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Los Angeles

Abolition of the Morphological Correlate of Long-Term Facilitation in *Aplysia*

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Philip Young-woo Sun

2013

ABSTRACT OF THE THESIS

Abolition of the Morphological Correlate

of Long-Term Facilitation in *Aplysia*

by

Philip Young-woo Sun

Master of Science in Physiological Science

University of California, Los Angeles, 2013

Professor David L. Glanzman, Chair

Long-term Sensitization (LTS) and Long-term facilitation (LTF) are accompanied by an increase in the number of varicosities in the presynaptic sensory neurons in *Aplysia* (Bailey and Chen, 1983, 1988a; Glanzman et al., 1990). Both LTS and LTF are subject to erasure by inhibiting PKM Apl III, a PKM isoform in *Aplysia*, or by disrupting reconsolidation in *Aplysia* (Cai et al., 2011, 2012). To examine the effect of the two protocols of memory erasure on the morphological correlate of LTF, individual SN varicosities were tracked in confocal microscope images of a sensorimotor synapse that mediates the siphon/gill withdrawal reflex (SWR) in *Aplysia*. While the morphological correlate of LTF persisted up to 48 hours after 5X5-HT training, inhibiting PKM Apl III by chelerythrine or disrupting reconsolidation by reactivation with 1X5-HT immediately followed by protein synthesis inhibition with anisomycin (ANISO) at 24-hr

reversed the presynaptic growth during 24-48 hr. Since chelerythrine did not change the number of SN varicosities in cocultures without prior 5X5-HT training, PKM Apl III inhibition selectively erased the structural growth underlying long-term memory. Furthermore, the abolition of the morphological correlate of LTF invariably resulted from inhibition of new growth during 24-48 hr as well as nonspecific removal of SN varicosities based on their formation history, suggesting that the engram for LTF is not localized to specific synapses.

The thesis of Philip Young-woo Sun is approved

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University of California, Los Angeles

2013

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List of Symbols

= Zeta

= Epsilon

List of Acronyms

SN = sensory neuron

MN = motor neuron

SWR = siphon/gill withdrawal reflex

STS = short-term sensitization

LTS = long-term sensitization

STF = short-term facilitation

LTF = long-term facilitation

LTP = long-term potentiation

5-HT = 5-hydroxytryptamine (serotonin)

1X5-HT = a single application of 5-HT

5X5-HT = five spaced applications of 5-HT

AMPAR = -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ANISO = anisomycin

Ap/AF = *Aplysia* form activation factor

ApC/EBP = *Aplysia* form CAAT box enhancer binding protein

ApCPEB = *Aplysia* form cytoplasmic polyadenylation element binding protein

Chel = chelerythrine

CRE = cyclic adenosine monophosphate response element

CREB = cyclic adenosine monophosphate response element-binding protein

cAMP = cyclic adenosine monophosphate

eGFP = enhanced green fluorescent protein

GluR2 = glutamate receptor 2 subunit of the -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

MAPK = mitogen-activated protein kinase

PDK1 = phosphoinositide-dependent protein kinase

PKA = protein kinase A

PKC = protein kinase C zeta

PKC Apl III = protein kinase C *Aplysia* III

PKM = protein kinase M zeta

PKM Apl III = protein kinase M *Aplysia* III

ZIP = zeta inhibitory peptide

I. Introduction

To gain a better understanding of the formation, maintenance, and erasure of memory, various animal models have been developed. *Aplysia californica* has proven useful as a model system for understanding the cellular mechanisms that underlie the intricate processes of learning and memory. *Aplysia* has a nervous system consisting of 20,000 neurons clustered into ten ganglia, the central nervous system of the animal. Moreover, *Aplysia*'s individual neurons are easily identifiable because of their large size (0.02~1mm in diameter) (Frazier et al., 1967), distinct shades of orange, and consistent locations across animals. Those morphological characteristics of the neurons in *Aplysia* allow us to define the neuronal circuits that mediate specific behaviors and also to record the electrophysiological activities of specific neurons individually. Moreover, when a sensory neuron (SN) and a motor neuron (MN), which naturally form synaptic connections *in vivo*, are retrieved and placed in cell coculture, they recover robust synaptic connections in the artificial environment within 48 hours. This unique ability of *Aplysia* neurons makes it possible to conduct various *in vitro* studies, enabling us to elucidate molecular mechanisms that underlie various processes of learning and memory.

The siphon/gill withdrawal reflex (SWR) is a behavioral response commonly used to study the molecular mechanisms of learning and memory in *Aplysia* (Kandel, 2001). In response to mechanical stimulation of the siphon or mantle shelf, the animal retracts the gill and the siphon as a defense mechanism (Pinsker et al., 1970). The SNs of the synapses are responsive to mechanosensation and innervate the siphon, tail, and body wall. Their cell bodies are located in pleural ganglia. The MNs innervated by the SNs have their cell bodies located in the abdominal ganglia and control the retraction of the siphon, the gill, and the mantle. This reflex in the tractable animal model provides us with quantifiable evidence of implicit learning; in response to

the presence of danger, the animal increases the duration of the reflex. This phenomenon is known as sensitization, which will be discussed in detail below.

Sensitization is a type of implicit, non-associative learning that *Aplysia* demonstrates when the animal learns the presence of a disturbing stimulus. In response to a potentially disturbing stimulus, the animal exhibits sensitization, the enhancement in the duration of SWR. The behavioral expression of SWR is graded; the length of its retention period changes with the number of disturbing stimuli. A single noxious stimulus to the tail induces short-term sensitization (STS), which lasts minutes to hours, and multiple stimuli produce long-term sensitization (LTS), which can last for up to three weeks (Pinsker et al., 1973). LTS is commonly induced by repeated mild electrical shocks on the tail. Such disturbing stimuli cause serotonergic facilitatory interneurons to secrete serotonin (5-hydroxytryptamine; 5-HT) (Marinesco and Carew, 2002), which then acts on neighboring neurons, including the monosynaptic synapses that mediate SWR. The activity of 5-HT is necessary for tail shock-induced facilitation of the sensorimotor synapse in a reduced animal model of *Aplysia* (Glanzman et al., 1989), further supporting the necessity of 5-HT in the induction of LTS. My approach is to re-construct the sensorimotor synapse in cell coculture and bathe it with 5-HT to artificially induce memory acquisition in the synapse and study the molecular mechanisms associated with the process.

The effect of sensitization on the monosynaptic synapse can be replicated in dissociated cell culture; here, serotonin, a modulatory neurotransmitter normally released by sensitizing stimuli, substitutes for the tail shock used during behavioral training in the intact animal (Mantarolo et al., 1986). In this *in vitro* system, learning is reflected by the enhancement or facilitation of the postsynaptic response. Like sensitization, the facilitation is graded; its strength varies according to the number of 5-HT applications. Applying a single application of 5-HT

(1X5-HT) induces short-term facilitation (STF), lasting shorter than 30 minutes, and applying five spaced applications of 5-HT (5X5-HT) produce long-term facilitation (LTF), lasting more than 24 hours. Although synaptic facilitation in the monosynaptic synapse that mediates LTS lasts for up to 72 hours in cocultures (Cai et al., 2012), its role in mediation of LTS has been confirmed previously (Frost et al., 1985). Therefore, if we study the molecular mechanisms that underlie LTF *in vitro*, then we are, in fact, elucidating molecular mechanisms that mediate the LTS of SWR in *Aplysia in vivo*.

Various studies have demonstrated that in converting short-term memory to long-term memory, the synapse runs additional molecular processes (Goelet et al., 1986). The key difference between the short-term change and the long-term change is that the short-term change involves post-translation modification of preexisting proteins only, whereas the long-term change requires synthesis of new proteins. In 1964, Agranoff and Klinger showed that long-term memory induction requires new protein synthesis in goldfish. Studies in *Aplysia* were in agreement with their finding. While inhibitors of transcription or translation did not block the induction of STS in the animal or STF in dissociated cell culture (Montarloro et al., 1986; Schwartz et al., 1971), they blocked the induction of LTS in the semi-intact animal (Castellucci et al., 1989) and LTF in dissociated cell culture (Montarolo et al., 1986). Altogether, these studies unequivocally support the idea that expression of new genes is necessary for the behavioral and cellular changes associated with the maintenance of long-term memory, but not short-term memory.

Further studies have provided us with deeper insights into the molecular processes of short-term and long-term memory. For short-term memory, common components of cytoplasmic signaling pathways, such as amplifier enzymes (i.e. adenylyl cyclase), cytoplasmic messengers

(i.e. cyclic adenosine monophosphate, Ca^{2+}), and protein kinases, are used to modify already existing target proteins that govern synaptic transmission, such as postsynaptic glutamate receptors (Goelet et al., 1986). While an array of other biochemical reactions simultaneously take place to induce and maintain STF, the absence of new protein synthesis explains why there is no known structural growth associated with STF.

