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ROLE OF SYNAPSIN IN LONG-TERM SYNAPTIC FACILITATION IN APLYSIA

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ROLE OF SYNAPSIN IN LONG-TERM SYNAPTIC FACILITATION IN *APLYSIA*

by

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ROLE OF SYNAPSIN IN LONG-TERM SYNAPTIC FACILITATION IN *APLYSIA*

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Anne Netek Hart, B.S., B.S.

Houston, Texas

May 2011

DEDICATION

This work is dedicated to my family

“The family - that dear octopus from whose tentacles we never quite escape, nor, in our inmost hearts, ever quite wish to.” -Dodie Smith

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ABSTRACT

ROLE OF SYNAPSIN IN LONG-TERM SYNAPTIC FACILITATION IN *APLYSIA*

Publication No. _____

Anne Netek Hart, B.S., B.S.

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Enhanced expression of the presynaptic protein synapsin has been correlated with certain forms of long-term plasticity and learning and memory. However, the regulation and requirement for enhanced synapsin expression in long-term memory remains unknown. In the present study the technical advantages of the marine mollusc *Aplysia* were exploited in order to address this issue. In *Aplysia*, learning-induced enhancement in synaptic strength is modulated by serotonin (5-HT) and treatment with 5-HT in vitro of the sensorimotor synapse induces long-term facilitation (LTF) of synaptic transmission, which lasts for days, as well as the formation of new connections between the sensory and motor neuron.

Results from immunofluorescence analysis indicated that 5-HT treatment upregulates synapsin protein levels within sensory neuron varicosities, the presumed site of neurotransmitter release. To investigate the mechanisms underlying increased synapsin expression, the promoter region of the *Aplysia synapsin* gene was cloned and a cAMP response element (CRE) was identified,

raising the possibility that the transcriptional activator cAMP response element-binding protein-1 (CREB1) mediates the 5-HT-induced regulation of synapsin. Results from Chromatin Immunoprecipitation (ChIP) assays indicated that 5-HT treatment enhanced association of CREB1 surrounding the CRE site in the *synapsin* promoter and led to increased acetylation of histones H3 and H4 and decreased association of histone deacetylase 5 surrounding the CRE site in the *synapsin* promoter, a sign of transcriptional activation. In addition, sensory neurons injected with an enhanced green fluorescent protein (EGFP) reporter vector driven by the *synapsin* promoter exhibited a significant increase in EGFP expression following treatment with 5-HT. These results suggest that synapsin expression is regulated by 5-HT in part through transcriptional activation of the *synapsin* gene and through CREB1 association with the *synapsin* promoter. Furthermore, RNA interference that blocks 5-HT-induced elevation of synapsin expression also blocked long-term synaptic facilitation. These results indicate that 5-HT-induced regulation of synapsin is necessary for LTF and that synapsin is part of the cascade of synaptic events involved in the consolidation of memory.

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CHAPTER I. BACKGROUND AND INTRODUCTION

INTRODUCTION

The processes that contribute to memory acquisition and consolidation of long-term memories are extraordinarily complex. Long-term memory can be subdivided into two general categories: explicit and implicit memory (Squire and Zola, 1996). Explicit, or declarative, memory is the memory for the conscious recall of facts and events. Implicit, or nondeclarative, memory is the memory for procedural (skills and habits), priming, classical conditioning and nonassociative (sensitization and habituation) learning.

One of the great challenges of studying the underlying processes of learning and memory is the ability to determine where the memory is stored. Once this is identified, then the relevance of molecular, genetic, biophysical and anatomical properties and plastic changes in these properties can be examined. Although many of the structures in the mammalian brain associated with both explicit and implicit memory have been identified (Squire and Zola, 1996), the mammalian brain is quite complex and the contribution and relevance of these factors can be difficult to discern. Herein lies the greatest advantage of utilizing simple systems such as *Aplysia* in the study of learning and memory. The simple nervous system of the marine mollusc contains large, identifiable neurons and is comprised of approximately 20,000 nerve cells, as compared to 10^{12} nerve cells in mammals, and these cells are clustered into ten ganglia. Behaviors of this animal can be modified to display multiple types of implicit learning including sensitization, habituation and dishabituation as well as both classical and operant conditioning. The identification and accessibility of *Aplysia* nerve cells allows for the characterization and modeling

of the neuronal circuits and pathways that mediate these behaviors resulting in more extensive analysis of the molecular cascades and biophysical properties of cells contributing to these processes (Byrne and Kandel, 1996).

LONG-TERM SENSITIZATION AND LONG-TERM FACILITATION IN *APLYSIA*

The extensively studied siphon-elicited siphon-gill withdrawal and tail-elicited tail-siphon withdrawal reflexes in *Aplysia* can undergo an elementary form of nonassociative learning called sensitization. Sensitization is the enhancement of a response to a weak stimulus, after a previous presentation of noxious stimuli and can occur after behavioral training of the animal (Pinsker et al., 1973). Behavioral sensitization, a learned fear response induced by a strong stimulus, is accompanied by facilitation at the monosynaptic connections between sensory neurons and motor neurons (Castellucci et al., 1970; Walters et al., 1983; Frost et al., 1985; Cleary et al., 1998). Strong evidence indicates that serotonin (5-hydroxytryptamine, 5-HT) is the neuromodulator that induces facilitation of the sensorimotor synapse (Glanzman et al., 1989; Levenson et al., 1999; Marinesco and Carew, 2002) and that this connection is glutamatergic (Dale and Kandel, 1993; Chin et al., 2002a; Antzoulatos and Byrne, 2004). During sensitization training, 5-HT levels in the hemolymph are elevated (Levenson et al., 1999) and depletion of 5-HT by the addition of the neurotoxin, 5, 7-DHT, blocks sensitization (Glanzman et al., 1989). The application of 5-HT *in vitro* mimics behavior training and induces facilitation (Montarolo et al., 1986; Mauelshagen et al., 1998).

5-HT or behavioral training can result in temporally graded, distinct phases of facilitation and/or memory (Mauelshagen et al., 1998; Sutton et al., 2002). Short-term sensitization (STS), induced by a single tail shock, and short-term facilitation (STF), induced by one pulse of 5-HT, lasts for minutes to hours and is not sensitive to inhibitors for transcription or protein synthesis (Pinsker et al., 1970; Montarolo et al., 1986). However, long-term sensitization (LTS), induced by spaced training, and long-term facilitation (LTF), induced by repeated pulses of 5-HT, lasts from days to weeks and requires both transcription and new protein synthesis (Pinsker et al., 1973; Montarolo et al., 1986; Emptage and Carew, 1993; Mauelshagen et al., 1996; Cleary et al., 1998; Sutton et al., 2002; Wainwright et al., 2002).

One major advantage of using this model system is that the sensorimotor synapse can be reconstituted in culture (Rayport and Schacher, 1986; Angers et al., 2002; see also Fig. 1.1). The ease of culture preparation has made this system widely accessible for detailed analysis as sensory and motor neurons are readily identifiable based on their size and position within the ganglion (Walters et al., 1983; Rayport and Schacher, 1986).

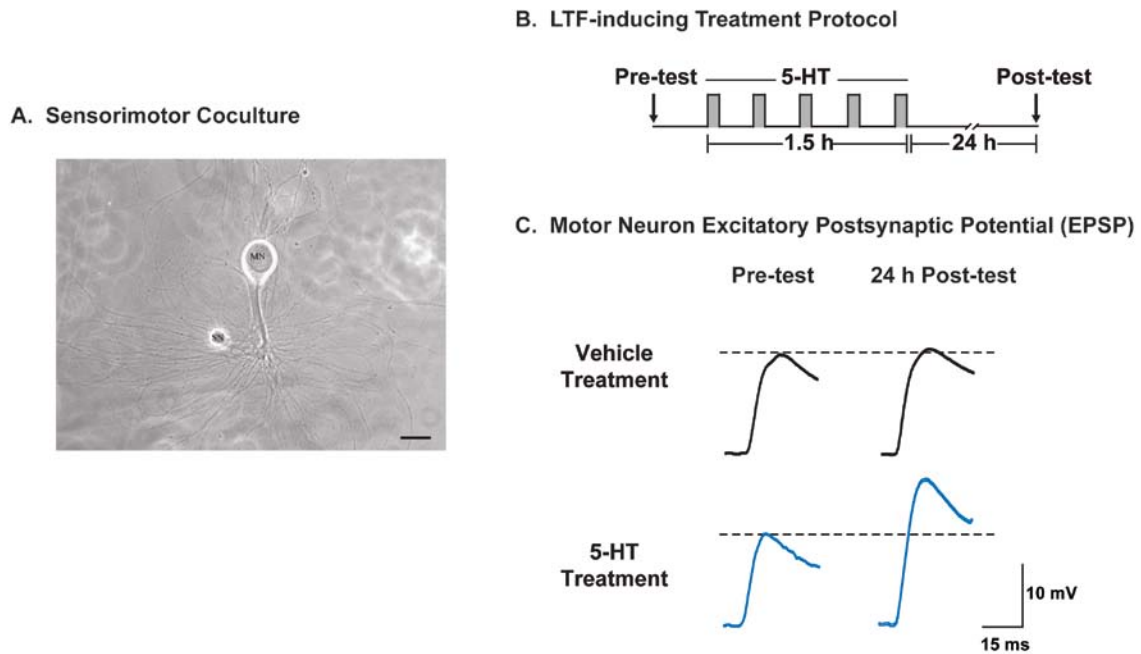


Figure 1.1. Example of long-term synaptic facilitation of a sensory and motor neuron connection in culture. **A**, Phase-contrast image of a fixed sensorimotor coculture on Day 6. The cell body of the sensory neuron (SN) and motor neuron (MN) are labeled and the extended neuritic processes are visible. Synaptic strength is assessed by extracellularly stimulating the SN and recording the change of potential in the MN, the excitatory postsynaptic potential (EPSP), using an intracellular electrode. Scale bar, 100 μm . **B**, LTF can be induced in culture by treating the culture with 5, 5 min pulses of 5-HT with an interstimulus interval of 20 min. **C**, The extent of facilitation is assessed by comparing the amplitude of the EPSP from the post-test (24 h after treatment) to the pre-test (prior to treatment). The amplitude of the EPSP is not different before and after treatment when the culture is treated with vehicle, but the EPSP amplitude is greater 24 h after treatment with 5-HT, compared to the pre-test. Hart and Byrne, unpublished observations.

MOLECULAR MECHANISMS OF LONG-TERM FACILITATION

Long-term changes in synaptic plasticity require the modification of gene expression and new protein synthesis (Castellucci et al., 1986; Montarolo et al., 1986; Castellucci et al., 1989; Miniaci et al., 2008). Binding of 5-HT to sensory neuron receptors induces multiple cascades of events that lead to an enhanced response (Hawkins et al., 2006; Fig. 1.2). In sensory neurons, 5-HT elevates cyclic adenosine monophosphate (cAMP) (Bernier et al., 1982; Ocorr and Byrne, 1985) through an adenylyl cyclase coupled receptor (Lee et al., 2009). Persistent activation of cAMP-dependent protein kinase PKA (Hegde et al., 1993; Chain et al., 1995) leads to the activation (phosphorylation) of the transcriptional activator, cAMP response element-binding protein-1 (Bartsch et al., 1998). CREB1 activates gene transcription by binding to a cAMP response element (CRE) in the promoter region of target genes (Dash et al., 1990) via its basic leucine zipper (bZIP) domain. Injection of CRE oligonucleotides into the sensory neuron competes for CREB1 binding to DNA and blocks 5-HT-induced LTF, but not STF, at the *Aplysia* sensorimotor synapse (Dash et al., 1990). In addition, expression of a reporter gene containing CRE sequences in the promoter region is enhanced in sensory neurons after 5-HT treatment (Liu et al., 2008). Interestingly, injection of phosphorylated (activated) CREB1 into the sensory neuron, without additional treatment, is able to initiate LTF in culture (Bartsch et al., 1998). Blocking the function of CREB1 through antibody injection (Liu et al., 2008), or blocking the 5-HT-induced CREB1 expression using RNA interference (Liu et al., 2011b) blocks

LTF, further supporting the necessary role of CREB1 in long-term synaptic facilitation.

Previous work in the Byrne lab described the time course of 5-HT-induced CREB1 mRNA and protein levels; CREB1 mRNA and protein levels are elevated up to 24 h after treatment (Liu et al., 2008). Interestingly, the lab found that CREB1 may regulate its own expression through a positive feedback loop in which CREB1 protein binds to and activates CREB1 gene expression in a 5-HT-induced manner (Mohamed et al., 2005).

Other types of memory also recruit the cAMP-PKA-CREB pathway, including an explicit form of memory, mammalian hippocampal-dependent long-term memory (Pittenger et al., 2002), as well as another implicit form of memory, olfactory learning in *Drosophila* (Yin et al., 1994). These types of memory are also dependent on transcription and translation (Bourtchuladze et al., 1994; Tully et al., 1994; Alberini et al., 1995; Alberini, 2008). Many similarities exist between the molecular mechanisms of certain forms of long-term plasticity in *Aplysia* and mammalian hippocampal long-term potentiation (Alberini et al., 1995; Bailey et al., 1996; Pittenger and Kandel, 2003; Lee et al., 2008).

5-HT and PKA activation also activate extracellular signal-regulated kinase (ERK, also known as p42/44 mitogen-activated protein kinase, MAPK) resulting in its translocation to the nucleus (Martin et al., 1997; Michael et al., 1998). Like the cAMP-PKA-CREB pathway, MAPK activation and signaling has also been implemented in multiple types of mammalian synaptic plasticity and memory (for review see: Sweatt, 2001). Results from Michael et al. (1998) and Bartsch et al.

(1995) indicate that MAPK phosphorylates CREB2, a transcription factor, inhibiting its function as a transcriptional repressor. Indeed, inhibiting CREB2 by either injection of anti-CREB2 antibody or RNA interference (RNAi) in the sensory neuron decreased the threshold for LTF induction and can convert STF to LTF (Bartsch et al., 1995; Lee et al., 2003). In addition, overexpressing CREB2 blocks 5-HT-induced LTF (Lee et al., 2003). Previous work from the lab revealed that 5-HT treatment of ganglia and sensory neuron cultures induced an increase in CREB2 protein immediately after treatment followed by a decrease in CREB2 levels 12 h after treatment (compared to control treatment) (Liu et al., 2011). Although the *Aplysia creb2* promoter contains a CRE sequence to which CREB1 and CREB2 associate with under basal conditions, 5-HT does not appear to regulate CREB2 transcription immediately after 5-HT treatment (Mohamed et al., 2005).

These results indicate that the combined effect of activating CREB1 and repressing CREB2 lead to the transcriptional induction of genes necessary for LTF (Kandel, 2001). However, less is known about the downstream effector genes that are regulated by CREB and contribute to LTF. Results from Guan et al. (2002) indicate that the transcription factor, *Aplysia* CCAAT enhancer binding protein (ApC/EBP), is regulated by CREB1. *ApC/EBP* is an immediate early gene, rapidly upregulated in response to cAMP or 5-HT (Alberini et al., 1994) and is another common memory-associated transcription factor shared between *Aplysia* and vertebrates (Alberini, 2009). Immediate early genes are not sensitive to protein synthesis inhibitors and their increased expression occurs as an initial step in response to stimuli. Multiple approaches have indicated that C/EBP is necessary

for LTF. For example, injection of double stranded RNA (dsRNA), antisense RNA targeting C/EBP (Lee et al., 2001) or an anti-C/EBP antibody (Alberini et al., 1994) into the sensory neuron independently inhibited 5-HT-induced LTF.

