molecular mechanisms within the cell but lacking in giving us the ability to study RNA dynamics. An example of just how helpful studying RNA dynamics in response to LTP can be comes from the study of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein). After stimulation, samples were fixed at 30min, 1hr and 2hrs, and processed for *in situ* hybridization to visualize Arc mRNA[106]. The ability to follow the mRNA over time, even using large time windows, provided us with a better idea of the mechanism by which Arc mRNA trafficked to activated synapses. A major question that still persists in the field of LTP is: how are newly synthesized transcripts enriched at their final subcellular localization? Two main possibilities exist; the first being that mRNAs are specifically targeted to the activated synapses through their localization sequences and RNABPs, and the second being that mRNAs are trafficked out distally throughout the neuron and are then anchored only at activated synapses. New techniques that enable live cell imaging of RNA dynamics hold the promise of allowing scientist to answer this question. One of these techniques involves the use of what are known as exciton-controlled hybridization-sensitive fluorescent oligodeoxynuclotide (ECHO) probes. ECHO probes can be designed to hybridize with a specific mRNA and contain a dye that is covalently linked to the oligo. What makes ECHO probes so unique from traditional oligo

probes used for fixed fluorescence in-situ hybridization (FISH) samples is that ECHO probes in a non-hybridized state emit almost zero fluorescence. It is only when ECHO probes hybridize with their specific target sequence that they will strongly emit fluorescence. While these ECHO probes have been already proven to provide excellent specificity in fixed samples, able to distinguish single nucleotide mismatches, the fact that these probes don't require non-hybrized probe to be washed away means they can be used to track RNA dynamics in the live cell[107]. Another new technique that promises to allow for RNA tracking *in vivo* comes from Dr. Samie Jaffrey's group; here a specific RNA sequence (termed an aptamer) can be incorporated into your RNA of interest. The modified RNA can then be expressed in the cell and detected by the addition of a fluorophore, DMHBI, which recognizes and binds to the aptamer, only then emitting fluorescence. Much like the ECHO probes, this technique would allow for live imaging of RNA in vivo due to the fact that there is no need to wash away unbound DMHBI. It is with techniques like ECHO probes and RNA aptamers that scientists will be able to study the dynamics of RNA within the living cell both before and after LTP induction, thus giving a better understanding of the mechanism behind RNA localization and how and when the RNA is utilized within the cell.

Synapse to Nuclear Signaling

The synapse to nuclear signaling of CRTC1 in *Aplysia* neurons, the focus of chapter three, was carried out in order to answer three key questions: one, is the requirement for CRTC1 during long-term synaptic plasticity conserved in non-mammalian species. Two, does CRTC1 differentially respond to activity-dependent versus activity-independent synaptic activity? And three, is CRTC1 synapto-nuclear translocation required in the pre- or postsynaptic neuron? We chose to focus on CRTC1 because it regulated CREB activity, which has been shown to be required for long-term, learning-related plasticity, and because its activity is entirely regulated by nuclear-cytoplasmic trafficking[52,59,61,63].

In cell types throughout the body, CREB can be activated by phosphorylation at serine 133 (pCREB)[57]. However pCREB doesn't always correlate with CREB-binding protein (CBP) recruitment to DNA and thus probable transcriptional activation [108]. This raises the question of what other factors could be important in activation of CREB and recruitment of CBP to DNA? And could these factors provide transcriptional specificity, allowing the cell to appropriately activate a subset of CRE containing genes in response to a particular stimulus?

The discovery of CRTC as a potent coactivator of CREB, independent of CREB phosphorylation at serine 133, and its requirement in L-LTP provided a potential answer of how a neuron might activate a subset of genes in response to synaptic stimuli[59,63]. Following up on these findings, Toh Hean Ch'ng in the Martin lab found that CRTC1 is indeed present in dendrites and spines in hippocampal neurons. Under basal conditions CRTC is tethered outside of the nucleus by 14-3-3 proteins, but upon stimulation CRTC1 accumulates in the nucleus of excitatory neurons. CRTC1 that accumulated in the nucleus of activated neurons was shown to result from pre-existing CRTC1 that translocated from synapses that were specifically activated. Of particular interest was the finding that upon CRTC1 knowdown induction of some, but not all, CREB target genes were attenuated[64]. Taken together, this data shows that CRTC1 can bind CREB and modulate its output. The fact that CRTC1 is present at synapses and can translocate to the nucleus makes it a great candidate for selectively modifying the activity of CREB and other possible bZIP transcription factors in response to LTP-inducing stimuli, and thus only activating the subset of genes required. The data presented in chapter three adds to our previous understanding of the CRTC mechanism by showing that it is conserved outside of mammals, can respond differentially to activity-dependent versus activity-independent synaptic plasticity, and that its functionally required in the post-synaptic neuron. CRTC1, while most likely not the only modifier of CREB output in response to LTP, could help

give us an understanding of the signaling pathways used to differentially activate target genes after LTP induction.

In his review of the current state and future role of LTP in learning and memory research, Rob Malenka states the theory of 'different LTP induction protocols result in mechanistically distinct types of NMDA-dependent LTP…which currently enjoys only weak evidence', asks what the functional significance of each would be[20]. As transcription plays such a key role in long-term plasticity, determining if these different protocols activate different sets of target genes could provide support that different long-term plasticity mechanisms do exist. One possibility is that through CREB interacting with other proteins or through phosphorylation of sites besides Ser133, this could allow CREB to activate different target genes. Following along these lines, identifying CREB and/or CRTC1 binding partners that regulate gene expression in response to long-term plasticity would be extremely important. If these types of investigations are carried out in the future, collecting high throughput RNA-sequencing data from neuronal preparations treated with different stimuli and/or different time-courses of these stimuli would provide clear evidence of whether distinct or similar sets of genes are up-regulated in response to the various stimuli. Looking at different time-courses would be critical so as to not overlook the possibility of different long-term plasticity stimuli activating the same core pathways just at different times due to the inclusion/exclusion of different cursory pathways. Treating neuronal preparations with long-term plasticity inducing stimulation and looking for gene expression changes has certainly been attempted in the past, but these studies have relied on now outdated microarray technology (which don't allow for looking at splicing changes, alternative poly-adenylation, ect) and never in a comprehensive manner, comparing multiple type of long-term plasticity inducing stimuli[109-113]. After comparing the gene expression changes, if differences do exist, correlating these changes with experiments assaying the gene occupancy of CREB and CREB binding partners could provide an explanation via differential binding of

target genes. In order to generate this type of data chromatin immunoprecipitation of DNA binding proteins like CREB, could be coupled with high-throughput sequencing (ChIP-Seq) in order to determine where in the genome these factors are bound. With this large amount of information it would also be possible to elucidate transcription factor networks to see which pathways are being activated in response to a given stimulation. The advantages of the *Aplysia* model system, such as isolation of specific cell-types and ability to induce both homo- and heterosynaptic forms of long-term plasticity, would otherwise make it a great system in which to attempt these experiments. Unfortunately due to the current state of the *Aplysia* genome, the more appropriate model system for performing such genome-wide approaches would be in the mammalian brain (i.e. mouse).

The requirement of transcription and translation to convert E-LTP to L-LTP opens up LTP to a huge amount of cellular regulation (transcription factor binding, splicing, miRNA, transcript stability, ect). Through this cellular regulation it is possible to achieve tight control over the spatial and temporal response of the neuron. The dissection of the regulatory pathways will provide invaluable insight into and drive the field of LTP for years to come.

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