During LTF, on the other hand, new protein synthesis occurs to bring about a longer-lasting effect. First of all, the conversion of short-term memory to long-term memory requires protein synthesis within a certain period of time. In 1964, Agranoff and Klinger showed that memories undergo a time-dependent process of consolidation, during which protein synthesis is required, and that the consolidated memories do not require translation for their maintenance. In 1986, when Montarolo and associates showed the necessity of new protein synthesis for the induction of LTF in *Aplysia*, they also found that the synapse exhibits a critical time window for its requirement of protein and ribonucleic acid synthesis. These findings suggest that the critical time window may be involved in determining the fate of memory, whether it will last short-term or long-term, and also that the induction and the maintenance of long-term memory involve distinct molecular processes as well.

Several lines of evidence demonstrate that transcription and translation trigger the activation or the enhancement in the functionality of already-present enzymes. When long-term memory is induced by 5X5-HT, the cAMP (cyclic adenosine monophosphate) - mediated pathway is activated to a greater extent than that induced by the 1X5-HT treatment. This level of activation by 5X5-HT is sufficient to activate enough PKA to activate mitogen-activated protein kinase (MAPK). Concomitantly, both kinases translocate to the nucleus and activate cyclic AMP response element-binding protein (CREB) (Bacskai et al., 1993; Bartsch et al., 1995; Dash et al.,

1990; Martin et al., 1997). CREB-1, a type of CREB, is a transcription factor that binds to a cAMP response element (CRE), a part of the promoters of different genes (Kandel, 2001). One result of the activation of immediate response genes by the binding of CREB-1 is enhanced production of ubiquitin hydrolase (Kandel, 2001), an enzyme that allows for the ongoing activity of the catalytic subunit of PKA by inducing proteolysis of the regulatory subunit of PKA (Hegde et al., 1993).

Transcription and translation during long-term memory induction involves not only modification of enzymatic activities, as presented previously, but also synthesis of structural proteins that mediate the growth of new synaptic connections. In 1983, Bailey and Chen first demonstrated that LTS is associated with structural changes in the presynaptic neuron of the synapse that partly mediates SWR. The structural growth can be recapitulated in cell cocultures by the 5X5-HT protocol (Glanzman et al., 1990), and blocking transcription or translation prevented the synapse from showing the structural growth (Bailey et al., 1992). CREB-1, which triggers synthesis of ubiquitin hydrolase as discussed previously, also triggers synthesis of CAAT box enhancer binding protein (ApC/EBP) in *Aplysia* (Albeirni et al., 1994). ApC/EBP then dimerizes with activation factor (Ap/AF) in *Aplysia* (Bartsch et al., 2000), and the heterodimer can activate the expression of late-response genes that ultimately lead to the formation of new synaptic connections (Kandel, 2001). Furthermore, this presynaptic structural growth can be achieved only when the postsynaptic neuron is present, suggesting that a retrograde messenger is necessary to induce the presynaptic growth associated with LTF (Glanzman et al., 1990). Later, Glanzman’s lab strengthened the retrograde signal hypothesis by showing that calcium ions and new protein synthesis in the postsynaptic neuron are necessary to induce LTF (Cai et al., 2008). In conclusion, these findings suggest that during the induction

phase of LTF, the synapse undergoes intricately organized intracellular and intercellular reactions that ultimately lead to the production of new proteins, and that the newly synthesized proteins mediate a variety of biochemical and morphological changes associated with long-lasting synaptic facilitation.

Extensive studies have been done to elucidate the dynamics of the structural growth associated with LTF. Bailey and Chen showed that there are three types of re-structuring processes taking place during the induction phase of LTF. These are:

a) an increase in the number, size, and vesicle complement of SN active zones (Bailey and Chen, 1983; 1988a, b)

b) an increase in the total number of SN varicosities (Bailey and Chen, 1988a)

c) an increase in the axonal arbor. (Bailey and Chen, 1988b)

In 1989, Bailey and Chen studied the time course for each type of structural modification processes to ascertain their possible roles in LTF. While LTS can last up to three weeks (Pinsker et al., 1973), the increase in the active zone size and vesicle complement was reversed by one week (Bailey and Chen, 1989), indicating that these kinds of structural changes do not mediate the maintenance of long-term memory entirely. On the other hand, the increase in the numbers of SN varicosity and active zone was maintained for one week and only partially reversed by three weeks, paralleling the behavioral form of long-term learning. These findings suggest that the changes in the number of SN synapses can contribute to the long-term synaptic facilitation for the entire duration of LTS.

Although the increase in the number of SN varicosities parallels the entire duration of LTS, the earlier studies did not ascertain the functional contribution of the newly formed varicosities as well as that of the pre-existing ones. Further studies revealed that the induction of

LTF is accompanied by not only addition of new varicosities but also the conversion of pre-existing inactive varicosities into functional synapses. The two processes showed a different time course of activation. In 2003, Kim and associates showed that the activation of pre-existing silent varicosities, ascertained by the filling of synaptophysin-eGFP and synapto-PHluorin in varicosities that were originally devoid of those markers, occurred 3-6 hours after 5X5-HT treatment. Synaptophyin-eGFP labels presynaptic neurotransmitter-containing vesicles, and synapto-PHluorin labels active transmitter release sites (Miesenbock et al., 1998), so the filling of these fluorescent markers unequivocally shows the activation of silent synapses. Kim and associates also showed that the majority of the newly grown varicosities were formed during 12-18 hours after 5X5-HT treatment. They also showed that while both the activation of pre-existing silent synapses and the synthesis of new varicosities persisted by 24-hr after the 5-HT treatment, the newly formed varicosities accounted for 68% of the newly activated synapses at 24-hr, suggesting that the synapse's primary strategy to enhance synaptic transmission during 0-24 hr is the formation of new varicosities.

In conclusion, among different types of morphological changes that occur in response to the long-term memory-inducing training, the only ones that last for the entire duration of LTS are the increases in the number of SN varicosities and in the number of active zones. Biochemical analysis revealed that the increase in the number of active zones can be achieved by activating previously inactive varicosities and also by forming and activating new varicosities. It has been found that this morphological change contributes to LTS and LTF (Bailey and Chen, 1988 a, b, Glanzman et al., 1990). Given that the increase in the number of presynaptic vesicle release sites is achieved primarily by this strategy during LTF induction, understanding the molecular

mechanisms that underlie this morphological change will reveal insightful information on the process of long-term memory acquisition and maintenance.

More recent studies have focused on elucidating the molecular mechanisms of long-term memory maintenance. In 1964, Agranoff and Klinger showed that memories undergo a time-dependent process of consolidation, and that the consolidated memories do not require new protein synthesis for their maintenance. According to this traditional understanding of long-term memory maintenance, the structural change associated with long-term memory can be maintained by the newly produced proteins during the critical time window of memory consolidation. As discussed previously, some of those proteins trigger learning-related presynaptic and postsynaptic structural growth (Bailey and Chen, 1983, 1988a, b; Glanzman et al., 1990) or modification of activities of already existing enzymes (Goelet et al., 1986; Kandel, 2001).

One field that has attracted attention currently is the role of constitutively active molecules found to be necessary to mediate the maintenance of long-term memory. A strong candidate for such persistently active molecules whose role is implicated in long-term memory maintenance is protein kinase M (PKMζ). PKMζ is a constitutively active fragment of the mammalian atypical Protein Kinase Cζ (PKCζ). Long-term potentiation (LTP) is the synaptic facilitation that lasts more than 30 minutes in *Drosophila melanogaster* and mammals. It has been found that PKMζ participates in some ongoing process(es) necessary to maintain LTP in *Drosophila melanogaster* (Drier et al., 2002) and mammals (Ling et al., 2002; Pastalkova et al., 2006; Shema et al., 2007). The involvement of PKMζ in long-term memory has been verified in *Aplysia*, also. Inhibition of PKM Apl III, a PKM isoform in *Aplysia*, with either zeta inhibitory peptide (ZIP), a selective PKMζ inhibitor (Ling et al., 2002; Pastalkova et al., 2006; Shema et al., 2007), or chelerythrine, a specific PKM Apl III inhibitor (Ling et al., 2002; Villareal et al., 2009), abolishes both LTS and LTF in *Aplysia* (Cai et al., 2011). What will be of interest is to

understand how inhibition of PKM Apl III mechanistically abolishes LTS and LTF. One possible study is to verify if PKM Apl III inhibition reverses the presynaptic structural growth associated with LTF. As LTF underlies LTS (Frost et al., 1985), this *in vitro* imaging study will enable us to verify whether PKM Apl III is required to maintain the physical trace of both LTF and LTS.

Another merit of morphological studies is that one can trace individual varicosities' formation and erasure history. This fate analysis will help us learn whether individual synapses are tagged with respect to memory maintenance. According to this possible mechanism of memory maintenance, the newly formed varicosities during the induction of long-term memory will be labeled by the synapse, thereby making them distinguishable from the varicosities that existed prior to learning. One well-known hypothesis in conjunction with this idea is the cytoplasmic polyadenylation element binding protein (ApCPEB) hypothesis. It has been proposed that endogenous ApCPEB mediates the induction and the maintenance of long-term memory during 24-48 hr in *Aplysia* (Miniaci et al., 2008; Si et al., 2003a, 2010). Through polymerization, inactive ApCPEB monomers can become active and self-perpetuating, producing new ApCPEB proteins through rapamycin-dependent local protein synthesis (Si et al., 2003a, b). The ApCPEB hypothesis suggests that as ApCPEB-mediated protein synthesis takes place locally to synthesize new varicosities during long-term memory acquisition, the ApCPEB-mediated pathway directly or indirectly tags the varicosities to trigger on-site protein synthesis. If this hypothesis holds true, then those labeled varicosities, the loci of memory, should be selectively removed when memory erasure is induced.