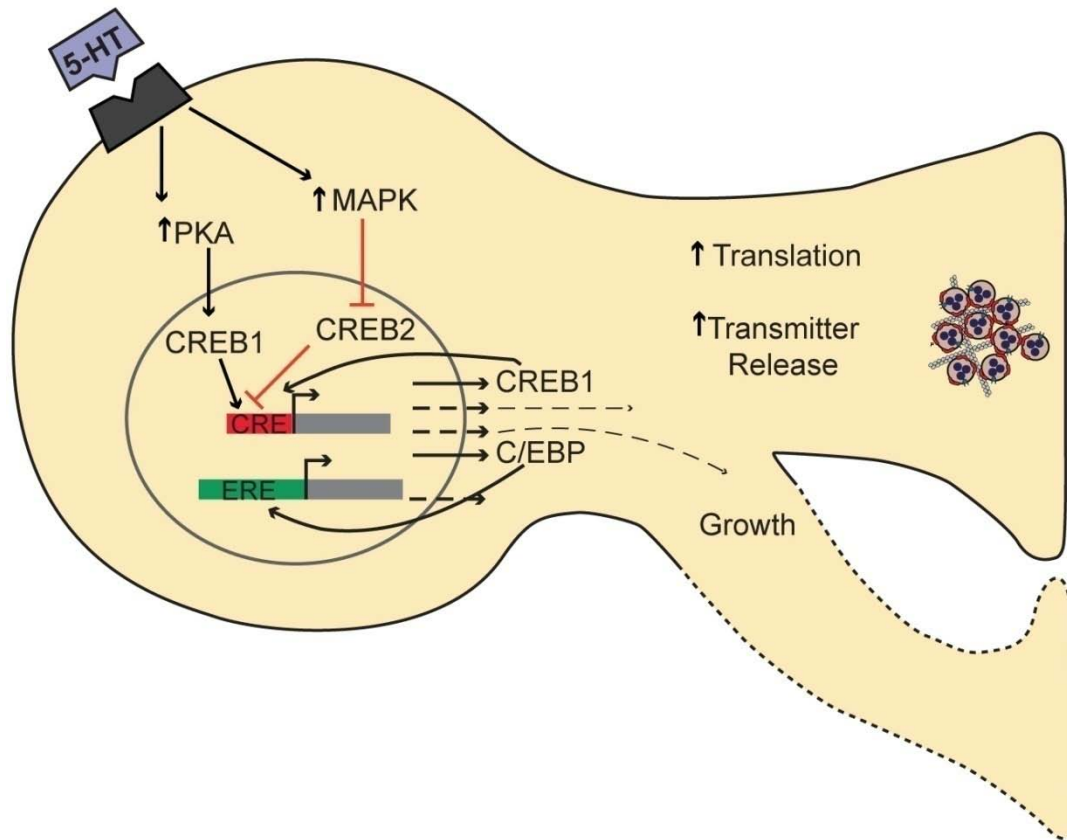


Figure 1.2. Simplified model of the molecular mechanisms underlying LTF in *Aplysia*. Five pulses of 5-HT induces LTF, which is dependent on both transcription and translation, leads to an increase in neurotransmitter release and sensory neuron growth. 5-HT binds to sensory neuron receptors and leads to the activation of multiple cascades resulting in the activation of PKA and MAPK. PKA phosphorylates CREB1, leading to activation of the transcription factor. Binding of CREB1 to CRE sites in the promoter regions of genes (e.g. *creb1*, *c/ebp*) induces transcription, a process necessary for LTF. MAPK phosphorylates CREB2, a transcriptional repressor, resulting in the inhibition of the ability of CREB2 to repress transcription. The combined activation of CREB1-mediated gene expression and inhibition of CREB2-mediated transcription suppression, leads to the activation of genes necessary for LTF. Although many of the transcription factors which are important for LTF have been identified, less is known about the genes regulated by these factors.

Although the time course of expression and requirement for transcription factors implemented in LTF has been examined, the identification of genes regulated by these remains to be better understood. In addition to C/EBP, another immediate early gene whose expression is induced by 5-HT and is necessary for LTF is ubiquitin C-terminal hydrolase (ApUch) (Hegde et al., 1997; Mohamed et al., 2005). ApUch cleaves the regulatory subunit of PKA and frees the catalytic subunit resulting in extended activation of this kinase (Hegde et al., 1993). Unlike C/EBP however, it does not appear that *ApUch* gene expression is directly regulated by CREB1 or CREB2 even though a CRE is present in the promoter region of the gene (Mohamed et al., 2005). However, recently the Byrne lab found that CREB2 exhibits enhanced binding to the *ApUch* promoter after long-term depression (LTD)-inducing protocols (Fioravante et al., 2008).

Results from another invertebrate model of long-term memory, aversive operant conditioning of aerial respiratory behavior in *Lymnaea stagnalis*, (Guo et al., 2010) indicated that the expression of the presynaptic proteins, syntaxin-1, a tSNARE protein, and dynamin-1, a protein involved in endocytosis and vesicle recycling, are regulated by CREB1. These results indicate that synaptic terminal-associated proteins may be an important target of CREB-regulated gene expression.

LEARNING-INDUCED SYNAPTIC GROWTH

One class of mechanisms underlying synaptic plasticity is the growth of new synaptic connections at the sensorimotor synapse. In many cases, both LTS and

LTF are associated with morphological changes in the sensory neuron (Bailey and Chen, 1988). For example, a 4-day sensitization training protocol leads to an overall increase in the total number of varicosities (the presumed presynaptic release site), sensory neurite outgrowth as well as an increase in the number, size and vesicle complement of presynaptic active zones (Bailey and Chen, 1983; Wainwright et al., 2002). In addition, an increase in varicosity formation and the number of branch points in the sensory neuron can be induced by cAMP injection indicating that the cAMP pathway is part of the induction mechanism contributing to long-term structural modifications (Nazif et al., 1991; O'Leary et al., 1995). Similar morphological changes have been observed in dissociated sensorimotor cocultures following 5-HT treatment (Glanzman et al., 1990; Hatada et al., 2000; Kim et al., 2003). It has been suggested that morphological changes associated with LTS and LTF, such as synaptogenesis and neurite outgrowth, may contribute to the stabilization (i.e. consolidation) of long-term memory (Bailey et al., 2004). However, there are cases where varicosity formation does not correlate with behavior or synaptic strength (Casadio et al., 1999; Hatada et al., 2000; Wainwright et al., 2002; Wainwright et al., 2004), indicating that a causal relationship may not exist and that further understanding of the contribution of morphological alterations to long-term memory and synaptic plasticity is needed.

THE ROLE OF SYNAPSIN IN LEARNING AND MEMORY, NEURONAL MORPHOLOGY AND SYNAPTIC PLASTICITY

The regulation of presynaptic proteins, including synapsin, syntaxin, and others, in learning and memory and particularly various forms of synaptic plasticity has been recently investigated (Hicks et al., 1997; Powell, 2006; Guo et al., 2010). The synapsin family of proteins are multi-functional proteins suggested to be involved with the establishment of synaptic connections, the regulation of neurotransmission, synaptic vesicle regulation and organization and learning and memory (Cesca et al., 2010). In mammals, three distinct synapsin genes (Syn I, II, III) encode ten different isoforms (Syn Ia, Ib, IIa, IIb, IIIa-f) whereas there is only one *synapsin* gene in *Aplysia* (Sudhof et al., 1989; Porton et al., 1999; Angers et al., 2002). Although it is still unknown which mammalian synapsin isoform *Aplysia* synapsin is most similar to, it appears that *Aplysia* synapsin has a similar domain arrangement to human synapsin Ia (Sudhof, 1990; Angers et al., 2002; summarized in Fig. 1.3). In general for the synapsin isoforms, the N-terminal, which consists of Domains A, B and C is more conserved whereas the C-terminal domain, Domains D-I, has more deviation across species and isoforms (Cesca et al., 2010).

Aplysia synapsin contains 28% sequence identity within Domain A compared to human synapsin I as well as the highly conserved PKA/CaMKI (Ca^{2+} /calmodulin-dependent protein kinase I) phosphorylation site (Angers et al., 2002) which modulates synapsin association with synaptic vesicles (Hosaka et al., 1999). *Aplysia* synapsin Domain B, considered a linker region for Domains A and C and not highly conserved, contains two MAPK phosphorylation sites, similar to mammalian synapsin I (Jovanovic et al., 1996; Matsubara et al., 1996; Cesca et al., 2010). Domain C, which is hypothesized to mediate the interaction of synapsin with

actin filaments and synaptic vesicle phospholipids as well as dimerization (Benfenati et al., 1989b; Benfenati et al., 1989a; Hosaka and Sudhof, 1999) is the most highly conserved domain between *Aplysia* synapsin and human synapsin Ia at 63% sequence identity (Angers et al., 2002). Similar to human synapsin Ia, *Aplysia* Domain D contains a MAPK phosphorylation site (Jovanovic et al., 1996; Matsubara et al., 1996) but lacks phosphorylation sites for CaMKII, p21-activated kinase (PAK and Cdk1 and Cdk5 (Angers et al., 2002; Cesca et al., 2010). At 20% sequence identity to human synapsin Ia, *Aplysia* synapsin contains Domain E, common to all 'a' isoforms and important for synapsin targeting to synaptic terminals (Gitler et al., 2004b), synaptic vesicle trafficking at the squid giant synapse (Hilfiker et al., 1998) as well as dimerization and synaptic vesicle clustering (Monaldi et al., 2010). *Aplysia* synapsin also contains many phosphorylation sites for protein kinase C (PKC), which are scattered throughout the various domains (Angers et al., 2002). Previous studies from the Byrne lab using kinase inhibitors indicate that one pulse of 5-HT leads to the phosphorylation of *Aplysia* synapsin which is dependent on both PKA and MAPK but independent of PKC activity (Angers et al., 2002).

Domains	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>
Function	SV association; actin binding	Linker region; SV association; actin binding	Association with actin filaments, synapsin, SV phospholipids	Actin binding	Targeting to synaptic terminals; SV trafficking and clustering; dimerization
Human Synapsin Ia	PKA CaMKI/IV	MAPK	Tyr-kinase Src	MAPK CaMKII PAK Cdk1, Cdk5	
<i>Aplysia</i> Synapsin	PKA CaMKI/IV	MAPK PKC	PKC	MAPK	PKC
Sequence Identity	28%		63%		20%

Figure 1.3. Summary table indicating similarities between human synapsin Ia and *Aplysia* synapsin. Conservation within domains A-E has been identified and summarized here indicating the hypothesized function of each domain, the phosphorylation sites within each domain for both human synapsin Ia and *Aplysia* synapsin as well as the shared sequence identity within each domain (Angers et al., 2002; Cesca et al., 2010). It should be noted that the hypothesized functions of each domain are based on studies in other species (Cesca et al., 2010).

Knocking out all three mammalian *synapsin* genes does not produce a lethal phenotype and synapsin is not necessary for general neuronal development or basal synaptic function (Gitler et al., 2004a). However, the functional significance of different synapsin isoforms is dependent on the cell type and synapse where synapsin regulates synaptic transmission and synaptic vesicle pools (Ferreira et al., 1998; Ferreira et al., 2000; Gitler et al., 2004a; Gitler et al., 2008; Cesca et al., 2010).

The Role of Synapsin in Learning and Memory

Recent studies have provided evidence of a role for the synaptic terminal-associated protein in several different paradigms of learning and memory. During spatial learning in the Morris water maze task, animals use visual cues to find a hidden platform in murky water. This task induced synapsin I mRNA and protein expression in rats in the hippocampus compared to both control-active (animals that were matched for the same amount of swim time) and control-sedentary rats (Gomez-Pinilla et al., 2001). These results indicated that enhanced synapsin expression is correlated with learning. Knock-out studies suggest that inhibiting expression of synapsin impairs performance on spatial learning and memory tasks. For example, knocking out all three synapsin genes impairs spatial working memory in the radial arm maze task (Gitler et al., 2004a). However the importance of synapsin is less clear as Corradi et al. (2008) found that spatial learning impairment on the Morris water maze task in Syn II^{-/-}, but not Syn I^{-/-}, is dependent on age as young animals do not exhibit impairment in this task (Silva et al., 1996; Corradi et

al., 2008). In these experiments, learning deficiencies in synapsin knock-out mice were also correlated with enhanced age-dependent neuronal loss and astrogliosis in the hippocampus and cortex (Corradi et al., 2008). It is unknown how developmental compensatory mechanisms, basal expression of synapsin III and aging processes affected these results.

Fear conditioning is often used to explore mechanisms underlying emotional memory. In this paradigm animals learn to associate a non-harmful (cued) stimulus (i.e. tone or light) with a harmful stimulus (i.e. foot shock) and freeze upon presentation of the cued stimulus. Both *Syn I^{-/-}* and *Syn II^{-/-}* mice exhibit age-dependent impairment for memory of both the context and the cued stimulus (Corradi et al., 2008). In a separate experiment, severe impairment for context memory was identified in *SynII/II^{-/-}* mice but not *SynI^{-/-}* mutant mice (Silva et al., 1996). These results provide evidence of a role of synapsin in learning and memory, but also exemplify the complications that can be associated with synapsin knock-out studies which suggest that synapsin genes contribute differently to memory.

Other types of memory appear to be associated with changes in synapsin expression. For example, upregulation of synapsin I mRNA, as well as CREB and BDNF mRNA, in the hippocampus is correlated with the training period of operant conditioning (Rapanelli et al., 2009). In addition, synapsin expression and regulation may participate in the formation and/or consolidation of song-related memories in the zebra finch (Velho and Mello, 2008). Furthermore, a deficiency in synapsin expression in larvae and adult *Drosophila* impairs olfactory associative

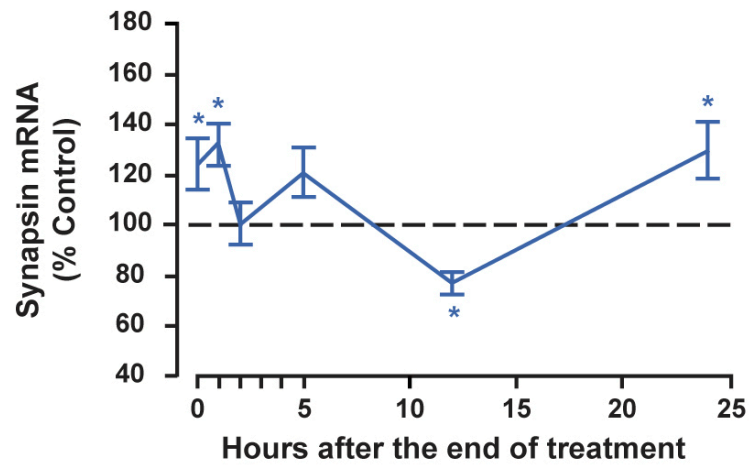
learning (Godenschwege et al., 2004; Michels et al., 2005). In *Aplysia*, synapsin protein is downregulated in synaptosomes from pleural-pedal ganglia 24 hours following LTD-inducing treatment in a proteosome-dependent manner (Fioravante et al., 2008). These results indicate that synapsin expression is regulated during one form of long-term memory in *Aplysia*. However, despite building evidence for an association between memory and synapsin, the mechanisms by which synapsin contributes to these processes remain unknown.

Interestingly, previous work from the Byrne lab has indicated that synapsin expression is regulated in pleural-pedal ganglia following treatment with 5-HT. In an initial experiment, the time course of synapsin mRNA expression in ganglia following treatment with 5-HT or vehicle was examined using qPCR. Six time points were examined: immediately (0 h), 1, 2, 5, 12 and 24 h after treatment. For each time point and treatment group, synapsin mRNA levels were normalized to 18S rRNA levels and a comparison was made between ganglia treated with vehicle (control) and 5-HT. Results indicated a complex pattern of 5-HT-induced synapsin mRNA regulation (Fig. 1.4A). Both immediately and 1 h after 5-HT treatment, synapsin mRNA levels were significantly increased compared to vehicle-treated ganglia (mean percentage control \pm SEM: 0 h, $124.3 \pm 10.3\%$, $n = 9$, $t_8 = 2.36$, $p < 0.05$; 1 h, $131.7 \pm 8.70\%$, $n = 9$, $t_8 = 4.77$, $p < 0.05$). This increase appeared transient because mRNA levels were not significantly different from vehicle at 2 h and 5 h following 5-HT treatment (2 h, $100.2 \pm 8.20\%$, $n = 9$, $t_8 = 0.49$, $p = 0.63$; 5 h, $120.5 \pm 9.72\%$, $n = 9$, $t_8 = 1.35$, $p = 0.21$). At 12 h after treatment, a significant decrease in synapsin mRNA levels was observed (12 h, $77.0 \pm 4.15\%$, $n = 9$, $t_8 =$

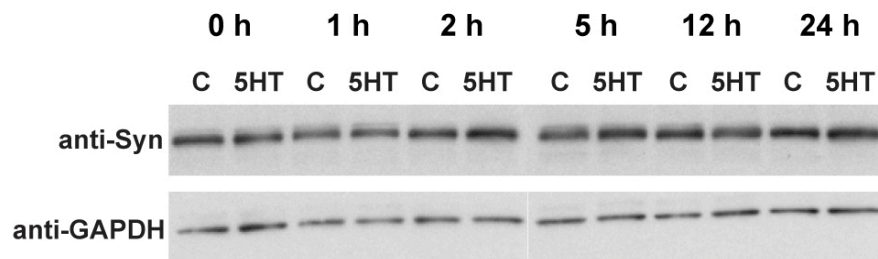
3.59, $p < 0.05$) followed by another significant increase at 24 h (24 h, $129.3 \pm 11.2\%$, $n = 9$, $t_8 = 2.86$, $p < 0.05$). Importantly, a one-way ANOVA indicated that there was not a significant effect of time on basal levels of synapsin mRNA ($F_{(5,53)} = 1.17$, $p = 0.34$). These results indicate that synapsin mRNA is dynamically regulated in ganglia after treatment with 5-HT.