Studying morphological changes associated with disruption of memory reconsolidation will reveal more information on the dynamics of memory maintenance. As Agranoff and Klinger showed in 1964, there is a critical time window during and shortly after training when short-term memory is consolidated into long-term memory, the form of memory resistant to disruption by

protein synthesis inhibition. However, it has been found later that in rodents, reactivation of hippocampal-dependent long-term memory put the memory back to the labile state, subject to erasure by protein synthesis inhibition (Nader et al., 2000). In addition, behavioral and electrophysiological evidence of reconsolidation have been found in *Aplysia* (Cai et al., 2012; Lee et al., 2012). An intriguing question is how the morphological correlate of LTF is affected by this memory-eliminating process.

One interesting aspect of memory reconsolidation is that the process appears to recapitulate the initial consolidation of long-term memory. Just like long-term memory consolidation, the process of reconsolidation exhibits a critical time window, following memory reactivation, during which the requirement for protein synthesis is graded temporally (Milekic and Alberini, 2002). Going further, reactivation of long-term memory involves facilitation of endocytosis of postsynaptic AMPARs (-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) (Rao-Ruiz et al., 2011; Rose and Rankin, 2006); The AMPAR is an ionotropic glutamate receptor. Since LTP involved the enhancement in the expression in postsynaptic sites via modulation in AMPAR trafficking (Bredt and Nicoll, 2003; Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir, 2002), the endocytosis process during reconsolidation appears to reverse this process. With the role of PKM Apl III in AMPAR trafficking confirmed previously (Sacktor, 2011), it is possible that memory reactivation and PKM Apl III inhibition show mechanistic convergence. The feasibility of this hypothesis can be gauged by examining whether the two memory erasure protocols, disrupting memory reconsolidation and inhibition of PKM Apl III, cope with the morphological correlate of LTF in a similar way. Specifically, do both of the protocols reduce the overall number of SN varicosities? If so, do they target a specific group of SN varicosities based on their formation history?

The main objective of the thesis project is to investigate the structural dynamics of presynaptic neurons during the induction and abolition of LTF in *Aplysia*. There are two specific questions. The first question is whether the physical engram of long-term memory, the increase in the total number of SN varicosities, is reversed during memory erasure. One plausible hypothesis is that the morphological correlate of long-term memory, known to be the primary contributor to synaptic facilitation (Kim et al., 2003), is reversed in response to PKM Apl III inhibition and to disrupting reconsolidation, as both memory erasure protocols reverse the electrophysiological and behavioral evidence of learning in *Aplysia*. The second question is how the synapse, if it does, reverses the morphological correlate of LTF during memory erasure. Does it specifically remove varicosities that were newly synthesized after the event of learning? Or, does it nonspecifically remove varicosities that existed prior to 5-HT training or newly formed within 24 hours after 5-HT training? Current information in the field does not yield a definite answer to this question, so it is difficult to establish a hypothesis for this question.

II. Materials and Methods

Cell culture and dye injection

The sensorimotor cocultures consisted of one pleural SN and one small siphon (LFS-type) MN from the same animal. *In vivo*, this sensorimotor synapse partly mediates SWR (Hawkins et al., 1983). Abdominal ganglia and pleural ganglia were first isolated from an adult *Aplysia*. The ganglia were then desheathed, and the neurons were individually dissociated from the CNS and paired in cell coculture. To allow the synapse to re-establish synaptic connection, the cultures were incubated for three days before the start of the experiments. On day 4, SNs and MNs were fluorescently labeled with intracellular dyes, dextran fluorescein (green) and with dextran rhodamine B (red), respectively, via pressure injection. Between the three image sessions at 0-hr, 24-hr, and 48-hr, the cocultures were incubated at 18°C. The first image session (0-hr) started on day 5.

Drug treatments

a. The effect of PKM Apl III inhibition on the morphological correlate of LTF

Immediately after the first episode of imaging at 0-hr, some of the cocultures were trained using five 5-min pulses of 5-HT separated by 15 minutes (100 μM, 5X5-HT training protocol). The rest of the cocultures (control group) were infused with the perfusion solution alone at 0-hr. Immediately after the second imaging session at 24-hr, the trained cocultures received no treatment (5-HT group) or 10 μM chelerythrine for one hour (5-HT-Chel group). 10μM chelerythrine selectively reduces PKM Apl III activity to less than 20% (Villareal et al., 2009). The untrained control group, as in the serotonin-trained group, received no treatment or 10 μM chelerythrine for one hour at 24-hr. Following the chelerythrine treatment, the drug was washed out with normal perfusion medium.

Then, the perfusion medium was replaced with culture medium, and the cocultures were returned to the incubator.

b. The effect of disrupting reconsolidation on the morphological correlate of LTF

Immediately after the first episode of imaging at 0-hr, some of the cocultures were trained using five 5-min pulses of 5-HT separated by 15 minutes (100 μM, 5X5-HT training protocol). The rest of the cocultures (control group) were infused with the perfusion solution alone at 0-hr. Immediately after the second imaging session at 24-hr, the trained cocultures received no treatment, anisomycin (ANISO, 10 μM) alone for 2 hours, or reminder training, consisting of one 5-min pulse of serotonin (100 μM, 1X5-HT protocol) immediately followed by ANISO for 2 hours. Anisomycin is a potent protein synthesis inhibitor. 100 μM anisomycin nonspecifically reduces protein synthesis to 19% (Fulton et al., 2005). Control cocultures, which did not receive 5-HT training at 0-hr, were given no treatment or anisomycin (ANISO, 10 μM) alone for 2 hours at 24-hr. Following the anisomycin treatment, the drug was washed out with normal perfusion medium. Then, the perfusion medium was replaced with culture medium, and the cocultures were returned to the incubator.

Cell imaging and criteria for varicosity counting

The fluorescently labeled sensorimotor cocultures were imaged by a LSM Pascal confocal laser scanning microscope at 0-hr, 24-hr, and 48-hr. The images were analyzed by Axiovision 4.8.2 (Zeiss, Thornwood, NY). The counter was blind to the experimental conditions. The total number of presynaptic varicosities in clear contact or overlap with postsynaptic structures (soma, major neurite, and fine processes) was determined for each SN. The majority of the varicosities contacted the MN on the soma or the initial segment. Only those with a punctate shape—those in which the oval-shaped main body could be distinguished by the narrowing of the neurite on

either side— and a measured area of 10 μm^2 or greater were counted. If a large fluorescent varicosity appears to consist of several visible punctuate varicosities, those small varicosities were counted individually; if not, the structure was treated as a single varicosity.

Fate analysis of varicosities

In fate analysis, the counted varicosities were first categorized into three groups based on their formation history. Those formed prior to the 5-HT training at 0-hr and hence present at the first image session are called the Original Varicosities. Those that appeared between the first and the second image sessions (within 24 hours after the 5-HT training) are called the Induced Varicosities. Lastly, those that were formed between the second and the third image sessions (24-48 hours after the 5-HT training) are called the New Varicosities. Next, the fates of the Old Varicosities and the New Varicosities during 24-48 hr were determined individually.

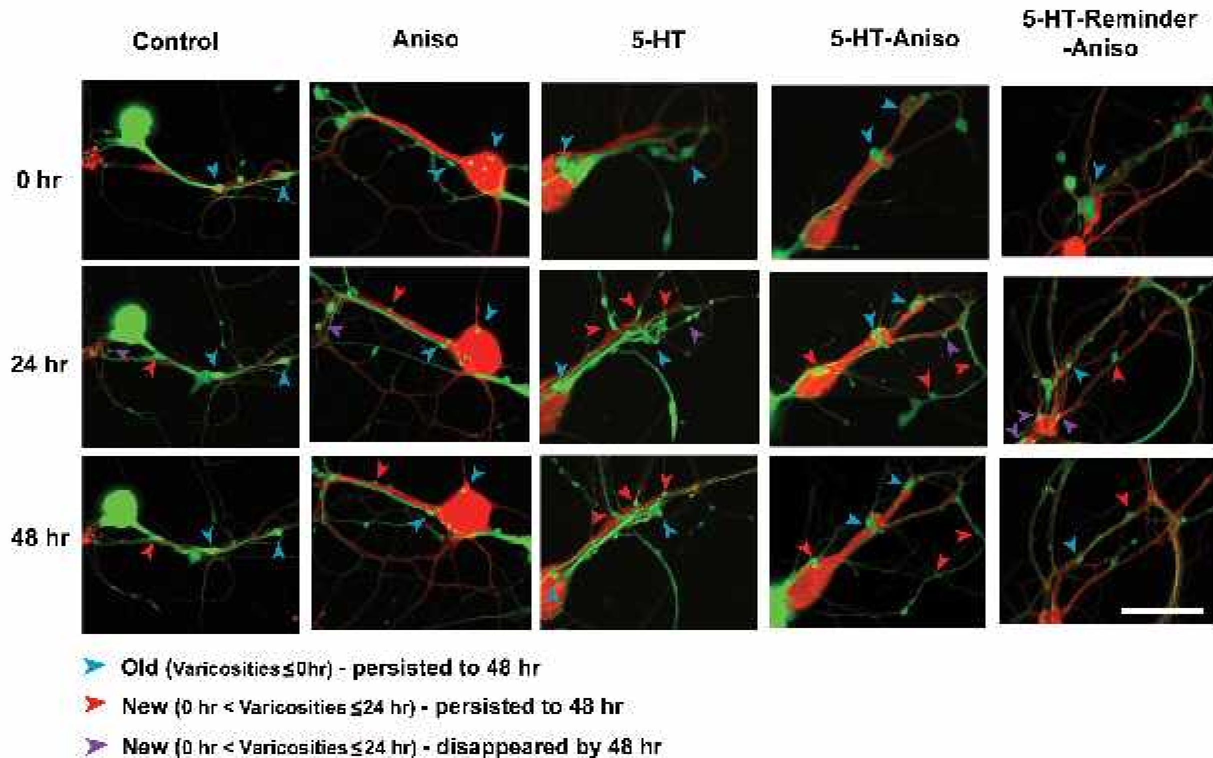

Figure s1. Confocal fluorescence micrograph of sensorimotor synapses from five experimental groups: A representative sensorimotor synapse from each experimental group for the morphological analysis on the effect of disruption of reconsolidation is presented here. **Blue arrowheads** indicate stable varicosities that persisted during 0-48 hr; **red arrowheads** indicate new varicosities that appeared during 0-24 hr and persisted during the 24-48 hr period; and **purple arrowheads** indicate varicosities that appeared during 0-24 hr period but disappeared during the 24-48 hr period.

Statistical analysis

All statistical tests were conducted using Prism 4.0 for Macintosh (GraphPad, La Jolla, CA). For appropriate comparison of presynpatic structural changes across coculture dishes, the total number of SN varicosities at 24-hr and 48-hr were normalized to the total number at 0-hr. To

assess the statistical significance between two data sets, unpaired Student's t-tests were done at the significance level of 0.05 ($p < 0.05$). Multiple group comparisons were done by one-way analysis of variance (ANOVA), and subsequently by Student-Newman-Keuls post-hoc tests for pairwise comparisons. The significance level was set at 0.05 ($p < 0.05$) for all tests.

III. Results

Part 1. Inhibition of protein kinase M disrupts maintenance of the persistent presynaptic structural changes that accompany long-term facilitation in *Aplysia*

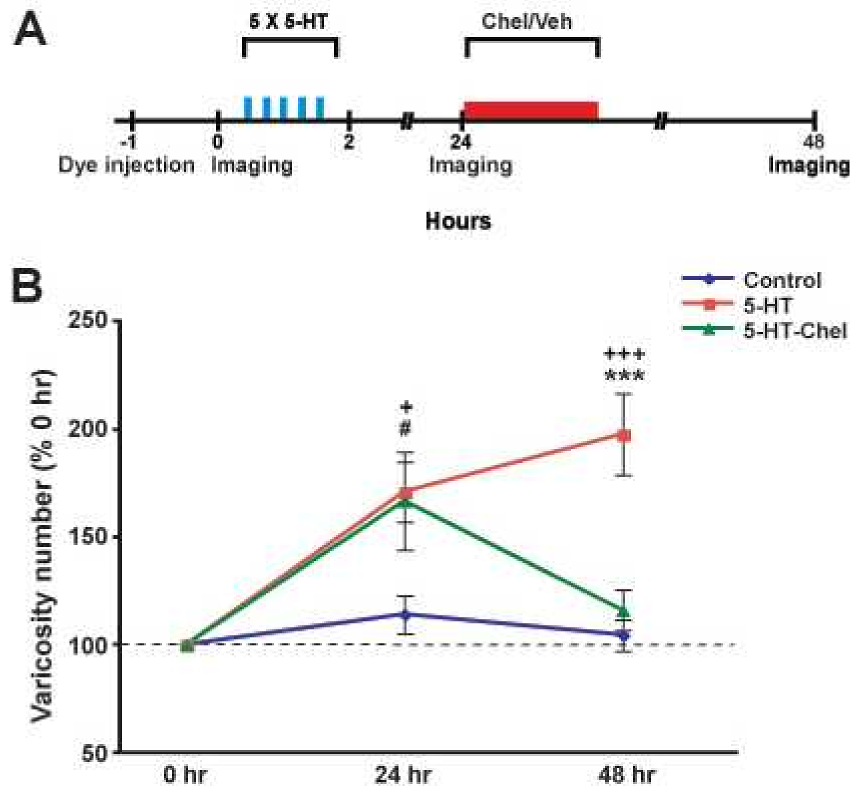

Figure 1. PKM Apl III inhibition reverses the enhancement in the overall number of varicosities.

(A) Experimental protocol (see Materials and Methods for details).

(B) Mean normalized SN varicosity number at 24-hr and 48-hr. An overall number of SN varicosities was measured at 24-hr and 48-hr from three experimental groups - Control (n = 14), 5-HT (n = 20), 5-HT-Chel (n = 14). The counts were normalized to the overall number of varicosities present at 0-hr in each sensorimotor synapse. A one-way ANOVA of the normalized values indicated that the group differences were significant at 24-hr ($p < 0.05$) and at 48-hr ($p < 0.0001$). 5X5-HT training induced an increase in the overall varicosity number at 24-hr in all 5-HT-trained groups, as can be seen by the increased number of varicosities in the 5-HT group (p <

0.05) and the 5-HT-Chel group ($p < 0.05$). While the increase in the varicosity number is still significant in the 5-HT group compared to the Control group at 48-hr ($p < 0.001$), chelerythrine treatment 24 hours after 5X5-HT training produced a reduction in the number of SN varicosities. At 48-hr, 5-HT-Chel group shows an overall number of varicosities significantly less than that of the 5-HT group ($p < 0.001$) but not significantly different from that of the Control group. Some of the control cocultures received chelerythrine at 24-hr, but the treatment did not change the number of SN varicosities. Plus signs indicate significance of the difference between the 5-HT group and the Control group, pound signs indicate significance of the difference between the 5-HT-Chel group and the Control group, and asterisk signs indicate significance of the difference between the 5-HT group and 5-HT-Chel groups. Error bars represent ± SEM.

Inhibition of protein kinase M 24 hours after 5X5-HT training causes an overall reduction in the number of varicosities at 48-hr

As previously reported (Glanzman et al., 1990), there was an increase in the number of SN varicosities in the cocultures that received the 5X5-HT training at 24-hr. Figure 1B shows that this morphological correlate of LTF is sustained until 48-hr in the serotonin-trained cocultures that received no treatment at 24-hr. By contrast, in the trained cocultures that were given chelerythrine at 24-hr, the overall number of varicosities decreased to the extent that it is no longer significantly greater than that of the Control group. Chelerythrine did not reduce the number of SN varicosities in cocultures that did not receive the 5X5-HT training at 0-hr. Therefore, inhibition of PKM Apl III selectively abolished the presynaptic structural growth that primarily underlies LTF (Kim et al., 2003).

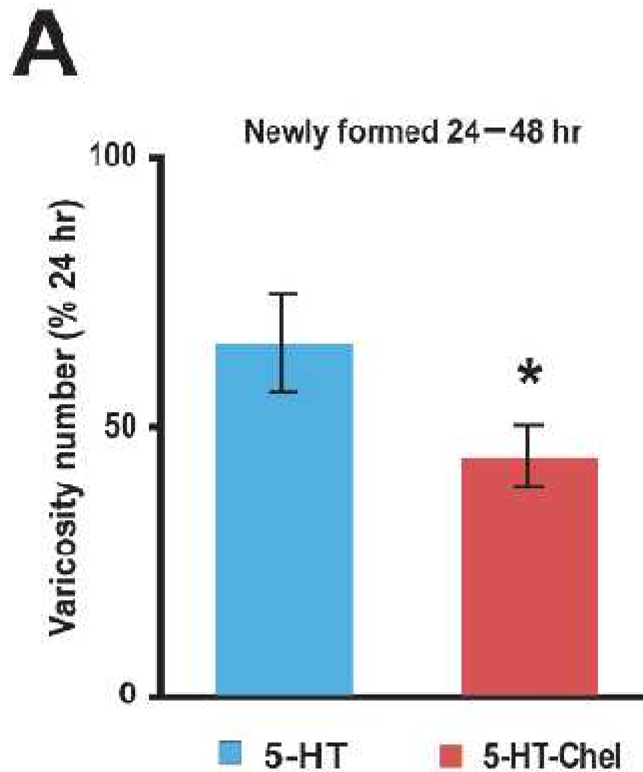

Figure 2. PKM Apl III inhibition reduces the synthesis of new varicosities.