In addition, the time course of synapsin protein expression in ganglia following treatment with 5-HT or vehicle was examined using Western Blot analysis (Fig. 1.4B). This experiment was performed to determine whether changes in synapsin mRNA corresponded to changes in protein levels in ganglia at the same time points examined previously. For each treatment and time point, synapsin levels in ganglia were quantified and normalized to levels of GAPDH. The results indicated that the dynamics of 5-HT-induced synapsin protein expression was similar to what was observed with synapsin mRNA. Compared to vehicle-treated ganglia, synapsin protein levels from 5-HT-treated ganglia peaked at 2 h after treatment, decreased at 12 h, and then returned to baseline at 24 h (mean percentage control \pm SEM: 0 h, $102 \pm 11\%$, $n = 6$, $t_5 = 0.01$, $p = 0.50$; 1 h, $121 \pm 15\%$, $n = 6$, $t_5 = 1.40$, $p = 0.11$; 2 h, $153 \pm 30\%$, $n = 6$, $t_5 = 2.02$, $p < 0.05$; 5 h, $129 \pm 26\%$, $n = 6$, $t_5 = 0.99$, $p = 0.18$; 12 h, $81.0 \pm 5.8\%$, $n = 6$, $t_5 = 3.67$, $p < 0.05$; 24 h, $104 \pm 20\%$, $n = 6$, $t_5 = 0.29$, $p = 0.39$). A one-way ANOVA indicated no significant effect of time on basal levels of synapsin protein ($F_{(5,30)} = 1.18$, $p = 0.34$). These results indicate that 5-HT treatment leads to the regulation of synapsin protein levels in ganglia suggesting that synapsin may be an important component of 5-HT-induced LTF at the sensorimotor synapse.

A.



B1. Synapsin Protein



B2.

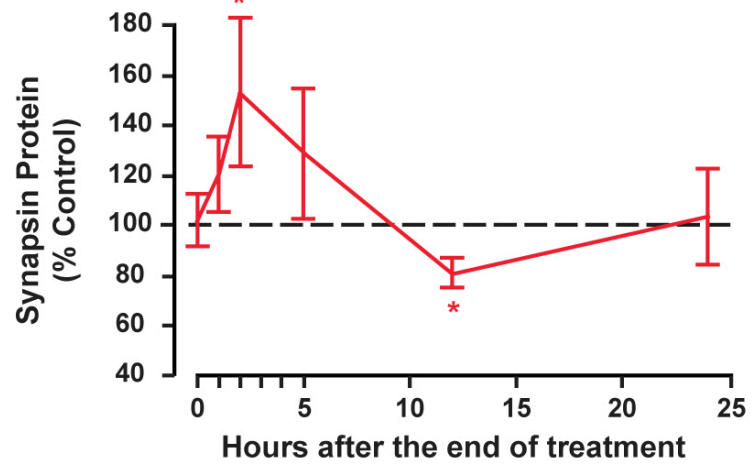


Figure 1.4. 5-HT-induced dynamics of synapsin mRNA and protein in ganglia.

A, Summary data from qPCR showing synapsin mRNA levels immediately (0), 1, 2, 5, 12 and 24 h after 5-HT treatment compared to vehicle-treated, time-matched controls. Synapsin mRNA levels were significantly elevated immediately, 1 and 24 h after 5-HT treatment but significantly decreased at 12 h after 5-HT treatment (* $p < 0.05$). These results indicate that 5-HT-treatment regulates synapsin mRNA levels in ganglia. **B1**, Representative Western blots from ganglia treated with vehicle (control, C) or 5-HT and lysed at the indicated times. Membranes were probed with anti-synapsin and anti-GAPDH antibodies. **B2**, Summary data from Western blot analysis depicting synapsin protein levels immediately (0), 1, 2, 5, 12 and 24 h after 5-HT treatment compared to vehicle-treated, time-matched controls. Synapsin protein was normalized to levels of GAPDH. Synapsin protein levels were significantly increased 2 h following 5-HT treatment, significantly decreased at 12 h and returned to baseline 24 h after treatment (* $p < 0.05$). These results indicate that 5-HT treatment regulates synapsin protein levels in ganglia.

The Role of Synapsin in Neuronal Morphology

Previous studies have implicated a role for synapsin in the establishment of synaptic connections. For example, synapsin I expression coincides with synaptogenesis during rat neuronal development (Melloni et al., 1993; Melloni and DeGennaro, 1994; Zurmohle et al., 1996). However, this is also the case for other synaptic vesicle proteins (Leclerc et al., 1989; Berton et al., 1997; Shimohama et al., 1998). Extensive studies using cultured hippocampal neurons from synapsin knock-out mice indicated that synapsin I deficiency leads to delayed neurite outgrowth and impaired synaptogenesis (Chin et al., 1995; Ferreira et al., 1998) whereas synapsin II deficiency results in impaired axon formation and differentiation and cytoskeletal organization (Ferreira et al., 1998). In these examples however, with time, synapsin deficient cells were similar to wild type. The morphological impairments of suppressing synapsin III in cultured hippocampal neurons appear to be dependent on a developmental time course, where only initial expression is necessary for proper axonal differentiation and elongation (Ferreira et al., 2000).

In some cases, overexpressing synapsin can induce morphological changes. For example, overexpressing synapsin IIb in neuroblastoma cells can accelerate morphological and functional changes associated with development (Han et al., 1991) and exogenous expression of synapsin I or II can lead to the reorganization of actin filaments in nonneuronal cells so that the cells form elongated processes (Han and Greengard, 1994). Furthermore, synapsin II depletion by the injection of antisense oligonucleotides targeting synapsin II in cultured rat hippocampal neurons results in abnormal distribution of filamentous actin (F-actin) (Ferreira et al., 1994).

However, in *Aplysia* sensory neurons, overexpression of synapsin alone does not increase the number of varicosities per 100 μm of neurite (Fioravante et al., 2007). It is possible then that while synapsin would not be the driving force behind morphological changes, it could be part of the mechanisms which contribute to 5-HT-induced synaptogenesis.

The Role of Synapsin in Synaptic Plasticity

Synapsin is also implicated in neurotransmitter release, and plays an important role in various forms of synaptic plasticity (Greengard et al., 1993; Hilfiker et al., 1999; Angers et al., 2002; Evergren et al., 2007; Fioravante et al., 2007). While the function of synapsin appears to be synapse-specific, a general role for synapsin has been proposed at the synaptic terminal in which synapsin reversibly associates with itself, synaptic vesicles, actin and other cytoskeletal elements in a phosphorylation-dependent manner (Cesca et al., 2010). Knock-out studies have indicated that inhibition of synapsin expression affects excitatory and inhibitory synapses differently. For example, synapsin deficiency at excitatory synapses did not affect basal synaptic transmission evoked by one stimulus but led to an increase in the rate of depression evoked by several stimuli. However, at inhibitory connections, synapsin deficiency resulted in a reduction in basal synaptic transmission but did not affect synaptic depression (Gitler et al., 2004a).

Post-tetanic potentiation (PTP) is a short-term form of synaptic plasticity but decreases in PTP magnitude have been correlated with deficits in learning and memory (Powell, 2006). For example, decreased PTP in the hippocampus of

Synapsin II^{-/-} and Synapsin I/II^{-/-} mice is associated with impaired memory in fear conditioning (Silva et al., 1996). In *Aplysia* cholinergic synapses in the buccal ganglion, synapsin antibody injection does not affect the amplitude of the inhibitory post-synaptic current (IPSC), but it does reduce PTP (Humeau et al., 2001), indicating that synapsin may affect vesicle mobilization. When mammalian synapsin I was injected into the presynaptic cells of the buccal ganglion, there was no change in basal IPSC amplitude or kinetics, however Humeau and colleagues observed a significant enhancement in PTP amplitude indicating that synapsin may play a role in regulating the size of the reserve pool or transition of vesicles from the reserve pool to the releasable pool.

Recently, the lab examined the role of synapsin in the glutamatergic, excitatory *Aplysia* sensorimotor synapse (Fioravante et al., 2007). Interestingly, overexpressing synapsin resulted in decreased basal synaptic strength and enhanced synaptic depression as well as enhanced 5-HT-induced facilitation of a depressed synapse. These results indicated that overexpressed synapsin levels affected vesicle mobilization in both homosynaptic and heterosynaptic forms of short-term plasticity. However, synapsin expression has not been examined in long-term sensitization or facilitation of the sensorimotor synapse.

Although not extensive, previous work has examined synapsin expression in long-term forms of synaptic plasticity in vertebrates. In rats, long-term potentiation (LTP) in the dentate gyrus, a subregion of the hippocampus, results in elevated synapsin protein levels 3 h after LTP-inducing stimulation (Lynch et al 1994). During transcription- and translation-dependent LTP in the rat hippocampus,

increased synapsin mRNA and protein levels are correlated to persistent enhancement of synaptic activity up to 8 hours after LTP induction (Hicks et al., 1997; Morimoto et al., 1998; Sato et al., 2000). It should be noted however, that certain forms of LTP can be induced in synapsin I and II knock-out models (Rosahl et al., 1995; Spillane et al., 1995).

Interestingly, basal synapsin levels appear to regulate protein levels of other synaptic-terminal associated proteins as well as synaptic vesicle density in the terminal. Double knock-out *Synapsin I/II* mice have about a 50% decrease in the number of synaptic vesicles in the hippocampus and visual cortex compared to wild-type animals in addition to a decrease in protein expression of synaptotagmin 1 and 2, synaptophysin 1, synaptoporin 2, synaptobrevin 2 (VAMP2) and SV2 in total brain lysate (normalized to NMDA-R1 levels) (Rosahl et al., 1995). However, in these studies there was no change in the levels of mRNA corresponding to these proteins indicating that a post-translational mechanism or premature protein degradation was most likely the cause as post-translational regulation of synaptic protein expression occurs during development (Daly and Ziff, 1997). In a separate set of experiments, whole brain lysates from triple synapsin knock-out mice revealed a significant decrease in VAMP2, synaptophysin 1 and synaptotagmin 1 (Gitler et al., 2004a). However, because these were knock-out experiments, it is not clear how developmental processes accommodate the absence of synapsin. It is possible that various synaptic terminal-associated proteins are regulated by different mechanisms during learning. For example, during LTD in *Aplysia*, synapsin downregulation occurs independently from VAMP (Fioravante et al.,

2008), suggesting that synapsin expression may be regulated separately from other synaptic vesicle-associated proteins. Cumulatively, these results indicate that synapsin contributes to multiple types of synaptic plasticity and suggest that synapsin would be an ideal target to focus on in the study of mechanisms which regulate long-term synaptic facilitation.

Guided by these previous experiments which indicate that synapsin is involved in learning and memory, neuronal morphology and synaptic plasticity, the goal of this research was to determine the role of synapsin in long-term synaptic facilitation in *Aplysia*. The general hypothesis was that synapsin expression contributes to LTF. Overall, there were three main questions that were asked in order to elucidate the role of synapsin in LTF: 1. Is synapsin expression regulated in response to 5-HT and/or behavioral training? 2. What are the mechanisms that regulate synapsin expression? 3. Is synapsin regulation necessary for LTF? Determining the expression of, and the requirement for, synapsin in neural plasticity and learning will significantly enhance understanding of the mechanisms underlying long-term memory.

CHAPTER II. SYNAPSIN EXPRESSION IS REGULATED AFTER BEHAVIORAL TRAINING OR TREATMENT WITH SEROTONIN

INTRODUCTION

The role of synapsin in learning, memory and long-term synaptic plasticity remains unclear. Knock-out studies have aimed to isolate the functional significance of the synapsin but little is known about the compensatory mechanisms that come in to play during development. Previous studies have observed a positive relationship between enhanced synapsin expression and learning. For example, synapsin expression and regulation may participate in the formation and/or consolidation of song-related memories in the zebra finch (Velho and Mello, 2008). In spatial learning, as measured by the Morris water maze learning paradigm, synapsin I expression is induced in rats (Gomez-Pinilla et al., 2001) and is linked to cognitive enhancement (John et al., 2009). Upregulation of synapsin I expression is also correlated with improvement in passive-avoidance memory associated with food restriction diet (Deng et al., 2009) and enhanced synapsin I mRNA levels are observed during training of operant conditioning (Rapanelli et al., 2009). Furthermore, increased synapsin mRNA and protein levels were correlated to persistent enhancement of synaptic activity during LTP in the rat hippocampus (Morimoto et al., 1998; Sato et al., 2000). However, the functional contribution of enhanced synapsin expression in these paradigms remains unknown. Therefore, as a first step, it was necessary to define the temporal profile of synapsin expression induced by 5-HT and behavioral training.

The lab previously conducted studies that drew relationships between behavioral training, treatment of ganglia and treatment of cultured sensory neurons

and found that similarities exist in expression patterns of the transcription factors CREB1 and CREB2 (Liu et al., 2008; Liu et al., 2011). Therefore, based on these results and initial experiments indicating that synapsin is regulated by 5-HT treatment in ganglia (Fig. 1.4), as well as previous experiments examining synapsin expression in other systems, the hypothesis was that synapsin would be regulated by treatment with 5-HT, the *in vitro* analogue of behavioral training, and this regulation would be similar in sensory neurons to that observed in ganglia.

Previous results indicated that synapsin mRNA and protein levels are dynamically regulated in ganglia in response to 5-HT treatment. These results indicated that in addition, synapsin protein was increased in ganglia from animals that underwent behavioral training 2 hours after the end of training. This time point corresponds to the observed increase in synapsin protein in ganglia treated with 5-HT. Furthermore, synapsin protein levels were also regulated by 5-HT in cultured sensory neurons. Because both behavioral training and the *in vitro* analogue of training regulate synapsin expression, synapsin expression could be an underlying mechanism contributing to long-term facilitation in *Aplysia*.

METHODS

Behavioral Training. The protocol for one-day sensitization training has been described previously (Wainwright et al., 2002) and was performed by a postdoctoral fellow in the lab, Gregg Phares, PhD. Briefly, a train of 10 strong AC electric shocks

(1 Hz) was applied diffusely to the lateral body wall of one randomly chosen side of the animal. Four trains were presented with 30 min ISIs with a total training time of 90 minutes. Two h after the end of training, a pair of pleural-pedal ganglia from each animal were removed, one ipsilateral (trained) and one contralateral (control) to the trained side. Protein from ipsilateral and contralateral ganglia was extracted and processed for western blot analysis (described below). Behavior was not assessed in these animals.