(A) Mean normalized number of SN varicosities that newly formed during 24-48 hr from the 5-HT-treated groups – 5-HT (n = 21), 5-HT-Chel (n = 14). New Varicosities, the varicosities that appeared during 24-48 hr, were counted at 48-hr, and the count was normalized to the overall number of varicosities present at 24-hr in each sensorimotor synapse. Compared to the 5-HT group, the 5-HT-Chel group shows a significantly lower rate of formation of new varicosities during 24-48 hr ($p < 0.05$, one-tailed). Error bars represent ± SEM.

Inhibition of protein kinase M 24 hours after 5X5-HT training causes inhibition of 5-HT-induced growth during 24-48 hr

Given that the morphological correlate of LTF can be abolished through disrupting the persistent activity of PKM Apl III, what is of interest is to understand how the synapse reduces the overall

number of varicosities to the original level during this process. One possible way to bring about the retraction is to discourage the synapse from synthesizing new varicosities. Figure 2A shows that the reversal of the morphological correlate of LTF through PKM Apl III inhibition involves a reduction in the number of 5-HT-induced varicosities (Induced Varicosities) formed during 24-48 hr. Compared to the 5-HT only group, which did not receive any treatment at 24-hr, the 5-HT-Chel group shows a significantly lower rate of formation of new varicosities during 24-48 hr. Therefore, PKM Apl III inhibition abolished the morphological correlate of LTF, at least in part, by intervening with molecular processes that underlie formation of new varicosities.

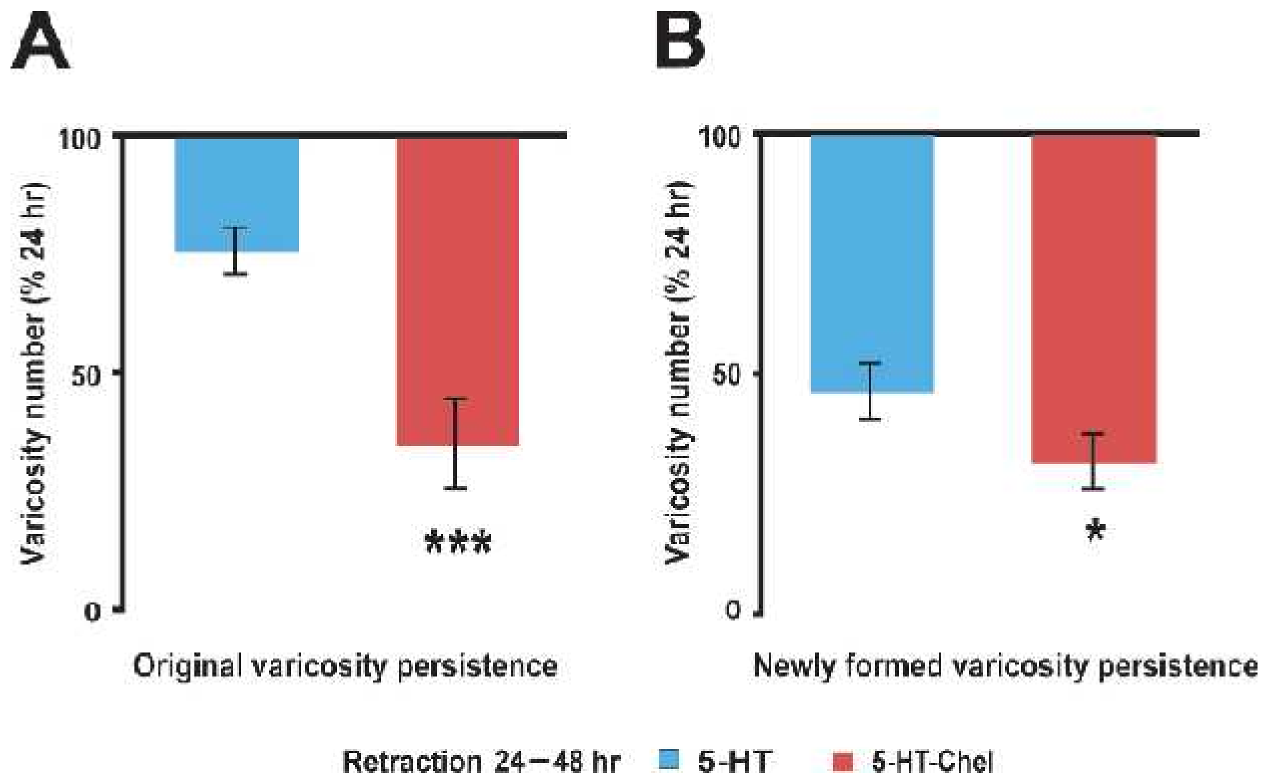

Figure 3. PKM Apl III inhibition reduces persistence of varicosities that existed prior to 5-HT training or newly formed within 24 hr after 5-HT training.

(A) Mean persistence rate of Original Varicosities during 24-48 hr from the 5-HT-treated groups – 5-HT (n = 19), 5-HT-Chel (n = 13). The persistence rate of Old Varicosities, the varicosities that existed prior to 5-HT treatment, during 24-48 hr was measured. From each pair, the number of Old Varicosities that persisted during 24-48 hr was normalized to the number of Old Varicosities present in each sensorimotor synapse at 24-hr. Compared to the 5-HT group, the 5-HT-Chel group shows a significantly lower persistent rate of Old Varicosities during 24-48 hr ($p < 0.001$). Error bars represent ± SEM.

(B) Mean persistence rate of Induced Varicosities during 24-48 hr from the 5-HT-treated groups – 5-HT (n = 20), 5-HT-Chel (n = 14). The persistence rate of Induced Varicosities, the varicosities that formed within 24 hours after 5-HT treatment, during 24-48 hr was measured.

From each pair, the number of Induced Varicosities that persisted during 24-48 hr was normalized to the number of Induced Varicosities present in each sensorimotor synapse at 24-hr. Compared to the 5-HT group, the 5-HT-Chel group shows a significantly lower persistent rate of Induced Varicosities during 24-48 hr ($p < 0.05$, one-tailed). Error bars represent ± SEM.

Inhibition of protein kinase M 24 hours after 5X5-HT training causes enhanced retraction of pre-learning varicosities as well as 5-HT-induced growth during 24-48 hr

Figure 2A shows that the reversal of the morphological correlate of LTF through PKM Apl III inhibition involves inhibition of 5-HT-induced growth. Our next question is whether PKM Apl III inhibition also triggers varicosity erasure to bring about the retraction. Then, taking a step further, does this destabilization process, if it exists, target a specific group of presynaptic varicosities based on their formation history? Figure 3A shows that the reversal of the morphological correlate of LTF through PKM Apl III inhibition involves enhanced removal of Original Varicosities, the varicosities that existed prior to 5X5-HT treatment. Compared to the 5-HT only group, which did not receive any treatment at 24-hr, the 5-HT-Chel group shows a significantly higher rate of erasure of Old Varicosities during 24-48 hr. Next, Figure 3B shows that the reversal of the morphological correlate of LTF through PKM Apl III inhibition also involves enhanced retraction of Induced Varicosities, the varicosities that were formed within 24 hours after 5X5-HT treatment. Compared to the 5-HT only group, which did not receive any treatment at 24-hr, the 5-HT-Chel group shows a significantly greater rate of removal of Induced Varicosities during 24-48 hr. Therefore, blocking persistent activity of PKM Apl III abolished the morphological correlate of LTF, in part, by facilitating elimination of presynaptic varicosities regardless of whether their formation was induced by serotonin training.

Part 2. Disruption of reconsolidation disrupts maintenance of the persistent presynaptic structural changes that accompany long-term facilitation in *Aplysia*

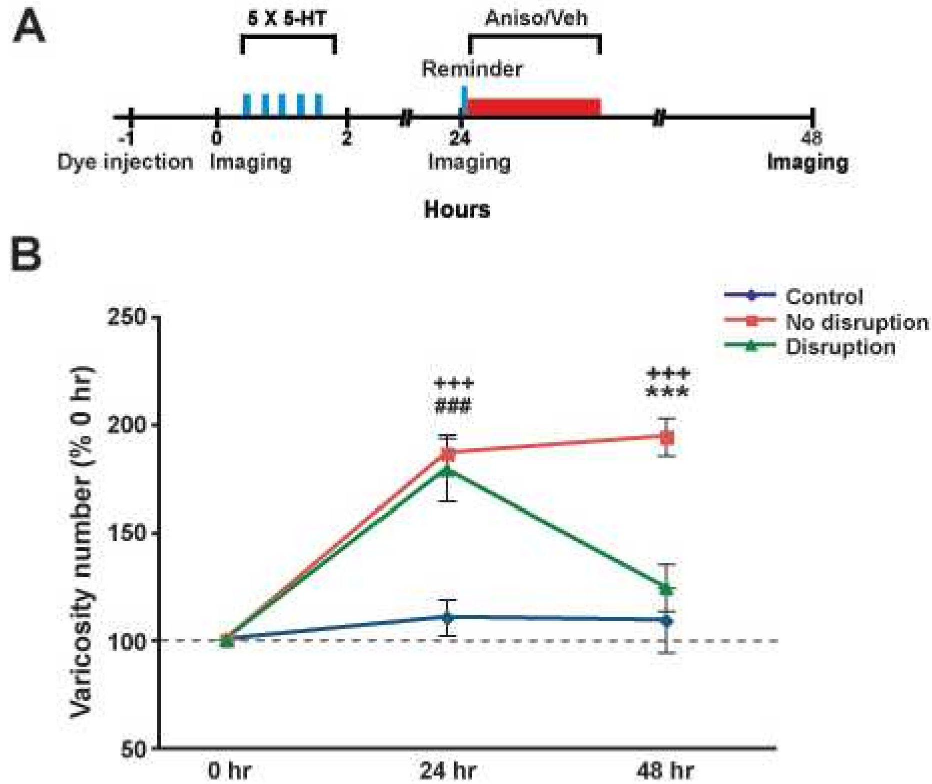

Figure 4. Disruption of reconsolidation of LTF reverses the enhancement in the overall number of varicosities.