Western Blot Analysis. Ganglia from behavioral training were homogenized in lysis buffer [10% SDS, 10 mM Tris, 10 mM EDTA, 1 mM DTT, 1% protease inhibitor cocktail (Sigma), 10 mM sodium fluoride, 5 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 0.5 μ M okadaic acid]. Lysate protein concentrations were determined using a protein assay (BioRad, Hercules, CA). Thirty μ g of protein from each lysate were resolved by SDS-PAGE and transferred to nitrocellulose membrane. After confirmation of equal loading with Ponceau staining (BioRad, Hercules, CA) and blocking in 5% non-fat dry milk, blots were incubated with a rabbit primary polyclonal antibody directed against *Aplysia* synapsin (Cocalico Biologicals Inc., see also Angers et al., 2002) overnight at 4 °C. After incubation of the membrane with HRP-conjugated secondary antibody (Zymed, San Francisco, CA) for 1 h at room temperature (RT), immunoreactive bands were visualized by Enhanced Chemiluminescence (ECL; GE Healthcare, Pittsburgh, PA) and measured by densitometry (ImageQuant 5.0 software, GE Healthcare Life Sciences, Piscataway, NJ). As a loading control, membranes were

re-blotted with a rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (IMGENEX, San Diego, CA) and GAPDH immunoreactive bands were quantified as described above. Densitometry measurements from synapsin immunoreactive bands were normalized to GAPDH immunoreactive bands and comparisons were made between 5-HT-treated and vehicle-treated ganglia using two-tailed Student's t-tests at each time point with SigmaStat software (Systat Software, Inc., San Jose, CA).

Immunofluorescence Analysis. Culturing procedures were performed as described previously (Martin et al., 1997; Chin et al., 1999; Angers et al., 2002). Sensory neurons from pleural ganglia from 60-100 g animals (NIH-*Aplysia* resource facility, University of Miami, Miami, FL) were cultured on coverslips in dishes for 5 days as described previously (Angers et al., 2002). For each experimental preparation ($n = 1$), two dishes of sensory neurons were used. One dish was treated with 5, 5 min pulses of vehicle (50:50% L15:ASW) and the other dish was treated with 50 μ M 5-HT with an interpulse interval (ISI) of 20 min (Liu et al., 2008). Immediately following the end of treatment, cultures were washed with L15:ASW and stored at 18°C for the indicated time. In individual experiments, cells were fixed 2, 12 or 24 h after treatment with 4% paraformaldehyde in phosphate buffered saline (PBS) containing 30% sucrose and blocked for 30 min at RT in Superblock blocking buffer (Pierce, Holmdel, NJ) supplemented with 0.2% Triton X-100 and 3% normal goat serum as previously described (Liu et al., 2008). Fixed cells were incubated with primary antibody (1:500; polyclonal anti-synapsin antibody) diluted in blocking buffer

overnight at 4°C followed by incubation with secondary antibody (1:100 dilution in blocking buffer; goat anti-rabbit conjugated to Cy-3; Jackson ImmunoResearch Laboratories, West Grove, PA). Because it was not possible to image the entire sensory neuron including processes at high power, a single field was initially chosen at low power that contained the maximum number and length of neurites for each neuron. For each dish, 4-8 neurons were analyzed. A z-series of optical sections was obtained with a Zeiss LSM 510 confocal microscope using a 63x oil immersion lens. Image saturation was prevented by measuring the intensity of the image during image capture using LSM 510 software. Image stacks through ~10 µm were collected at 0.5 µm increments and projected into a single image (Metamorph Offline software, Universal Imaging Corporation, Downingtown, PA). Synapsin immunoreactive intensity was measured within individual varicosities, which are swellings along the SN neurite that are greater than 1.5 times the diameter of the neurite (Bailey et al., 1979; Wainwright et al., 2002). To determine if a swelling qualified as a varicosity, the diameter of the neurite (the number of pixels counted) before and after the swelling was measured, then the diameter of the swelling was measured. Using a tracing tool in MetaMorph Offline software, each varicosity was manually outlined and the average intensity within the varicosity region was recorded. Approximately 10 varicosities per image (neuron) were measured this way. For each neuron, the intensity measurements for all varicosities were averaged to give one intensity measurement. Four to 8 neurons on each coverslip were analyzed in this manner and the measurements for each cell in a dish were averaged. For each time point, intensity measurements from the dish treated with

5-HT was compared to that from the dish treated with vehicle using a two-tailed Student's t-test. Treatment and analysis were both performed in blind fashion in order to eliminate potential bias during imaging and analysis.

In addition to analysis of synapsin immunoreactivity in sensory neurons, these images were also used to examine synapsin immunoreactivity within sensory neuron processes. Using MetaMorph Offline software, the background of the image was measured and subtracted, resulting in the average intensity for the sensory neuron processes. For each time point, the intensity measurements from 5-HT-treated dishes were compared to those from vehicle-treated dishes using a two-tailed Student's t-test.

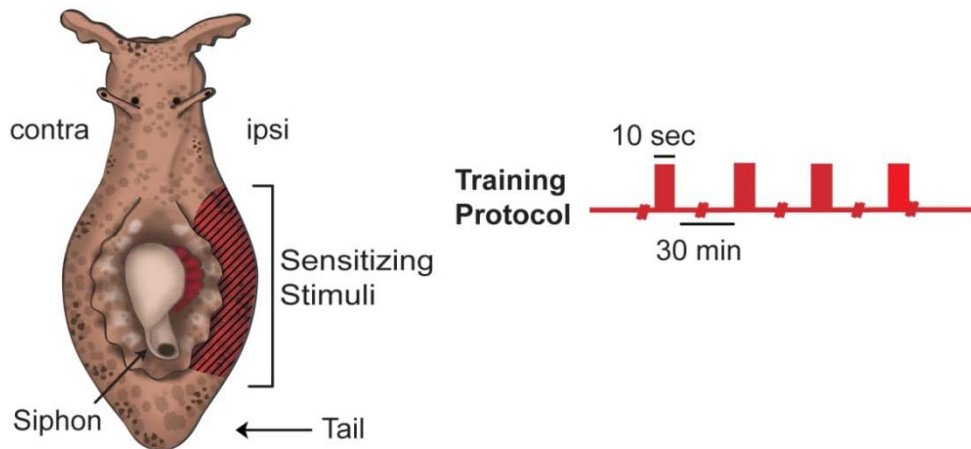
RESULTS

Behavioral training regulates synapsin protein levels in ganglia

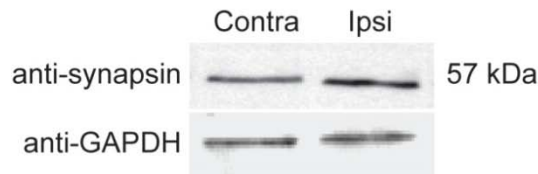
Although 5-HT treatment is a widely used *in vitro* analogue of behavior training (Montarolo et al., 1986; Zhang et al., 1997; Mauelshagen et al., 1998), it is not known if behavioral training leads to the same dynamic expression of synapsin as observed in ganglia treated with 5-HT (Fig. 1.4). To address this issue, synapsin protein levels in ganglia from behaviorally trained animals were measured 2 h after the end of training in order to determine if the regulation of synapsin observed after 5-HT treatment would be recapitulated by behavioral sensitization training of the intact animal. Two h after training, ganglia were isolated and synapsin protein levels were examined using Western blot analysis. Because only one side of the

animal received sensitizing stimuli, and sensitization is lateralized and restricted to the trained side (Cleary et al., 1998; Wainwright et al., 2002; Antzoulatos et al., 2006; Antzoulatos and Byrne, 2007), ganglia from the untrained side of the animal were used as control. Synapsin levels in ganglia from the trained side (ipsilateral) were compared to those in ganglia from the untrained side (contralateral). Results indicated that behavior training produced a significant increase in synapsin protein levels 2 h after training (Figure 2.1) (ipsi vs. contra, mean percentage control \pm SEM: $152 \pm 23.5\%$, $n = 8$, $t_7 = 2.386$, $p < 0.05$), confirming the physiological relevance of the 5-HT treatment as an analogue to behavioral sensitization training.

A. Sensitization Training



B1. Western Blot



B2.

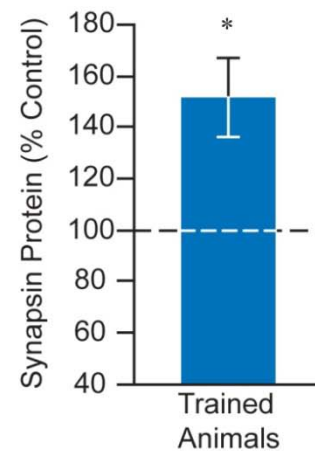


Figure 2.1. Synapsin protein levels are increased in ganglia 2 h following behavioral training. **A**, Schematic of Behavioral Sensitization Training. A train of 10 strong AC electric shocks (1 Hz) was applied diffusely to the lateral body wall of one randomly chosen side of the animal. Four trains were presented with 30 min ISIs with a total training time of 90 minutes. Two h after the end of training, a pair of pleural-pedal ganglia from each animal were removed, one ipsilateral (trained) and one contralateral (control) to the trained side. **B1**, Representative Western blot illustrating synapsin and GAPDH protein levels 2 h after training in ganglia from both the contralateral side of the animal (contra) and from the ipsilateral (ipsi) side of the animal with respect to training. **B2**, Summary data indicating that synapsin protein levels are significantly increased 2 h after behavioral training (* $p < 0.05$). Synapsin protein levels, as measured by densitometry, were normalized to GAPDH protein levels for each ganglion. The ratio of ipsilateral to contralateral side (with respect to training) of each animal was used to quantify training-induced changes in the levels of synapsin protein in pleural ganglia.

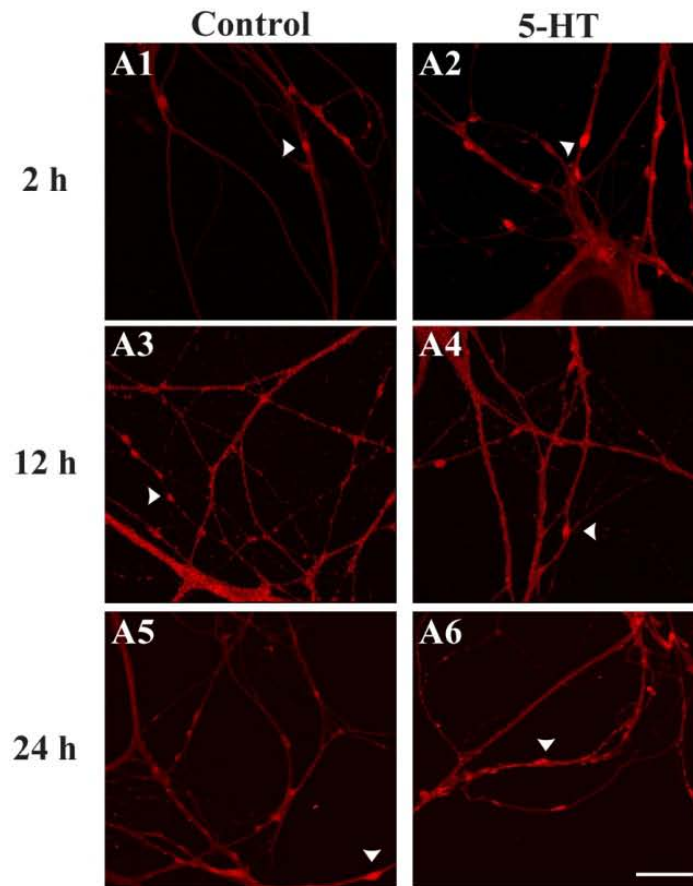
5-HT regulates synapsin protein levels in cultured sensory neurons

The experiments described above examined synapsin regulation in whole ganglia, which are comprised of various neuronal subtypes. To determine whether synapsin is specifically regulated in sensory neurons, the presynaptic site associated with LTF, the effect of 5-HT on synapsin protein in isolated sensory neurons was examined using immunofluorescence. Because synapsin is a synaptic vesicle-associated protein that is primarily localized to synaptic terminals (Angers et al., 2002), the regulation of synapsin expression was initially investigated in axonal varicosities, the presumed sites of neurotransmitter release (Bailey and Chen, 1988). Synapsin immunoreactivity in sensory neurons was examined at 2, 12 and 24 h after treatment with vehicle or 5-HT. Consistent with the results from ganglia, synapsin expression was increased in the varicosities of sensory neurons 2 h after treatment with 5-HT (mean percentage of vehicle-treated cells \pm SEM: $127 \pm 4.9\%$, $n = 3$, $t_2 = 4.30$, $p < 0.05$) (Fig. 2.2A1, A2, B). At 12 h after 5-HT treatment, no significant difference in synapsin levels was observed indicating that levels were at baseline (mean percentage of vehicle-treated cells \pm SEM: $105 \pm 8.1\%$, $n = 5$, $t_4 = 2.78$, $p = 0.69$) (Fig. 2.2A3, A4, B). However, an increase in synapsin immunoreactivity in varicosities of sensory neurons was observed 24 hours after treatment (mean percentage of vehicle-treated cells \pm SEM: $119 \pm 5.0\%$, $n = 5$, $t_4 = 2.78$, $p < 0.05$) (Fig. 2.2A5, A6, B).

To determine if the increase in synapsin immunoreactivity was the result of local rearrangement within the sensory neuron as compared to a *bona fide* increase in expression, synapsin immunoreactivity within the sensory neuron processes was

also examined. The results confirmed the previous findings where synapsin immunoreactivity was increased at 2 h (mean percentage of vehicle-treated cells \pm SEM: $214.2 \pm 18.5\%$, $n = 3$, $t_2 = 6.97$, $p < 0.05$), at baseline at 12 h (mean percentage of vehicle-treated cells \pm SEM: $109.9 \pm 7.5\%$, $n = 5$, $t_4 = 1.40$, $p = 0.23$) and increased at 24 h after treatment (mean percentage of vehicle-treated cells \pm SEM: $130.2 \pm 11.3\%$, $n = 5$, $t_4 = 2.90$, $p < 0.05$) (not shown). Thus, the dynamics of synapsin expression observed in 5-HT-treated sensory neurons follow a similar trend compared to 5-HT-treated ganglia. These results suggest that changes in synapsin expression could alter the dynamics of synaptic transmission and LTF.

A. Synapsin immunoreactivity



B. Synapsin immunoreactivity in SN varicosities

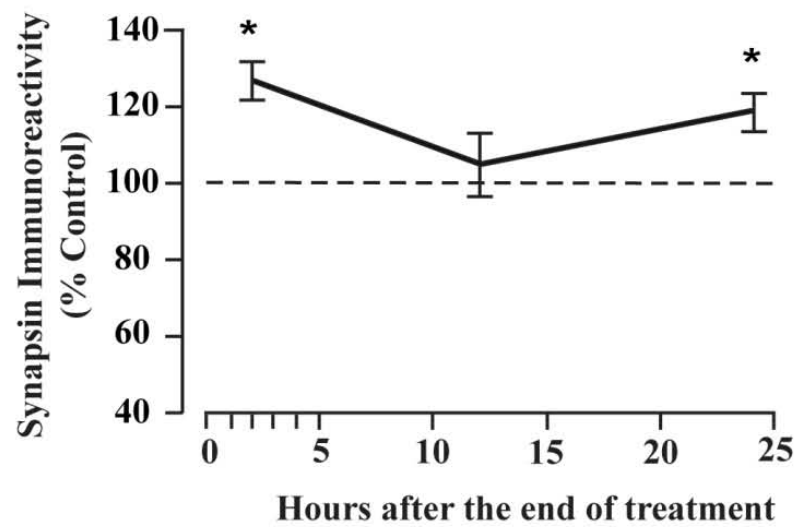


Figure 2.2. 5-HT-induced synapsin protein levels in isolated sensory neurons.

A, Synapsin immunoreactivity in the varicosities of cultured sensory neurons treated with vehicle (control) or 5-HT and fixed 2 h (**A1-A1**), 12 h (**A3-A4**) or 24 h (**A5-A6**) after treatment. Arrows point to synapsin-immunoreactive varicosities along neurites. Scale bar, 25 μm . **B**, Summary data. Plot of average immunofluorescence intensity ($\pm\text{SEM}$) in the 5-HT-treated groups (normalized to control) assayed at 2, 12 and 24 h after treatment. A significant increase in synapsin immunoreactivity within varicosities was observed at both 2 and 24 h after 5-HT (* $p < 0.05$) but not at 12 h after 5-HT ($p = 0.69$).