(A) Experimental protocol (see Materials and Methods for details).

(B) Mean normalized SN varicosity number at 24-hr and 48-hr. An overall number of SN varicosities at 24-hr and 48-hr was measured from three experimental groups - Control (n = 21), No Disruption (n = 40), Disruption (n = 26). The No Disruption group consists of the 5-HT group and the 5-HT-ANISO group, and the Disruption group consists of the 5-HT-Reminder-ANISO group only. The counts were normalized to the overall number of varicosities present at 0-hr in each sensorimotor synapse. A one-way ANOVA of the normalized values indicated that

the group differences were significant at 24-hr ($p < 0.001$) and at 48-hr ($p < 0.001$). 5X5-HT training induced an increase in the overall varicosity number at 24-hr in all 5-HT-trained groups, as can be seen by the increased number of varicosities in the No Disruption group ($p < 0.001$) and the Disruption group ($p < 0.001$), both of which received 5X5-HT training at 0-hr. While the increase in the varicosity number is still significant in the No Disruption group at 48-hr compared to the Control group at 48-hr ($p < 0.001$), the Disruption group, which received the reminder-ANISO treatment 24 hours after 5X5-HT training, showed a significant reduction in the number of SN varicosities. At 48-hr, the Disruption group shows an overall number of varicosities significantly less than that of the No Disruption group ($p < 0.001$) but not significantly different from that of the Control group. ANISO treatment alone has no effect on the varicosity number in the control or the 5X5-HT-trained cocultures during 24-48 hr (only some of the control cocultures received ANISO treatment at 24-hr). Plus signs indicate significance of the difference between the No Disruption group and the Control group, pound signs indicate significance of the difference between the Disruption group and the Control group, and asterisk signs indicate significance of the difference between the No Disruption group and the Disruption group. Error bars represent ± SEM.

Disrupting the reconsolidation of LTF 24 hours after 5X5-HT training causes an overall reduction in the number of varicosities at 48-hr

As previously reported (Glanzman et al., 1990), there was an increase in the number of SN varicosities in the cocultures that received the 5X5-HT training at 24-hr. As shown in Figure 4B, this morphological correlate of LTF lasts to 48-hr in the serotonin-trained cocultures that received no treatment at 24-hr. By contrast, in the trained cocultures that were given 1X5-HT

immediately followed by ANISO at 24-hr, the overall number of varicosities decreased to the extent that it is not different from that of the Control group at 48-hr. ANISO alone did not reduce the number of varicosities in control or 5X5-HT-treated cocultures during 24-48 hr. Therefore, reactivation of the long-term monosynaptic memory with a reminder of 1X5-HT immediately followed by protein synthesis inhibition selectively abolished the presynaptic structural growth that primarily underlies LTF (Kim et al., 2003).

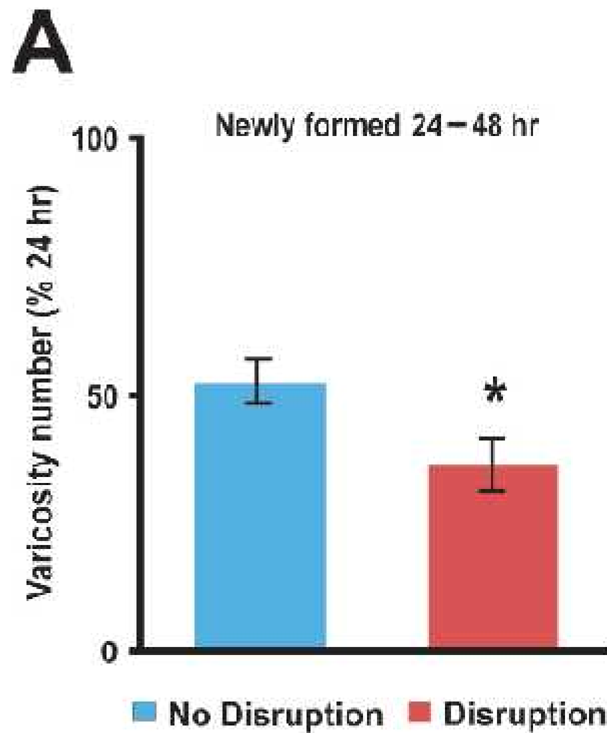

Figure 5. Disruption of reconsolidation of LTF reduces the synthesis of new varicosities.

(A) Mean normalized number of SN varicosities that newly formed during 24-48 hr from the 5-HT-treated groups – No Disruption (n = 40); Disruption (n = 26). New Varicosities, the varicosities that appeared during 24-48 hr, were counted at 48-hr, and the count was normalized to the overall number of varicosities present at 24-hr in each sensorimotor synapse. Compared to the No Disruption group, the Disruption group shows a significantly lower rate of formation of new varicosities during 24-48 hr ($p < 0.05$). Error bars represent ± SEM.

Disrupting the reconsolidation of LTF 24 hours after 5X5-HT training causes inhibition of 5-HT-induced growth during 24-48 hr

While it has been verified that the increase in the total number of SN varicosities, which accompanies LTF, can be abolished through disrupting the reconsolidation of LTF (Figure 4), what is of interest is to study how this reversal process takes place. One possible way to bring

about the reduction in the overall number of varicosities is to discourage the synapse from forming new varicosities. Figure 5A shows that the reversal of the morphological correlate of LTF through disrupting reconsolidation involves a reduction in the number of 5-HT-induced varicosities (Induced Varicosities) synthesized during 24-48 hr. The Reminder-ANISO treatment at 24-hr significantly lowered the rate of formation of new varicosities during 24-48 hr in the trained cocultures. ANISO alone did not change the rate of the 5-HT-induced growth during 24-48 hr. Therefore, disrupting the reconsolidation of LTF abolished the morphological correlate of LTF, at least in part, by inhibiting formation of new varicosities.

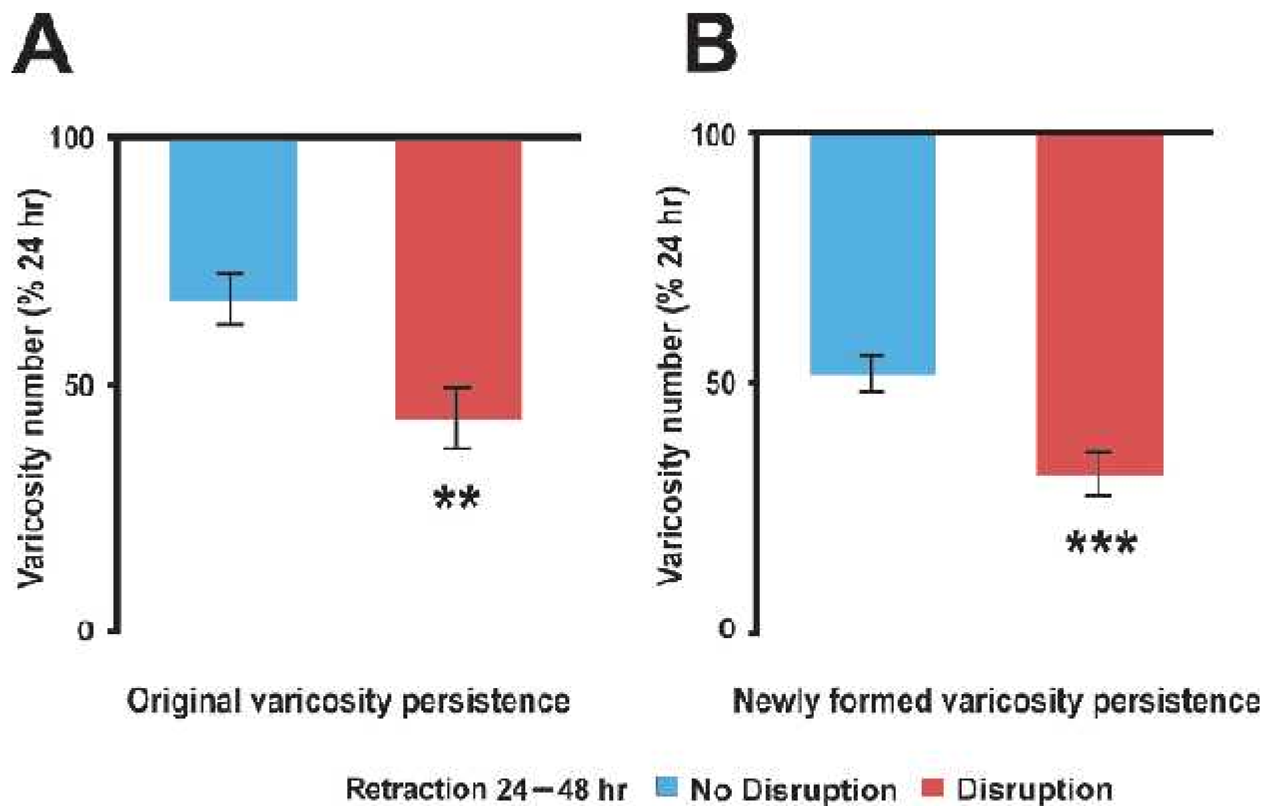

Figure 6. Disruption of reconsolidation of LTF reduces persistence of varicosities that existed prior to 5-HT training or newly formed within 24 hr after 5-HT training.

(A) Mean persistence rate of Original Varicosities during 24-48 hr from the 5-HT-treated groups – No Disruption (n = 40); Disruption (n = 26). The persistence rate of Old Varicosities (OVs), the varicosities that existed prior to 5-HT treatment, during 24-48 hr was measured. From each pair, the number of Old Varicosities that persisted during 24-48 hr was normalized to the number of Old Varicosities present in each sensorimotor synapse at 24-hr. Compared to the No Disruption group, the Disruption group shows a significantly lower persistent rate of Old Varicosities during 24-48 hr ($p < 0.01$). Error bars represent ± SEM.

(B) Mean persistence rate of Induced Varicosities during 24-48 hr from the 5-HT-treated groups – No Disruption (n = 40); Disruption (n = 26). The persistence rate of Induced Varicosities, the varicosities that formed within 24 hours after 5-HT treatment, during 24-48 hr was measured.

From each pair, the number of Induced Varicosities that persisted during 24-48 hr was normalized to the number of Induced Varicosities present in each sensorimotor synapse at 24-hr. Compared to the No Disruption group, the Disruption group shows a significantly lower persistent rate of Induced Varicosities during 24-48 hr ($p < 0.001$). Error bars represent ± SEM.

Disrupting the reconsolidation of LTF 24 hours after 5X5-HT training causes enhanced retraction of pre-learning varicosities as well as 5-HT-induced growth during 24-48 hr

Figure 5A shows that disrupting the reconsolidation of LTF reverses the presynaptic structural change associated with LTF by inhibiting 5-HT-induced growth. Our next question is whether disrupting the reconsolidation also triggers removal of existing varicosities. If so, then does this structural retraction process specifically remove the presynaptic varicosities that were formed within 24 hours after 5X5-HT treatment (Induced Varicosities). Or, does it nonspecifically remove Induced Varicosities as well as Original Varicosities, the varicosities that existed prior to 5X5-HT treatment? First, Figure 6A shows that the reversal of the morphological correlate of LTF through disrupting reconsolidation involves enhanced removal of Original Varicosities. The Reminder-ANISO protocol at 24-hr significantly increased the removal rate of Old Varicosities 24-48 hr in the 5-HT-trained cocultures. ANISO alone did not affect the erasure rate of these Old Varicosities during 24-48 hr, suggesting that memory reactivation, not protein synthesis inhibition, is necessary to trigger the retraction. Next, Figure 6B shows that disrupting reconsolidation also enhances the retraction rate of Induced Varicosities. The Reminder-ANISO treatment at 24-hr significantly increased the erasure rate of Induced Varicosities during 24-48 hr in the 5-HT-trained cocultures. Just as we saw in Old Varicosities, ANISO alone did not affect the removal rate of Induced Varicosities during 24-48 hr. Therefore, disrupting the

reconsolidation of LTF brings about the abolition of the morphological correlate of LTF, at least in part, by facilitating elimination of presynaptic varicosities regardless of whether their formation was induced by 5X5-HT training.

IV. Discussion

Persistent activity of PKM Apl III is necessary to maintain the morphological correlate of LTF in Aplysia

A morphological correlate of LTF, the increase in the number of presynaptic varicosities, was abolished when PKM Apl III activity was inhibited. In the cocultures that received the 5X5-HT training only, the increase in the overall number of varicosities was maintained for 48 hours. This is consistent with the previous finding that LTF is maintained for 48 hours in cocultures after 5X5-HT treatment (Cai et al., 2011). Specific inhibition of PKM Apl III at 24-hr, however, reversed the morphological correlate of LTF. This new finding clearly indicates that PKM Apl III plays a critical role in maintaining the learning-related morphological change. Since chelerythrine decreased the overall number of presynaptic varicosities only in the dishes that received prior 5-HT training, the action of PKM Apl III is specific for newly established memory. The necessity of persistent activity of PKM Apl III in maintaining LTF and LTS during 24-48 hr after 5-HT training has been verified previously (Cai et al., 2011). Therefore, the currently reported data provide strong evidence that PKM Apl III mediates synaptic facilitation, which underlies LTS, through maintaining the learning-related presynaptic growth. Moreover, the increase in the number of SN varicosities parallels the maintenance of LTS in *Aplysia* (Bailey and Chen, 1989), further supporting the idea that PKM Apl III maintains long-term memory by maintaining the morphological correlate of long-term memory.

5-HT-induced varicosities are not the only loci of long-term memory

Fate analysis of individual varicosities provides us with more information on how the synapse reverses the morphological correlate of learning during memory erasure. The fate analysis

showed that the PKM Apl III inhibition brought about the recovery of the original number of varicosities through at least two distinguishable strategies. First, inhibiting PKM Apl III reduced the rate of new growth. This suggests that PKM Apl III participates in some persistent molecular processes that mediate the formation of new varicosities during 24-48 hr. More studies are to be done to unveil the role of the enhanced presynaptic growth during this time period. Since it has been more than 24 hours since the synapse learned, this process is unlikely to be involved in memory acquisition process. It is possible that the chelerythrine-induced blockade of new varicosity formation prevented the synapse from undergoing late-phase consolidation of the long-term memory that does not depend on new protein synthesis. Secondly, inhibiting PKM Apl III nonspecifically facilitated removal of varicosities that formed prior to or after 5-HT training. This nonspecific action of PKM Apl III suggests that it is involved in a synaptic-tagging free mechanism that mediates varicosity maintenance. Elucidating the molecular mechanisms by which PKM Apl III maintains both Old Varicosities and Induced Varicosities will provide us with more information on therapeutic strategies for treating memory deficit disorders.

Molecular mechanisms by which PKM maintains long-term memory

Various recent studies have revealed the molecular mechanisms by which PKM maintains long-term memory. In rats, constitutively active PKM molecules maintain long-term memory by modulating the trafficking of GluR2-containing AMPARs (Migues et al., 2010). Also, in mouse anterior cingulate cortex, synaptic potentiation that lasted two weeks paralleled upregulation of the phosphorylated form PKM , while the enhanced expression of unphosphorylated PKM lasted for less than a week (Li et al., 2010). Later, in 2011, Sacktor suggested that PKM participates in a positive-feedback loop, in which it becomes phosphorylated by

phosphoinositide-dependent protein kinase (PDK1) and mediates phosphorylation of the C-terminal end of AMPAR or associated protein while interacting with protein interacting with C kinase (PICK1) dimers. In this pathway, PKM can facilitate the delivery of AMPARs to postsynaptic sites. The resulting event, the enhancement in the expression of AMPARs on postsynaptic sites, is known to be the molecular engram of LTP in mammals (Blair et al., 2001; Chitwood et al., 2001; Morris et al., 2003; Roberts and Glanzman, 2003; Rumpel et al., 2005). Our fate analysis in *Aplysia* shows that PKM Apl III inhibition eliminated both the Old Varicosities and the Induced Varicosities. This nonselective nature of the erasure process suggests that the molecular engram of long-term memory, which is maintained by PKM Apl III, does not lie in the Induced Varicosities only.

Disrupting the reconsolidation of LTF at 24-hr reversed the morphological correlate of LTF

The physical reversal of the learning-related presynaptic growth is in agreement with the elimination of behavioral and electrophysiological forms of long-term memory induced by disrupting reconsolidation of LTF in *Aplysia* (Cai et al., 2012). The consistent pattern of abolition strengthens the validity of conducting morphological analysis to study the molecular mechanisms that underlie reconsolidation of nonassociative long-term memory using the sensorimotor synapse *in vitro*. Also, the failure to disrupt the morphological correlate of LTF by ANISO alone is in agreement with the previous finding that LTF maintenance does not depend on new protein synthesis from 24 hours after learning in *Aplysia* (Cai et al., 2011). Furthermore, in cocultures that were not given prior 5-HT training at 0-hr, ANISO alone did not cause a decrease in the number of overall varicosities during 24-48hr, suggesting that protein synthesis is not required to maintain the baseline number of varicosities prior to learning.