DISCUSSION

5-HT treatment or behavior training regulate synapsin expression

5-HT treatment has been used extensively as an *in vitro* analogue to behavioral training in both ganglia and culture (Mohamed et al., 2005; Liu et al., 2008; Liu et al., 2011). The lab previously published a paper that describes the time course of CREB2 expression after behavioral training, 5-HT-treated ganglia and 5-HT-treated cultured sensory neurons (Liu et al., 2011). The lab also examined CREB1 expression and activation in ganglia after 5-HT treatment and in 5-HT-treated sensory neurons (Liu et al., 2008). In both cases, the expression patterns and time course of these two transcription factors were relatively similar. Therefore, the hypothesis that the time course of synapsin expression following behavior training, 5-HT-treatment in ganglia and 5-HT-treated cultured sensory neurons would also follow a similar trend was tested.

Results indicated that treatment with 5-HT dynamically regulates the expression of synapsin at the level of both mRNA and protein in ganglia (Fig. 1.4). Initially and 1 h after 5-HT treatment, synapsin mRNA levels are transiently increased, decreased by 12 h followed by another increase at 24 h after treatment (Fig 1.4A). Treatment with 5-HT lead to an increase in synapsin protein at 2 h, decrease at 12 h and return to baseline at 24 h (Fig. 1.4B). In support of the hypothesis, an increase in synapsin protein levels in ganglia at 2 h after the end of behavior training (Fig. 2.1) and cultured sensory neurons treated with 5-HT (Fig. 2.2A1, A2, B) was observed. Interestingly, synapsin protein levels in ganglia and cultured sensory neurons treated with 5-HT differed at 12 and 24 h after treatment;

at 12 h after treatment synapsin levels were decreased in ganglia but were at baseline in cultured sensory neurons whereas 24 after treatment, synapsin levels were at baseline in ganglia but increased in cultured sensory neurons. There are many potential explanations for these differences.

1. The observed differences could be a result of cell type heterogeneity in ganglia (Kandel, 1979; Cleary et al., 1995). While synapsin expression in cells within the pleural-pedal ganglia is relatively low compared to that observed in sensory neuron processes located in the neuropil (Angers et al., 2002), synapsin regulation and expression in these cells may be the root of these differences. Synapsin immunostaining is also present in the motor neuron (Hart and Byrne, unpublished observations). For this reason, it was important for us to examine protein levels in individual sensory neurons. Synapsin regulation in other cell types, particularly motor neurons, is of potential interest but would require further investigation that is beyond the scope of this project.

2. Differences between ganglia and culture could be due to variations in time courses of expression. Although the time course of CREB1 and CREB2 were similar between ganglia and culture, in certain cases the expression time course did not line up exactly (Liu et al., 2011; Liu et al. 2011b). It is possible that the increase in synapsin protein in ganglia does not occur until 25 or 26 h after 5-HT.

3. 5-HT treatment results in a decrease in synapsin expression from the 2 h time point to the 12 h time point in both ganglia (Fig. 1.4) and in cultured sensory neurons (Fig. 2.2). The decrease in synapsin levels in ganglia is below baseline at 12 h. However even though synapsin levels in cultured sensory neurons are

decreased compared to the 2 h time point, they do not go beyond baseline. This decrease in 5-HT-induced synapsin expression at 12 h could be the result of 5-HT-induced degradation rather than a short half-life as the estimated half-life of *Aplysia* synapsin is at least 24 h (see Fig. 4.3), which is in agreement with that of mammalian synapsin I (Daly and Ziff, 1997; Murrey et al., 2006). It is possible that 5-HT treatment induces ubiquitin-mediated degradation of synapsin as is seen with other proteins (Hegde et al., 1993; Zhao et al., 2003; Upadhyaya et al., 2004). However, it appears that synapsin degradation is transient and new translation of synapsin could occur in the later phase and result in the 24 h increase observed in sensory neurons. Because there is an increase in mRNA levels in ganglia treated with 5-HT at 24 h after treatment, transcriptional induction of synapsin during the later phase is also a possibility.

5-HT regulates synapsin in isolated sensory neurons

5-HT treatment altered synapsin levels in cultured sensory neurons that were devoid of a postsynaptic target. This is somewhat surprising considering that structural changes (i.e., increase in number of varicosities and neurite outgrowth) induced by 5-HT require the presence of a postsynaptic target (Glanzman et al., 1990). Nevertheless, this result is consistent with previous studies showing that mechanisms that regulate gene expression during LTF (i.e. CREB1 activation, induction of C/EBP, MAPK translocation) function in isolated sensory neurons regardless of the absence of a postsynaptic motor neuron (Alberini et al., 1994;

Martin et al., 1997; Liu et al., 2008). The results indicate that motor neurons are not required for 5-HT to increase synapsin expression for at least 24 h after treatment.

The time course of synapsin expression

The time course of synapsin expression is particularly interesting with regards to 5-HT-induced long-term synaptic facilitation and morphological changes, both of which express a biphasic temporal profile similar to that of synapsin (Mauelshagen et al., 1996; Kim et al., 2003). In ganglia, five pulses of 5-HT leads to an initial enhancement of synaptic strength that decays back to baseline by 3 h after treatment, followed by an additional increase in strength beginning 10-15 h after treatment (Mauelshagen et al., 1996). These results are similar to the time course of LTF in culture (Ghirardi et al., 1995). Interestingly, PKA activity follows a similar pattern where repeated application of 5-HT leads to an initial increase in activity which falls back to baseline by 3 h, and rises again by 20 h after treatment (Muller and Carew, 1998). This biphasic regulation is also observed with CREB1 levels in sensory neurons, where CREB1 levels are elevated at 2h, back to baseline at 12 h and elevated again at 18 and 24 h after 5-HT treatment (Liu et al., 2011b).

The results also indicate that synapsin expression correlates with the different morphological phases of LTF. 5-HT leads to the activation of pre-existing synapses, as well as the formation of new synapses (Kim et al., 2003). Because the activation of pre-existing “silent” synapses occurs between 3-6 h, elevated levels of synapsin at 2 h after treatment may in part contribute to this phase of 5-HT-induced modifications. In addition, the increase in synapsin expression at 24 h

after 5-HT may contribute to the later phase of LTF, which corresponds with the formation of new synapses (Kim et al., 2003). It is also possible that the early increase in synapsin is also contributing to the formation of new synapses seen during the later phase of LTF. Continued varicosity formation and neurite outgrowth persists up to 48 h after 5-HT in culture (Hatada et al., 2000), therefore the increase in synapsin at 24 h may be an important component of the later phase of morphological changes.

Indeed, in cultured hippocampal neurons, synapsin I is expressed before the establishment of synaptic contacts (Fletcher et al., 1991) and the introduction of synapsin I or II in neuronal cells accelerates the rate of functional and structural synaptogenesis (Han et al., 1991; Lu et al., 1992). In this model, early-elevated synapsin levels may be a part of the initial pathway for 5-HT-induced synaptogenesis. The hypothesis that 5-HT-enhanced synapsin levels contribute to the morphological changes associated with LTF is addressed in Chapter 4.

The initial time course of synapsin regulation is somewhat similar to that seen following LTP in the dentate gyrus. Lynch et al. (1994) found that synapsin levels, along with other synaptic vesicle proteins, synaptophysin and synaptotagmin, were not increased 45 min after LTP induction but were significantly elevated 3 h after stimulation. These authors suggest that synaptic vesicle protein elevation is part of a presynaptic “framework” in which an increase in the number of synaptic contacts, and increase in transmitter release, complements the postsynaptic response. Other studies examining synapsin levels in the hippocampus also found that LTP induction results in the enhanced synapsin

expression (Morimoto et al., 1998; Sato et al., 2000) indicating that multiple forms of long-term enhancement of synaptic plasticity are associated with synapsin mRNA and protein regulation. The role and significance of stimulus-elevated synapsin levels is further discussed in Chapters 4 and 5.

CHAPTER III. THE *SYNAPSIN* PROMOTER IS TRANSCRIPTIONALLY ACTIVATED IN RESPONSE TO SEROTONIN

INTRODUCTION

Long-term changes in neuronal activity lead to alterations in gene expression and ultimately to modifications in neuronal function. Previous studies have identified CRE motifs in both the human and rat *synapsin I* gene promoters (Sauerwald et al., 1990; Sudhof, 1990) indicating that the *Aplysia synapsin* promoter may also contain a CRE motif. Genes that are regulated by transcription factors necessary for learning and memory (i.e. CREB), are of particular interest because the expression and function of these genes may underlie changes in neuronal properties that lead to memory formation and/or consolidation (Pfenning et al., 2007).

Although correlations have been made between synapsin levels and learning, memory and enhanced plasticity, the mechanisms by which synapsin transcription is regulated during these processes remains unknown. Therefore, potential regulatory element binding sites were identified in the promoter region of the *Aplysia synapsin* gene using Transcription Element Search System (TESS) software. This software scans the input sequence to identify potential cis-elements (short-sequences of DNA from 4-10 bp long) using the TRANSFAC database.

The binding of a transcription factor to a promoter sequence in DNA alters the expression of the gene leading to either enhanced or repressed expression of that gene. This is often accompanied by alterations in the structure of chromatin, the combined DNA-protein construct. During periods of little or no transcription, DNA is wrapped tightly around histone proteins (structurally identified as nucleosomes) leading to highly compacted DNA. There are four highly conserved,

core histone proteins, H2A, H2B, H3 and H4, which make up a histone octamer as the nucleosome core. Histone modification can occur through post-translational modification of the N-terminal histone tails of H3 and H4 altering the interaction of DNA with histones and other proteins. During transcription, histone modifications relax the chromatin structure and allow transcriptional machinery to interact with the DNA.

Previous work in *Aplysia* has examined the state of histone acetylation and transcriptional initiation following LTF and long-term depression (LTD)-inducing protocols (Guan et al., 2002; Fioravante et al., 2008). The acetylation of histone tails occurs by transferring an acetyl group from Acetyl-Coenzyme A to a positively charged histone tail, a process catalyzed by histone acetyltransferases (HATs) that neutralizes the positive charge. This process leads to a relaxed chromatin structure. The reverse action occurs when histone deacetylases (HDACs) remove the negatively charged acetyl groups from histone tails. Therefore, histone acetylation is associated with transcriptional activation and HDAC association is linked to transcriptional inhibition (Levenson and Sweatt, 2005).

Because the human and rat *synapsin I* promoter region contains a CRE binding sequence, the hypothesis that the *Aplysia synapsin* promoter may also contain a CRE sequence and that 5-HT-induced synapsin expression could be regulated by CREB1 was tested. TESS analysis indicated that the *Aplysia synapsin* promoter contains multiple potential binding sites for transcription factors associated with long-term memory and plasticity. Of these, CREB1 association and the state of histone acetylation surrounding the CRE site was examined. Results indicated that

CREB1 exhibits binding under basal conditions and this association is enhanced in response to 5-HT treatment. In addition, an increase in histone acetylation within this region, which is accompanied by a decrease in association of histone deacetylase 5 (HDAC5) was also observed. Furthermore, 5-HT treatment increases reporter gene expression under direction of the *synapsin* promoter. Results from these studies suggest that 5-HT treatment leads to the transcriptional activation of the *synapsin* gene.

METHODS

Cloning the synapsin promoter. This work was performed by W. Yao. *Aplysia* genomic DNA was isolated, digested and ligated with the Genome-Walker adaptor DNA (Universal GenomeWalker kit, Clontech, Mountain View, CA). The genomic fragments were used as templates for PCR with an adaptor primer (AP1, 5'-GTAATACGACTCACTATAGGGC-3', or AP2, 5'-ACTATAGGGCACGCGTGGT-3') and a synapsin gene-specific primer (CCGAACAATTGGTGAACCATTCCTTGAAACC and CATGTTGCCCTCTCAGTTCCACTCTCAGG). After electrophoresis, the PCR product was subjected to electrophoresis, extracted from agarose gel and subcloned into pCR 2.1 TOPO TA vector (Invitrogen, Carlsbad, CA). The cloned fragment was sequenced by SEQRIGHT (Houston, TX), using M13 universal primers.

Transcription Element Search System (TESS) Analysis. Predicted regulatory elements were identified using the TESS software (Computational Biology and Informatics Laboratory, University of Pennsylvania, School of Medicine; URL, <http://www.cbil.upenn.edu/cgi-bin/tess/tess>) that surveyed ~1.3 kbp of the *Aplysia synapsin* promoter region using default settings. Annotated sequences of the promoter region were obtained using a minimum log likelihood ratio (ta) of 6.0 and a maximum log likelihood deficit (td) of 8.0 (default; combined query option).

Synapsin promoter-EGFP reporter vector. To estimate transcriptional activation of *synapsin* during LTF, an EGFP reporter vector was constructed to contain a region of the *synapsin* promoter containing the CRE site. The vector was constructed from a pNEX3-EGFP vector (kind gift from B. Kaang, Seoul National University, Seoul, South Korea). The promoter region of the pNEX3-EGFP vector containing the AP-1 enhancer element and the RSV region was excised using HindIII restriction enzymes (Promega, Madison, WI). A 622 base pair region of the cloned *synapsin* promoter with flanking HindIII sites was inserted into the modified vector. The *synapsin* promoter-EGFP vector was sequenced by SEQWRIGHT (Houston, TX). Sensory neurons were cultured as described previously (Angers et al., 2002). To allow for basal levels of EGFP expression, on Day 3 in culture, the *synapsin* promoter-EGFP-pNEX3 plasmid (1 mg/ml) was injected into the nucleus of sensory neurons. Dextran-conjugated Texas Red fluorescence marker (70 kDa) was co-injected to monitor the efficiency and locus of injection. On Day 5, sensory neurons were treated with 5 pulses of either 5-HT or vehicle as described previously

(Chapter 2 Methods). Two h after the end of the treatment, cells were fixed with 4% paraformaldehyde solution and processed for immunofluorescence analysis as previously described (Chapter 2 Methods). Confocal optical sections through the middle of the cell body were obtained using a Zeiss LSM 510 confocal microscope and a 63X oil immersion lens and analyzed for mean fluorescence intensity using MetaMorph Offline software. EGFP signal in the cytoplasm and red fluorescence from injection dye in the nucleus were determined by tracing the outline of the cell body and nucleus. Mean fluorescence intensity for EGFP in the cytoplasm was normalized to the intensity of fluorescent dye injected into the nucleus. The fluorescent intensities from 3-5 injected neurons on each coverslip were analyzed, and measurements from neurons on the same coverslip were averaged. 5-HT-treated sensory neurons were compared to vehicle-treated sensory neurons. Results were analyzed by a paired, two-tailed Student's t-test.

Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were performed as previously described (Mohamed et al., 2005; Fioravante et al., 2008). For each assay, pairs of pleural-pedal ganglia were surgically removed from four anesthetized animals and the eight ganglia were divided into two groups so that the ganglia collected from one animal were never in the same group. Each group contained two ganglia from the right side of two animals and two ganglia from the left side of two animals. Each group was treated with either vehicle or 5-HT. Immediately following treatment, ganglia were treated with 1% paraformaldehyde for 30 min at RT with rotation to cross-link proteins bound to DNA. The reaction

was quenched with the addition of glycine (final concentration, 0.125 M). The pleural ganglia were isolated in ice-cold PBS containing protease inhibitors (protease inhibitor cocktail, Sigma, St. Louis, MO) and processed using a ChIP Assay Kit (Millipore, Bedford, MA) according to the manufacturer's instructions. After cell lysis, nuclei were recovered by low-speed centrifugation, resuspended, and sonicated to shear the genomic DNA to lengths of 0.3–1.3 kb. The lysates were then diluted with ChIP dilution buffer. A portion of the lysate was then stored at 4°C to be used for Input Control. Subsequently, the lysate was precleared with salmon sperm DNA/protein A-agarose beads, followed by brief centrifugation to pull down the beads. To measure association of CREB1 with the synapsin promoter, CREB1 antibody (Genemed, South San Francisco, CA) was added to precipitate DNA-protein complexes. For the histone acetylation assay, the following antibodies were used: anti-acetyl-histone H3 (Millipore, Bedford, MA) and anti-acetyl-Histone H4 (Millipore, Bedford, MA), and anti-histone deacetylase 5 (HDAC5) (Santa Cruz Biotechnology, Santa Cruz, CA). A ChIP assay was performed without the addition of an antibody as a negative control. Immune complexes were recovered by the addition of 60 µl of salmon sperm DNA/protein A agarose solution and incubated for 1 h at 4 °C with rotation. The beads were washed with low-salt, high-salt and lithium chloride washing buffer and eluted. To reverse cross-linking, NaCl (0.3 M) was added and the immune complexes were incubated overnight at 65 °C. Following treatment with Proteinase K for 2 h at 45 °C, DNA was recovered by phenol chloroform extraction and ethanol precipitation and resuspended in 20 µl of H₂O. DNA isolated from the assay was analyzed by PCR (33 cycles) using primers

to target a 272-bp fragment containing the CRE sequence of the *synapsin* promoter. The PCR primer sequences (5' to 3' direction) for the *synapsin* promoter were as follows: forward primer, 5'-CATGTTGCCCTCTCAGTTCCACTCTC-3' and reverse primer, 5'-GAGTACAAAGCAACAAGGTTGAGTG-3' (Integrated DNA Technologies, Inc., San Jose, CA). The PCR product was resolved on a 1% agarose gel followed by densitometry measurements using ImageQuant 5.0 software. The PCR product from antibody-precipitated DNA (anti- CREB1, H3, H4 and HDAC5 antibodies) was normalized to input control. Ganglia treated with 5-HT were compared to vehicle-treated ganglia and results were analyzed with a two-tailed Student's t-test.

RESULTS

Characterization of the *Aplysia synapsin* promoter

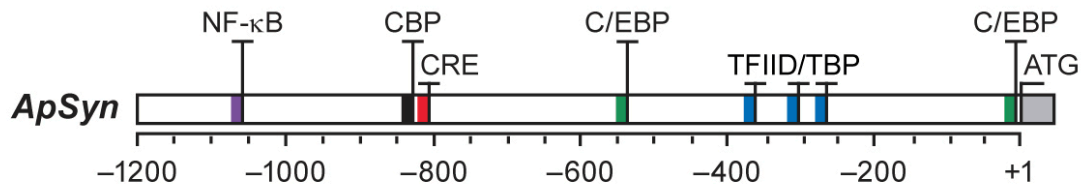
The *Aplysia synapsin* promoter was cloned by a former lab technician, W. Yao as described in the Methods. Using TESS software, potential consensus sequences were identified to determine transcription factor binding sites that might contribute to the dynamic regulation of *synapsin* expression. Figure 3.1 illustrates potential binding sites for transcription factors in the *synapsin* promoter identified by TESS software. These sites are indicated as the number of base pairs from the translation start site (ATG, +1) because the transcription start site is not known.

Several putative binding sites for transcription factors known to be involved in synaptic plasticity were identified. For example, putative binding sites for CCAAT-enhancer-binding protein (C/EBP) were located at -542 (5'-CTGAGAAAT-3') and at -18 (5'-CCTGTGGTCA-3') bp from the ATG (Fig. 3.1, green). C/EBP is an immediate early gene essential for LTF and is regulated by CREB1 in response to 5-HT treatment (Alberini et al., 1994; Guan et al., 2002). A putative site for nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), a plasticity-associated transcription factor, was also observed at -1071 bp (5'-GTAGAATCTCC-3') (Fig. 3.1, purple).

A cAMP response element (CRE) variant 5'-TGACGCAT-3' was also found in the promoter region of the *Aplysia synapsin* promoter, located at -818 bp from the ATG (Fig. 3.1, red). The presence of a CRE site is required for CREB-dependent gene induction (Montminy et al., 1986; Mayr and Montminy, 2001; Mohamed et al., 2005; Liu et al., 2008), which is necessary for 5-HT-induced LTF (Dash et al., 1990). Nineteen bp upstream of the CRE site is a CREB-binding protein (CBP) motif (5'-TCACGAGATA-3') at -837 bp from ATG (Fig. 3.1, black). CBP is a transcriptional co-activator that modulates CREB1-mediated gene expression through its actions as a bridging molecule (Ogryzko et al., 1996), a histone acetyltransferase (Bannister and Kouzarides, 1996), as well as through CREB acetylation (Lu et al., 2003).

In addition, potential binding sites for Transcription Factor II D (TFIID) and TATA Binding Protein (TBP) are located at -380 (5'-T/TTATC-3') and -273 (5'-T/TTATC-3') bp from the translation start site (Fig. 3.1, blue). An additional TFIID

potential binding site is located at -315 bp (5'-TTTGAA-3') from the ATG (Fig 3.1, blue). TFIID and TBP are part of a transcription preinitiation process in which the TFIID complex binds to a TATA box within the promoter region of the gene to allow for proper positioning of transcriptional machinery.



Motif	Start	Sequence
NF-κB	-1074	GTAGAATCTCC
CBP-1	-837	TCACGAGATA
CRE	-818	TGACGCAT
C/EBP	-542	CTGAGAAAT
TFIID/TBP	-380	T/TTATC
TFIID	-315	TTTGAA
TFIID/TBP	-273	T/TTATC
C/EBP	-18	CCTGTGGTCA

Figure 3.1. Potential regulatory element binding sites in the promoter region of *Aplysia synapsin*. ~1.3 kbp of the promoter region of the *Aplysia synapsin* gene was cloned and analyzed. Predicted regulatory elements were identified using TESS software. Potential binding sites for transcription factors were identified and labeled accordingly as bp from the translation start site (the ATG codon; +1) because the transcription start site remains unknown: NF-κB (purple, -1074), CBP (black, -837), CRE (red, -818), C/EBP (green, -542, -18) and TFIID/TBP (blue, -380, -315, -273).

***Synapsin* gene transcription is increased following treatment with 5-HT**

To examine whether the 5-HT-induced enhancement of synapsin mRNA and protein expression is regulated through the *synapsin* promoter, a pNEX3-EGFP expression vector was modified. This vector was chosen because pNEX expression vectors are a successful mechanism for exogenous gene expression in *Aplysia* (Kaang, 1996) and overexpression of EGFP was previously shown to not affect synaptic physiology (Fioravante et al., 2007). In addition, the lab has previously used the pNEX3-EGFP vector to construct a CRE-EGFP expression vector in order to examine 5-HT-induced CRE-mediated EGFP expression (Liu et al., 2008).

Therefore, to determine if 5-HT treatment leads to transcriptional activation of the *synapsin* gene, a *synapsin* promoter-EGFP expression vector was constructed. The *synapsin* promoter-EGFP expression vector was co-injected with injection dye into the nucleus of cultured sensory neurons 48 h prior to treatment. Two h after treatment with 5-HT or vehicle, cells were fixed and imaged using confocal microscopy (Fig. 3.2B). This time point corresponds to the largest increase in synapsin protein after 5-HT treatment observed in ganglia (Fig. 1.4) and sensory neurons (Fig. 2.2A1, A2) and in ganglia from behaviorally trained animals (Fig. 2.1). Mean fluorescence intensity for EGFP in the cytoplasm of the cell body was normalized to the amount of plasmid injected, indicated by the intensity of injected red fluorescent dye in the nucleus (Fig. 3.2C1). A two-tailed Student's t-test indicated that EGFP expression in 5-HT-treated cells was significantly different from vehicle-treated cells (expressed as percent of vehicle \pm SEM: $149 \pm 7.4\%$; $n = 6$, t_5

= 3.95; $p < 0.05$) (Fig. 3.2C2). These results suggest that the *synapsin* promoter is transcriptionally activated in sensory neurons by 5-HT and could support the increase in synapsin mRNA and protein after 5-HT treatment.

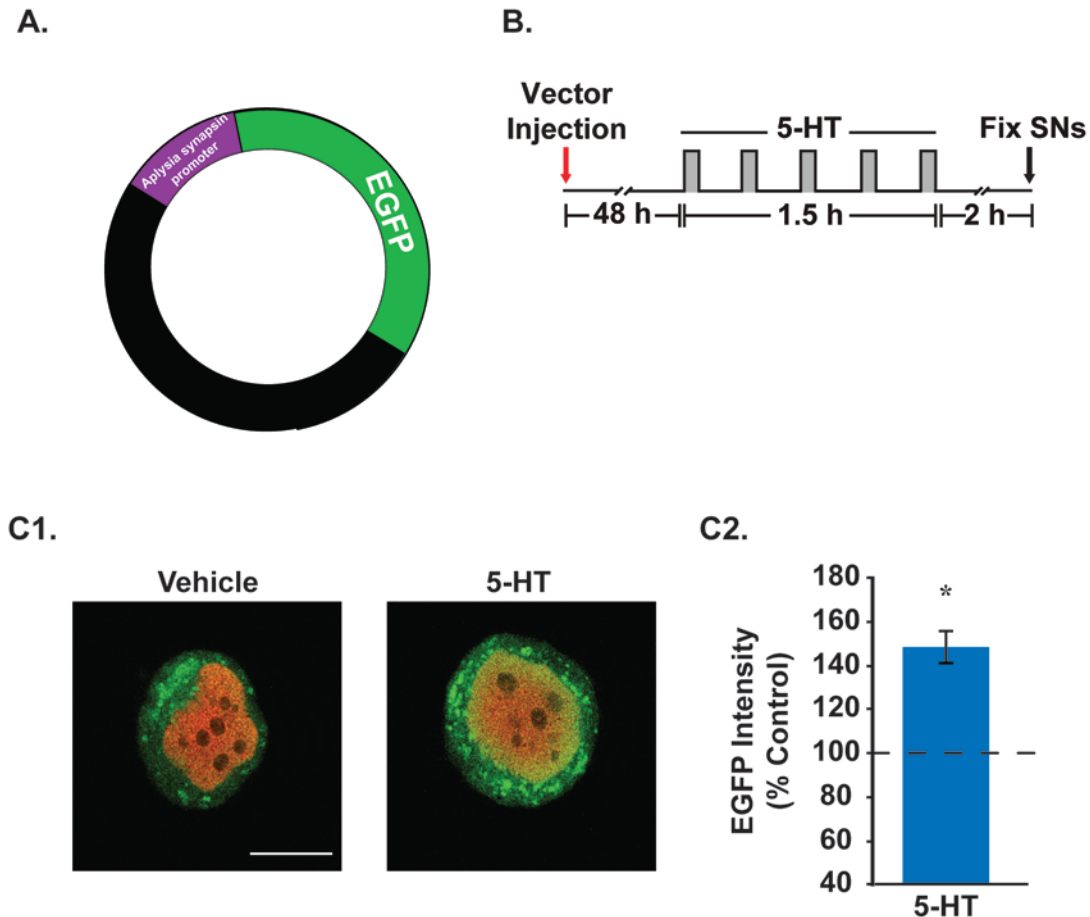


Figure 3.2. The synapsin promoter is transcriptionally activated in response to 5-HT. **A**, Schematic of reporter vector. **B**, Experimental Protocol. Sensory neurons (SNs) were co-injected with fluorescent dye and the expression vector on the third day of culture, treated on Day 5 (48 h after injected) and fixed 2 h after treatment for immunofluorescence analysis. **C1**, *Synapsin* promoter-driven EGFP expression in the cytoplasm of cultured sensory neurons 2 h after vehicle (left) or 5-HT treatment (right). EGFP intensity in the cytoplasm was normalized to red injection dye in the nucleus. Scale bar, 25 μ m. **C2**, Summary data from B1. Average EGFP immunofluorescence intensity (\pm SEM) in 5-HT-treated SNs (normalized to vehicle-treated cells). The significantly increased EGFP levels 2 h after 5-HT treatment (* $p < 0.05$) indicated that the *synapsin* promoter is activated by 5-HT.

5-HT- enhanced CREB1 association with the CRE site of the *synapsin* promoter

The observation that the promoter region of the *synapsin* gene contains a CRE site indicates that CREB1 may regulate the expression of *synapsin*. However, even though a CRE site is present does not mean that it is functional (Pfenning et al., 2007). ChIP assays have previously been used in *Aplysia* to examine the association of CREB1 with the promoter region of various genes and to assess the state of histone acetylation (Guan et al., 2002; Mohamed et al., 2005; Fioravante et al., 2008). To test the hypothesis that CREB1 is involved in the transcriptional regulation of *synapsin*, ChIP assays were performed to examine the association of CREB1 with the CRE site in the promoter region of *synapsin* under basal conditions (vehicle-treated) and after treatment with 5-HT. Based on the findings that synapsin mRNA levels in ganglia were significantly increased immediately and 1 h following treatment with 5-HT (Fig. 1.4A), the association of CREB1 with the *synapsin* promoter region immediately following treatment was assessed. Densitometry measurements of the amplified PCR product from the ChIP assays revealed that under basal conditions, CREB1 associated with the *synapsin* promoter and this association was significantly enhanced after treatment with 5-HT (Fig. 3.3) (mean percentage vehicle \pm SEM: $116.7 \pm 4.97\%$; $n = 6$, $t_5 = 2.01$, $p < 0.05$) (values were normalized to input control). These results provide additional support that synapsin is regulated directly by CREB1 during LTF.

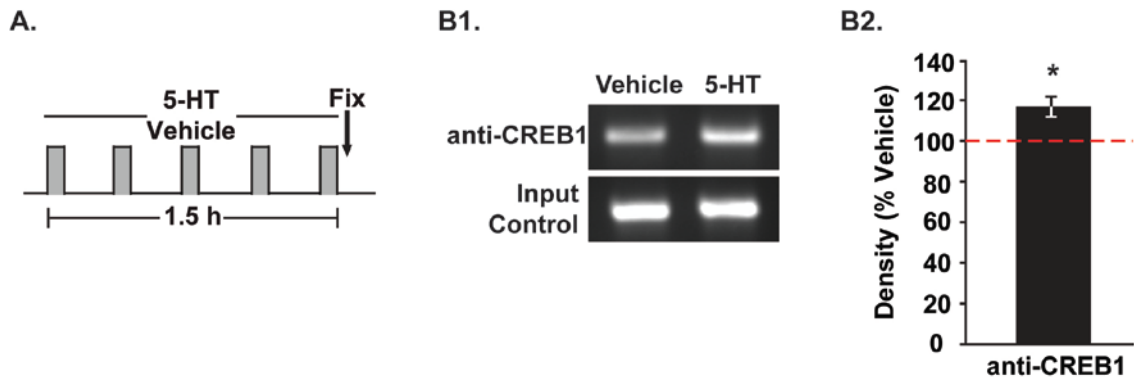


Figure 3.3. 5-HT enhances the association of CREB1 with the *Aplysia synapsin* promoter. **A**, Experimental Protocol. Ganglia were removed from the animal and treated with 5 pulses of 5-HT or vehicle and processed for the ChIP assay immediately following treatment. **B1**, Representative gel from PCR-amplified DNA resulting from ChIP assays examining the association of CREB1 with the CRE site of the *synapsin* promoter using an anti-CREB1 antibody on ganglia treated with vehicle or 5-HT. Densitometry measurements of bands were normalized to input control. CREB1 associates with the synapsin promoter under control conditions (left lane) and treatment with 5-HT led to increased association of CREB1 to the synapsin promoter (right lane). No change was observed in input control. **B2**, Summary data indicating that 5-HT treatment induced a significant increase in CREB1 association with the synapsin promoter (* $p < 0.05$).