According to our data, giving a reminder immediately prior to ANISO treatment was necessary to reduce the overall number of SN varicosities. Memory reactivation with a single set of serotonin immediately followed by ANISO reversed the serotonin-induced structural growth by 48-hr. This finding suggests that giving a reminder induces labilization of LTF by destabilizing individual varicosities, and the synapse undergoes reconsolidation, which requires some translation-dependent process(es), to maintain the morphological evidence of the original memory. Moreover, the physical reversal in the morphological correlate of LTF indicates that the disruptive effect of the 1X5-HT & ANISO does not result from impairment of memory retrieval, but from at least partial reversal of the physical memory trace.

Molecular relevance of disrupting memory reconsolidation to PKM Apl III inhibition

An intriguing question is whether disrupting memory reconsolidation involves inhibition of PKM Apl III or, instead, an independent mechanism that results in an analogous reversal of synaptic growth. The fate analysis reveals that the reversal of the morphological correlate of LTF by disrupting reconsolidation resulted from both inhibition of new growth during 24-48 hr and nonspecific removal of varicosities based on their formation history. The two strategies the synapse exhibits during disruption of reconsolidation are analogous to those triggered by PKM Apl III inhibition. Recent studies suggest that this phenotypic similarity in the dynamics of memory erasure arises from molecular relevance. It has been found previously that reactivation of long-term memory involves facilitation of AMPAR endocytosis in the MN (Rao-Ruiz et al., 2011; Rose and Rankin, 2006). With the involvement of PKM in modulation of the AMPAR trafficking ascertained by Sacktor in 2011, what will be of interest is to examine how disrupting memory reconsolidation destabilizes this PKM -mediated pathway. Understanding the molecular

mechanisms by which reactivation destabilizes long-term memory will help us find therapeutic targets for diseases characterized by abnormal memory retrieval, such as Post-Traumatic Stress Disorder.

Possible roles of ApCPEB in long-term memory maintenance

One of the most important findings in the fate analysis is that the reduction in the overall number of varicosities through PKM Apl III inhibition and through disrupting reconsolidation does not occur through simple retraction of the new varicosities induced by 5-HT training. This argues against the conventional belief that the engram for LTF is completely localized to the Induced Varicosities, the varicosities that formed within 24 hours after 5-HT treatment. It has been proposed previously that ApCPEB mediates tagging of Induced Varicosities, and this process is accompanied by the polymerization of ApCPEB and consequent local protein synthesis (Si et al., 2003a, b). Our morphology data argue against this hypothesis because memory erasure led to removal of Induced Varicosities as well as Old Varicosities that existed prior to 5-HT training. Furthermore, ANISO treatment alone did not perturb the enhancement in the number of varicosities, suggesting that the synapse does not require ongoing protein-synthesis mechanisms to maintain LTF during 24-48 hr. This argues against the previous finding that ApCPEB-mediated local protein synthesis is necessary to maintain LTF during 24-48 hr (Miniaci et al., 2008; Si et al., 2010). More future studies can be done to address the contradicting results.

Another indication of the differential roles of ApCPEB and PKM Apl III in maintenance of long-term memory is the different critical time windows for ApCPEB and PKM Apl III. In 2012, Cai and associates verified that persistent PKM Apl III activity is required to maintain LTS for 24-168 hours after sensitization training. ApCPEB activity, however, is required to maintain

LTF possibly during 0-24 hr (Cai et al., 2011) and certainly during 24-48 hr (Miniaci et al., 2008; Si et al., 2010), but not at 168 hr (Cai et al., 2011). These findings suggest that while PKM Apl III is required for maintenance of the early and the late phases of LTF, ApCPEB is required for the induction and the maintenance of the early phase of LTF only. Future studies can reveal whether ApCPEB and PKM Apl III interact during 24-48 hr after the 5-HT training, during which the ongoing activities of both are required to maintain LTF.

Elucidation of PKM Apl III is role in long-term memory maintenance illuminates potential to manipulate learning and memory function

Although studies using the model of *Aplysia* have focused predominantly on presynaptic changes there is ample evidence that postsynaptic neurons mediate synaptic plasticity in *Aplysia*. *In-vitro* studies have shown that a retrograde signal from the postsynaptic neuron, of which the secretion is mediated by postsynaptic Ca^{2+}, is necessary to induce the morphological correlate of LTF in *Aplysia* (Cai et al., 2008). It was also found that the expression of PKM Apl III is upregulated in the MN by 5-HT infusion (Villareal et al., 2009). The molecular mechanism by which PKM regulates the AMPAR trafficking in the postsynaptic neuron is not fully understood. Studying how PKM inhibition or disrupting reconsolidation affects the postsynaptic side of the synapse in *Aplysia* will help make the *Aplysia* literature more relevant to the mammalian literature, and, going further, to clinical research for human subjects.

Manipulation of PKM activity in mammals and *Aplysia* illuminates a pathway to artificially enhance one's memory or treat memory deficit problems. There is evidence that overexpression of PKM in rat neocortex enhanced the duration of degrading long-term memory (Shema et al., 2011). However, one caveat is that PKM is synthesized by different mechanisms

in mammals and invertebrates. In mammals, PKM is produced through transcription from an alternative start site within the atypical PKC gene (Sacktor, 2012), whereas in *Aplysia*, PKM Apl III is synthesized by post-translational calpain-dependent cleavage of PKC APl III, an ortholog of mammalian PKC (Bougie et al., 2009). Nevertheless, ZIP, a commonly used inhibitor of mammalian PKM , reversed 5-HT-induced LTF *Aplysia* also (Cai et al., 2011). The universal effectiveness of ZIP in blocking both mammalian and *Aplysia* isoforms of PKM suggests that the kinase appears to behave similarly in these groups of animals.

Analysis of the morphological study and additional questions to be addressed

In our study, we have shown that PKM Apl III inhibition or disrupting reconsolidation reversed the morphological correlate of LTF, the increase in the total number of SN varicosities. However, one should keep in mind that presynaptic growth is not the only mechanism by which the synapse induces LTF during 0-24 hr. Other events that accompany LTF, such as activation of silent presynaptic varicosities that existed prior to 5-HT training (Kim et al., 2003) contributes to synaptic facilitation as well. To gain a more comprehensive understanding of the dynamics of memory maintenance and erasure, it is critical to study how these seemingly distinct correlates of LTF are affected by PKM Apl III inhibition or disruption of reconsolidation of LTF in *Aplysia*.

One possible improvement in the morphological analysis is to devise a quantifiable method to account for SN varicosities' differential contributions to synaptic transmission based on their cross-sectional areas. There are two major limitations in this quantification process. First, the cross-sectional images are not necessary taken on the same plane in the three image sessions. The sensorimotor synapse, which floats in the medium of coculture bath, inevitably moves between image sessions. The mobility of the synapse skew the angles at which the cross-

sectional images of individual varicosities are taken, thereby making varicosity area not a reliable measure. Secondly, even if we can resolve the first limitation in quantification, there are too many variables that determine the amount of transmitter release from each varicosity, such as the number of transmitter-containing vesicles in the active zone of the varicosity, the presence of proteins that mediate the docking of the vesicles to the presynaptic membrane of the varicosity, the area of postsynaptic structures in contact with the varicosities, and the location on the MN to which the varicosities are attached. Elucidating how each of the abovementioned factors influences transmitter release will allow us to acquire a more precise measure of transmitter release from each SN.

V. Conclusion

Our morphological study shows that the morphological correlate of LTF, the primary contributor to synaptic facilitation (Kim et al., 2003), was abolished by inhibiting an isoform of PKM or disrupting reconsolidation of LTF in *Aplysia*. The abolition of the nonassociative synaptic memory involved inhibition in the synthesis of new varicosities and enhancement in the erasure of varicosities that existed prior to or after 5-HT training. These findings reveal insightful information on how the physical trace of long-term memory is eliminated during memory erasure in *Aplysia*. Previously, PKM inhibition and disruption of reconsolidation appeared to be related mechanistically, as can be seen by their common implications in the AMPAR trafficking (Migues et al., 2010; Rao-Ruiz et al., 2011; Rose and Rankin, 2006; Sacktor, 2011). In our study, the two memory erasure protocols triggered analogous structural dynamics of SNs, further supporting the idea that PKM-mediated pathway is affected by disruption of reconsolidation. Through the reductionist system capable of undergoing learning-related structural growth as well as reconsolidation, we should ascertain the role of PKM in reconsolidation. Moreover, we should study how PKM Apl III interacts with ApCPEB, a molecule also known to mediate the persistence of long-term memory in *Aplysia* (Miniaci et al., 2008; Si et al., 2003a, 2010).

VI. References

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