5-HT increases histone acetylation surrounding the CRE site of the *synapsin* promoter

The state of histone acetylation has been used previously as an indicator of transcriptional regulation (Levenson and Sweatt, 2005). Studies indicate that hyperacetylation of histone tails, accompanied by a decrease in the association of histone deacetylases (HDAC), leads to transcriptional activation of genes of interest (Guan et al., 2002; Fioravante et al., 2008; but see Shahbazian and Grunstein, 2007). Therefore, to determine whether the enhanced association of CREB1 with the *synapsin* promoter following 5-HT treatment is associated with transcriptional initiation, changes in histone acetylation and the association of histone deacetylase 5 (HDAC5) in the vicinity of the CRE site were investigated in 5-HT- or vehicle-treated ganglia. ChIP assays were performed with antibodies directed against the acetylated forms of histones, H3 and H4, as well as HDAC5 (Fig. 3.4). These antibodies have previously been used to describe CREB2-dependent activation of transcription of *ApUch* following an LTD-inducing protocol (Fioravante et al., 2008). Densitometry measurements of the amplified PCR product from the ChIP assays revealed that 5-HT treatment significantly induced acetylation of histones H3 (mean percentage vehicle \pm SEM: $308 \pm 84\%$, $n = 5$, $t_4 = 3.15$, $p < 0.02$) and H4 ($411 \pm 89\%$, $n = 5$, $t_4 = 4.99$, $p < 0.02$) and decreased association of HDAC5 ($27 \pm 12\%$, $n = 5$, $t_4 = 3.24$, $p < 0.02$) (values were normalized to input control) with the region surround the CRE site. These results suggest that transcriptional activation of the *synapsin* gene following 5-HT treatment may be regulated through the CRE site in the *synapsin* promoter.

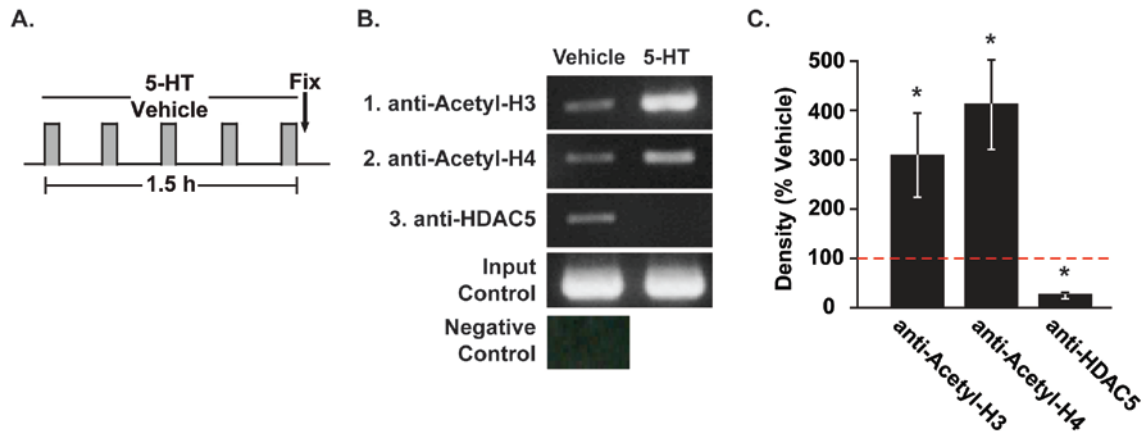


Figure 3.4. 5-HT-induced binding of CREB1 to *synapsin* promoter is accompanied by histone acetylation. **A**, Experimental Protocol. Ganglia were removed from the animal and treated with 5 pulses of 5-HT or vehicle and processed for the ChIP assay immediately following treatment. **B**, Representative gel from a ChIP assay assessing the state of histone acetylation of the *synapsin* promoter region containing the CRE site in ganglia treated with 5-HT or vehicle. Densitometry measurements of bands from antibodies against acetylated histone H3 (anti-acetyl-H3), acetylated histone H4 (anti-acetyl-H4) and anti-histone deacetylase 5 (anti-HDAC5) were normalized to input control. Basal association of acetylated histones (**B1**, **B2**, left lane) and HDAC5 (**B3**, left lane) was observed. 5-HT treatment led to increased acetylation of histones (**B1**, **B2**, right lane) and decreased association of HDAC5 (**B3**, right lane). No change in input control was observed. A negative control indicated that PCR-amplified product was dependent on immunoprecipitation with an antibody. **C**, Summary data indicating that there was a significant increase in association of acetylated H3 and H4 but a decreased association of HDAC5 with the *synapsin* promoter in ganglia treated with 5-HT (* $p < 0.05$).

DISCUSSION

To begin to identify potential mechanisms underlying the regulation of 5-HT-induced synapsin expression, the promoter region of the *synapsin* gene was cloned and analyzed using TESS software. Putative consensus sequences for various transcription factors were identified. Of particular interest were binding sites of transcription factors which have been previously been shown to be important for learning and memory.

For example, a consensus site for the transcription factor NF- κ B was identified. Although NF- κ B has been linked to hippocampal synaptic plasticity (Albensi and Mattson, 2000), 5-HT does not appear to regulate *Aplysia* NF- κ B activity (Povelones et al., 1997). Furthermore, injection of NF- κ B enhancer sequence oligonucleotides did not block 5-HT-induced LTF in sensorimotor coculture (Dash et al., 1990) indicating the *Aplysia* NF- κ B does not regulate gene expression necessary for LTF.

Of interest was the identification of two potential binding sequences for C/EBP, a transcription factor essential for LTF (Alberini et al., 1994). ChIP assays examining the *Aplysia* C/EBP promoter region revealed that 5-HT induces hyperacetylation of histones H3 and H4 as well as increased association of CREB1-CBP and decreased association of CREB2 (Guan et al., 2002). In addition, these results correlated with a 5-HT-induced increase in C/EBP mRNA levels indicating that enhanced histone acetylation and CREB1 binding are associated with transcriptional activation of the gene (Guan et al., 2002). However, both mammalian *synapsin I* and *synapsin II* gene promoters lack a CAAT box

(Sauerwald et al., 1990; Petersohn et al., 1995). Whether *synapsin* is regulated by C/EBP and what the functional significance of such regulation would be are potentially interesting questions for future studies.

Importantly, a CRE motif was found in the promoter region of *Aplysia synapsin* (Fig.3.1). Although many genes contain a CRE site, not all are functionally significant (Pfenning et al., 2007), therefore it was necessary to examine CREB1 association with the *synapsin* promoter. ChIP assays indicated that 5-HT treatment enhances the recruitment of CREB1 to the *synapsin* promoter near the CRE site (Fig. 3.3). Human and rat *synapsin I* promoter regions also contain CRE motifs (Sauerwald et al., 1990; Sudhof, 1990) however the *Aplysia synapsin* CRE motif, TGACGCAT, varies from the canonical CRE motif, TGACGTCA, but contains the central CpG dinucleotide, which is important for strong CREB binding (Smith et al., 2007). A positional bias for CRE at approximately -150 bp upstream of the transcription start site has been observed in vertebrates (Smith et al., 2007) however genes which appear to be regulated by CREB1 in *Aplysia* have a CRE in a region greater than -150 bp upstream (Mohamed et al., 2005). Therefore, it is possible that the *Aplysia synapsin* CRE, located at -818 bp upstream of the translation start site, is functional.

Previously, Jungling et al. (1994) and Hoesche et al. (1995) failed to find regulation of synapsin expression, in neuroblastoma and PC12 cell lines respectively, following treatments which activate the cAMP/PKA cascade. In *Aplysia* and in other systems (Karpinski et al., 1992; Jungling et al., 1994; Vecsey et al., 2007), relief of basal repression is additionally necessary for CREB-mediated

transcription to proceed (Bartsch et al., 1995; Guan et al., 2002; Guan et al., 2003; Mohamed et al., 2005). In addition to activation of the cAMP/PKA pathway, 5-HT application leads to the recruitment of ERK to deactivate the transcriptional repressor CREB2 (Bartsch et al., 1995; Martin et al., 1997). Because the cAMP/PKA cascade was selectively activated in these studies, it is likely that basal repression of CRE-mediated transcription of *synapsin I* was not relieved. Therefore, it is probably the combined effect of activation of CREB1 and removal of inhibitory constraints (such as CREB2-mediated repression) that allows induction of genes necessary for LTF (Kandel, 2001) and likely 5-HT-induced synapsin expression.

A CBP binding site near the CRE site in the *synapsin* promoter was also identified (Fig. 3.1). Two cases that examined CBP mutant mice (in which a dominant-negative allele of CBP was present or mice lacked one allele) found learning impairments that were rescued by the presence of an HDAC inhibitor (Alarcon et al., 2004; Korzus et al., 2004) indicating that learning requires a consorted balance between CBP and HDAC activity (Levenson and Sweatt, 2005). As a result, histone acetylation was examined as a monitor of transcriptional activation of the *synapsin* gene following 5-HT treatment. Acetylation of lysine residues on histone tails changes the structural conformation so that transcriptional regulators are able to access DNA and control gene expression. Such learning-induced modulation of chromatin structure has been associated with the regulation of gene expression during memory formation (Levenson et al., 2004; Levenson and Sweatt, 2005). Therefore, the finding that the hyperacetylation of histones H3 and H4 and the decreased association of HDAC5 surrounding the *synapsin* promoter

CRE site (Fig. 3.4) suggest 5-HT-induced transcriptional activation of the *synapsin* gene.

Finally, experiments using an EGFP reporter construct driven by the *synapsin* promoter indicated that treatment with 5-HT activated the *synapsin* promoter (Fig. 3.2). Together, these results suggest that upon treatment with 5-HT, CREB1, which is activated through the cAMP/PKA cascade and is necessary for LTF (Dash et al., 1990; Bartsch et al., 1998; Liu et al., 2008), is recruited to the promoter region of *synapsin*, and drives its expression. Future studies examining the regulation of synapsin expression through the *synapsin* promoter would be of particular interest at the later time points after treatment as well as determining if synapsin expression is regulated by the transcription factor, C/EBP.

CHAPTER IV. SEROTONIN-INDUCED SYNAPSIN EXPRESSION IS NECESSARY FOR LONG-TERM SYNAPTIC FACILITATION

INTRODUCTION

Synapsin knock-out studies have suggested that basal levels of synapsin are important for specific types of learning and memory (Silva et al., 1996; Gitler et al., 2004a; Godenschwege et al., 2004; Corradi et al., 2008; Knappek et al., 2010). In addition, there also appears to be a correlation between elevated synapsin levels and enhanced synaptic plasticity (Morimoto et al., 1998; Sato et al., 2000), learning and memory (Gomez-Pinilla et al., 2001; Velho and Mello, 2008; Rapanelli et al., 2009) and morphological changes (Han et al., 1991; Han and Greengard, 1994). However, the functional significance of enhanced synapsin expression associated with learning, plasticity and morphology remains unclear. Therefore, in this experiment, RNA interference was used to block the 5-HT-induced increase in synapsin levels to examine the functional significance of synapsin regulation for LTF as well as the morphological changes that accompany LTF.

RNAi is a commonly used tool which silences the expression of target genes in a variety of organisms and cell types, through a relatively well-known mechanism of action (Dykxhoorn and Lieberman, 2005). Small interfering RNAs (siRNAs), approximately 21-25 nucleotides, are introduced into the cytoplasm of the cell. The siRNAs form complexes with RISC (RNA-induced silencing complex) protein components and unwind in an ATP-dependent process. The activated RISC-complex then associates with target mRNA and cleaves endogenous complementary mRNA. Variations in model system, gene of interest, target accessibility (location of and folding of mRNA), thermodynamics of complementary

bond formation, siRNA sequences, and method of delivery are responsible for the different time dependencies of RNAi activity (Dykxhoorn and Lieberman, 2005).

RNAi has been a particularly critical instrument in discovering the molecular mechanisms that underlie changes in short- and long-term plasticity in *Aplysia*. For example, RNAi of the 5-HT receptor coupled to adenylyl cyclase blocks 5-HT-induced short-term plasticity, such as excitability and synaptic facilitation (Lee et al., 2009). In addition, injection of double stranded RNA (dsRNA) targeting CREB2 into the sensory neuron 24 h prior to treatment blocks LTF in sensorimotor cocultures (Lee et al., 2003) as does injection of dsRNA designed against C/EBP, injected 1 h prior to 5-HT treatment (Lee et al., 2001). Furthermore, the lab previously used small interfering RNA (siRNA) to block the 5-HT-induced increase in CREB1 without affecting basal CREB1 levels (Liu et al., 2011b).

Based on the successful employment of RNAi in *Aplysia*, the goal was to utilize siRNA directed against *Aplysia* synapsin to investigate the functional significance of synapsin regulation for LTF by specifically targeting and blocking the increase in synapsin protein after treatment with 5-HT. Based on the observation that 5-HT induces synapsin expression, potentially through CREB1, the hypothesis that synapsin expression is necessary for LTF was tested. In addition, the hypothesis that synapsin expression is necessary for the 5-HT-induced morphological changes that accompany LTF in culture was also tested.

Injecting synapsin siRNA 2.5 h prior to treatment blocks the 5-HT-induced synapsin expression as well as 5-HT-induced LTF. Control experiments to determine affects of synapsin siRNA on basic release mechanisms and plasticity

were also performed. Results indicated that synapsin siRNA does not affect two types of short-term plasticity. The effect of synapsin siRNA on 5-HT-induced morphological changes was also examined in order to make a connection between synaptic strength and sensory neuron morphology. However, previous results found in papers that examine 5-HT-regulated morphological changes were not able to be reproduced. Potential mechanisms are further discussed in Chapter 5.

METHODS

RNA interference. siRNA was custom-designed and prepared by Dharmacon to specifically target *Aplysia* synapsin mRNA. The Custom SmartPool contained 4 unique siRNAs to target specific areas of the synapsin sequence that should not interfere with the expression of other genes. The synapsin siRNAs were designed against the following sequences: ¹²⁹⁹CGATATCCACGTTTCAGAAA¹³¹⁷; ¹⁵³⁷CCAATGAGAGCGCCAGGTA¹⁵⁵⁵; ¹⁶⁵⁹GGTTAGGCAAAGAGTCGTT¹⁶⁷⁷; ²⁰¹⁸TCAAGTTGTGGGTGGACGA²⁰³⁶. Control siRNA (scrambled siRNA, also provided by Dharmacon) or synapsin siRNA (5 μ M) was injected into the cytoplasm of cultured sensory neurons, together with a fluorescent marker (2.5 mg/ml dextran-Alexa 488, 10 kDa) in a 100 mM KCl solution 2.5 h prior to treatment with vehicle or 5-HT. To assess the effects of siRNA on synapsin protein levels, cells were fixed and processed for immunostaining (see Chapter 2 Methods) at 6, 28 and 52 h after injection. All groups were compared to control siRNA-injected, vehicle treated

sensory neurons using a two-tailed Student's t-test. An additional experiment was performed to examine if siRNA is functional at 24 h after injection. Sensory neurons were injected with either control or synapsin siRNA, treated 20.5 h after injection with vehicle or 5-HT, and fixed 2 h after treatment (24 h after injection) and assessed using a two-tailed Student's t-test.

Electrophysiology: Sensorimotor cocultures were prepared as described previously (Angers et al., 2002; Liu et al., 2008). Synapsin or control siRNA was pressure injected into the cytoplasm of sensory neurons using the Eppendorf (Westbury, NY) microinjection system 2.5 h before the pretest. The efficacy of the injection was monitored by fluorescence. Prior to treatment with 5-HT or vehicle, basal synaptic strength was assessed by evoking an excitatory postsynaptic potential (EPSP) in the motor neuron by extracellular stimulation of the presynaptic sensory neuron using a patch electrode filled with L15:ASW (50:50 % volume) placed next to the sensory neuron cell body. EPSPs were recorded from motor neurons with 10–15 M Ω sharp electrodes filled with 3 M potassium acetate. Cocultures containing motor neurons with a resting membrane potential more positive than -40 mV and input resistance less than 10 M Ω were excluded. Resting membrane potential and input resistance were recorded during both the pretest and posttest. Prior to sensory neuron stimulation, the resting membrane potential of the motor neuron was current clamped at -90 mV. Responses were recorded using an Axoclamp-2B amplifier and pCLAMP 8.2 software (Molecular Devices, Sunnyvale, CA). EPSP amplitude was measured offline using Clampfit 9.0 (Molecular Devices, Union City,

CA). If the pretest EPSP was lower than 5 mV, the culture was also excluded from further use. Twenty-four h after the end of treatment, a posttest EPSP was recorded. For statistical analysis, the ratio of posttest/pretest amplitudes was calculated. Data were analyzed by a two-way ANOVA followed by post-hoc analysis with Student-Newman-Keuls tests.

In a separate set of experiments, short-term synaptic depression and facilitation after depression were assessed in cocultures injected with either control or synapsin siRNA. Twenty-eight h after siRNA injection, a train of 10 stimuli at 0.05 Hz was delivered. The first eight stimuli were designed to examine the magnitude of short-term synaptic depression. Depression was assessed by comparing the amplitude of the 8th EPSP (EPSP8) to that of the first EPSP (EPSP1). Immediately after the 8th stimulus a bolus of 5-HT (50 μ M) was added to the bath with a pipette to examine the extent of facilitation after depression and two additional stimuli were then delivered. Facilitation of a depressed synapse was assessed by comparing the amplitude of the 10th EPSP (EPSP10, 40 s after 5-HT application) to the 8th EPSP (before 5-HT). Results were analyzed using two-tailed Student's t-tests.

Morphological analysis of sensorimotor co-cultures. Immediately following the posttest, co-cultures were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in blocking buffer for 30 min, and incubated with mouse anti-synapsin (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and rabbit anti-VAMP (kind gift of Dr. K. C. Martin, University of California Los

Angeles) primary antibodies. The mouse monoclonal antibody was used to enable double-labeling. Cocultures were then incubated with anti-mouse secondary antibody conjugated to Cy-5 and anti-rabbit secondary antibody conjugated to Cy-3 to visualize the intracellular localization of synapsin and VAMP. Confocal analysis was performed two ways; the first employing a technique previously used in the lab to examine synapsin expression in cocultures (Angers et al., 2002) and the other which aimed to mimic techniques previously used in another lab to measure the number of sensory neuron varicosities along the main motor axon of the motor neuron (Glanzman et al., 1990).

Because it was not possible to image the entire coculture at high power, for the first type of analysis, an 8x8 grid (800 x 800 μm) containing 64 square regions (each 100 x 100 μm) was superimposed over a previously captured phase-contrast image of the sensorimotor coculture (Angers et al., 2002). A random number table created in Microsoft Excel indicated which 7 square regions would be imaged and analyzed. The number 7 was chosen because it represents greater than 10 percent of the entire region to be assessed. As previously described, a z-series of optical sections was obtained with a Zeiss LSM 510 confocal microscope using a 63x oil immersion lens. Image stacks through $\sim 10 \mu\text{m}$ were collected at 0.5 μm increments and projected into a single image using Metamorph Offline software. The total number of sensory neuron varicosities for each of the 7 images were counted and the average number of varicosities for each group was assessed using a two-way ANOVA.

In a second type of analysis, which employed a technique similar to that of Glanzman et al. (1990), a z-series of optical sections was obtained to assess the number of sensory neuron varicosities along the main motor axon. As before, the average number of varicosities for each group was assessed using a two-way ANOVA. Confocal image capture and analysis were both performed in blind fashion in order to eliminate potential bias during imaging and analysis.

RESULTS

Development of synapsin siRNA

The objective of this experiment was to specifically inhibit new expression of synapsin without significantly disrupting basal levels of synapsin. Because RNAi is specific to the gene of interest and preparation, the design and methods used in these experiments were independently tailored to the synapsin mRNA. A custom-designed mixture of four distinct synapsin siRNAs (Figure 4.1) was used to block the 5-HT-induced increase in synapsin expression. BLAST software identified no other gene targets for the synapsin siRNAs. A negative control for RNAi experiments, control siRNA, was also purchased from Dharmacon.


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1  atgtctttct ccaacttcaa ggacagtttc ggctccggca tgaactacct gcgccgccgc
61  ttctcctcgg gagatctcca gggggaggca agcgacaatg acgactcgcc caatgtgggc
121 ggactcaact ttcggaaagg cccaagtccc agcgcgccaa actccccgtc caaatcggcg
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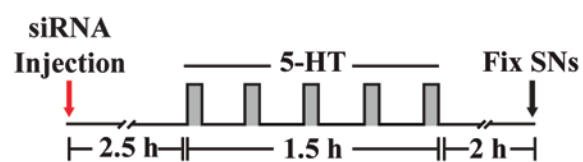
Figure 4.1. Identification of four synapsin siRNA target sequences. Synapsin mRNA sequence indicating the translation start site (green, ATG codon, +1) and the four unique synapsin siRNA target sequences (red) designed by Dharmacon.

Injection of synapsin siRNA 2.5 h prior to treatment with 5-HT blocks the 5-HT-induced increase in synapsin expression

An appropriate time point at which injection of synapsin siRNA would block the 5-HT-induced increase in synapsin expression at 2 h after treatment without affecting basal levels of synapsin expression was first established. Injecting the synapsin siRNA 2.5 h prior to treatment with 5-HT effectively blocked the 5-HT-induced increase in synapsin protein expression 2 h after 5-HT treatment without affecting basal protein levels (expressed as percent of control siRNA-injected, vehicle-treated cells: control siRNA+5HT: $127.3 \pm 4.9\%$; synapsin siRNA+5HT: $97.0 \pm 6.1\%$, $n = 5$, $t_4 = 3.66$, $p < 0.05$) (Fig. 4.2). These results validate the use of synapsin siRNA as a tool to block 5-HT-induced changes in synapsin protein levels.

An additional experiment was performed to assess the functional duration of synapsin siRNA. Results indicated that siRNA was stable in the cellular milieu and effective for at least 24 h post injection because it blocked the increase in synapsin protein in cells treated with 5-HT 20.5 h post injection and measured 2 h after treatment (expressed as percent of control siRNA-injected, vehicle-treated cells \pm SEM: control siRNA+5-HT: $124.8 \pm 2.4\%$; synapsin siRNA+5-HT: $99.3 \pm 5.7\%$, $n = 4$, $t_3 = 7.05$, $p < 0.05$; not shown).

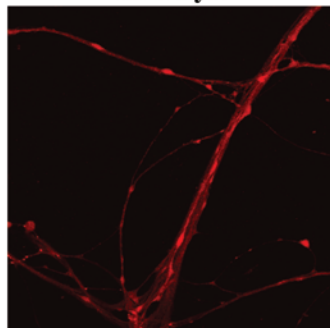
A.



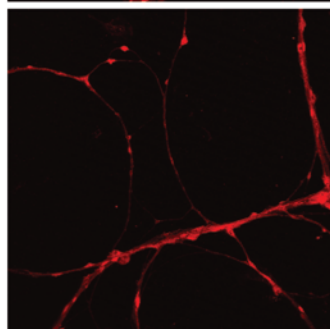
B1. Synapsin immunoreactivity

anti-Syn

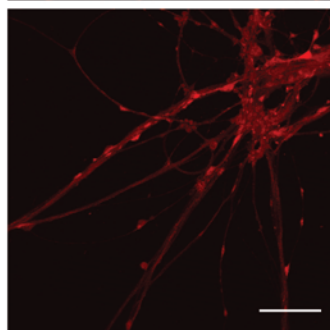
Con siRNA,
Veh



Con siRNA,
5-HT



Syn siRNA,
5-HT



B2. 6 h after injection

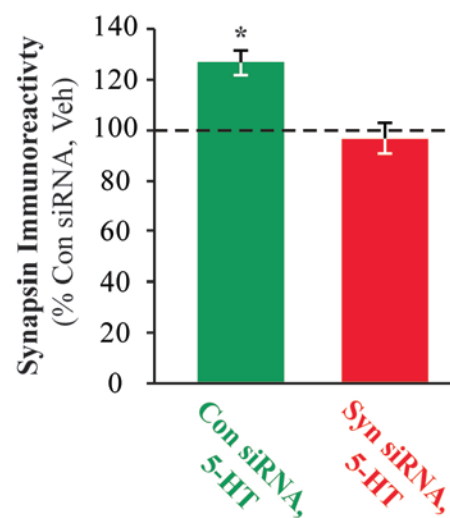


Figure 4.2. Synapsin siRNA blocks the 5-HT-induced increase in synapsin immunoreactivity 2 h post 5-HT treatment. **A**, Experimental Protocol. Synapsin or control siRNA is injected into cultured sensory neurons 2.5 h prior to treatment with vehicle or 5-HT. Two h post treatment, cells were fixed and processed for immunofluorescence. **B1**, Examples of sensory neurons injected with control siRNA and treated with vehicle (top panel), injected with control siRNA and treated with 5-HT (middle panel) and injected with synapsin siRNA and treated with 5-HT (bottom panel). Scale bar, 25 μ m. **B2**, Summary data from B1. Average fluorescence intensity (\pm SEM) of synapsin immunostaining in varicosities 2 h after 5-HT treatment in control siRNA- and synapsin siRNA-injected cells normalized to synapsin intensity in control siRNA-injected, vehicle-treated cells. Injection of synapsin siRNA blocked the 5-HT-induced increase in synapsin protein 2 h after treatment (* $p < 0.05$).

Knock-down of Synapsin Expression

RNAi activity and the extent of synapsin knock-down was assessed on basal levels of synapsin expression at 6, 28 and 52 h after injection using immunofluorescence analysis (Fig. 4.3). Cultured sensory neurons were injected with either control or synapsin siRNA and fixed at these time points. The results indicate that synapsin siRNA injection does not alter basal synapsin expression 6 h after injection (percent of control siRNA-injected cells \pm SEM: 6h, $99.9 \pm 1.63\%$, $n = 3$, $t_2 = 0.46$, $p = 0.69$).

The 28 h time point was also examined because it corresponds to 24 h after treatment, the time point when LTF is assessed, and is an important control in this study. Twenty-eight h after siRNA injection, there was no significant difference in synapsin immunoreactivity between control and synapsin siRNA-injected sensory neurons (percent of control siRNA-injected cells \pm SEM: synapsin siRNA: $96.0 \pm 3.5\%$, $n = 5$, $t_4 = 1.08$, $p = 0.34$). These results suggest that injecting synapsin siRNA 2.5 h before treatment will not affect basal synapsin levels at 24 h after treatment.

In addition, an extended time point of 52 h after injection was chosen to determine if synapsin siRNA can eventually knock-down basal levels. At 52 h post siRNA injection, basal synapsin immunoreactivity levels of synapsin siRNA-injected sensory neurons were decreased by 11% compared to control siRNA-injected cells (percent of control siRNA-injected cells \pm SEM: synapsin siRNA: $89 \pm 3.0\%$, $n = 5$, $t_4 = 3.65$, $p < 0.05$). These results indicate that, after an extended period of time, synapsin siRNA decreases basal synapsin levels.

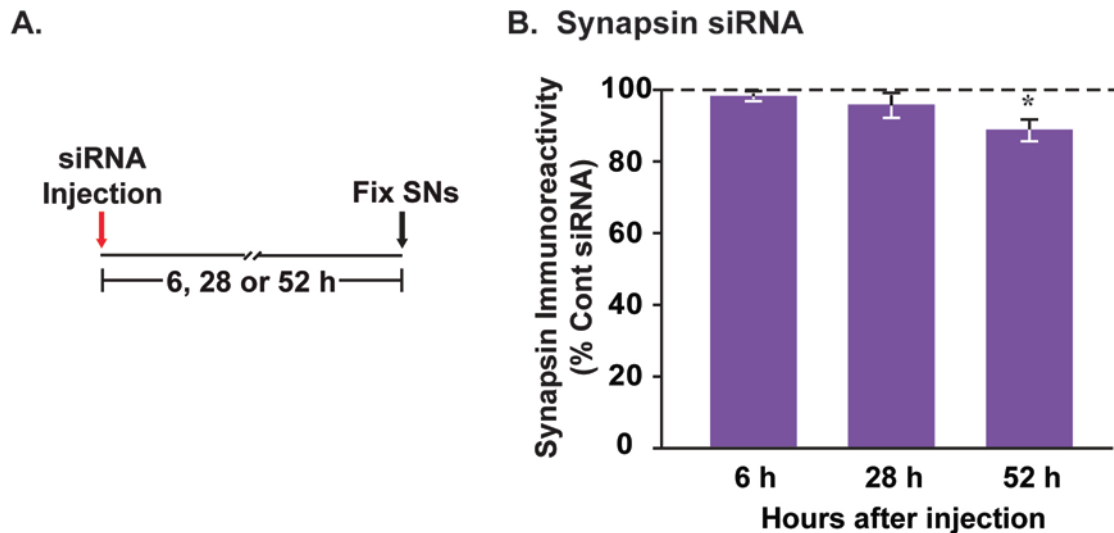


Figure 4.3. Synapsin siRNA does not affect basal synapsin levels at 6 or 28 h post injection. **A**, Experimental Protocol. Sensory neurons were injected with either control or synapsin siRNA and fixed either 28 or 52 h after injection. **B**, Plot of basal synapsin immunoreactivity (average fluorescence intensity (\pm SEM)) from synapsin siRNA-injected cells (normalized to control siRNA) 6 h, 28 h and 52 h post injection. No effect of synapsin siRNA injection on basal synapsin levels was observed at 6 ($p = 0.69$) or 28 h ($p = 0.34$). However, a significant difference was observed 52 h after injection ($*p < 0.05$) suggesting that basal synapsin levels are affected by synapsin siRNA at this time point.

5-HT-induced synapsin expression is necessary for LTF

Based on these preliminary experiments, the importance of 5-HT-induced synapsin expression for LTF was next examined using synapsin siRNA to block the increase in synapsin levels. Basal synaptic strength in sensorimotor cocultures (pre-test) was first assessed and either control siRNA or synapsin siRNA was injected into the cytoplasm of sensory neurons. Two-and-one-half h after the siRNA injections, cocultures were treated with either 5 pulses of 5-HT or vehicle and the extent of LTF was assessed 24 h after treatment (post-test) (Fig. 4.4A). A two-way ANOVA revealed a significant main effect of injection ($F_{(1,31)} = 9.31$, $p < 0.01$) and treatment ($F_{(1,31)} = 8.63$, $p < 0.01$) with a p value of 0.13 for the injection x treatment interaction ($F_{(1,31)} = 2.43$) 24 h after treatment (Fig. 4.4B; mean percentage post/pre \pm SEM: control siRNA+vehicle: $103.1 \pm 5.2\%$, $n = 9$; control siRNA+5-HT: $148.4 \pm 18.0\%$, $n = 7$; synapsin siRNA+vehicle: $88.1 \pm 8.0\%$, $n = 8$; synapsin siRNA+5-HT: 102.0 ± 7.8 , $n = 8$). Subsequent Student-Newman-Keuls post-hoc tests indicated that 5-HT induced significant facilitation in the control siRNA-injected cocultures (control siRNA: vehicle vs. 5-HT, $q = 4.48$, $p < 0.05$), but not in the synapsin siRNA-injected cocultures (synapsin siRNA: vehicle vs. 5-HT, $q = 1.39$, $p = 0.34$). In addition, the 5-HT-induced facilitation in the synapsin siRNA-injected cells was blocked compared to that of control siRNA-injected cells (5-HT: control siRNA vs. synapsin siRNA, $q = 4.47$, $p < 0.05$). Importantly, injection of synapsin siRNA did not significantly affect synaptic transmission over a 28 h period (vehicle: control siRNA vs. synapsin siRNA, $q = 1.54$; $p = 0.29$). These results suggest that the 5-HT-induced increase of synapsin levels in sensory neurons is necessary for LTF.

An additional analysis indicated that there was not a significant difference in initial synaptic strength (pre-test) among the four groups ($F_{(3,31)} = 3.01$, $p > 0.05$) suggesting that the observed differences in synaptic strength following treatments were not due to differences in the initial strengths of the sensorimotor synapses. Changes in motor neuron input resistance and resting potential were also assessed as an extra control since previous studies found that behavioral training does not lead to changes in the passive properties of motor neurons (Cleary et al., 1998; Antzoulatos and Byrne, 2007). In agreement with the results previously published, a two-way ANOVA indicated that, for input resistance, there was not a significant effect of injection ($F_{(1,31)} = 2.43$, $p = 0.13$), treatment ($F_{(1,31)} = 0.17$, $p = 0.68$), or injection x treatment interaction ($F_{(1,31)} = 0.37$, $p = 0.55$) (Fig. 4.5A). A two-way ANOVA also indicated that, for resting potential, there was not a significant effect for injection ($F_{(1,31)} = 2.78$, $p = 0.11$), treatment ($F_{(1,31)} = .002$, $p = 0.96$), or injection x treatment interaction ($F_{(1,31)} = 0.39$, $p = 0.56$) (Fig. 4.5B).

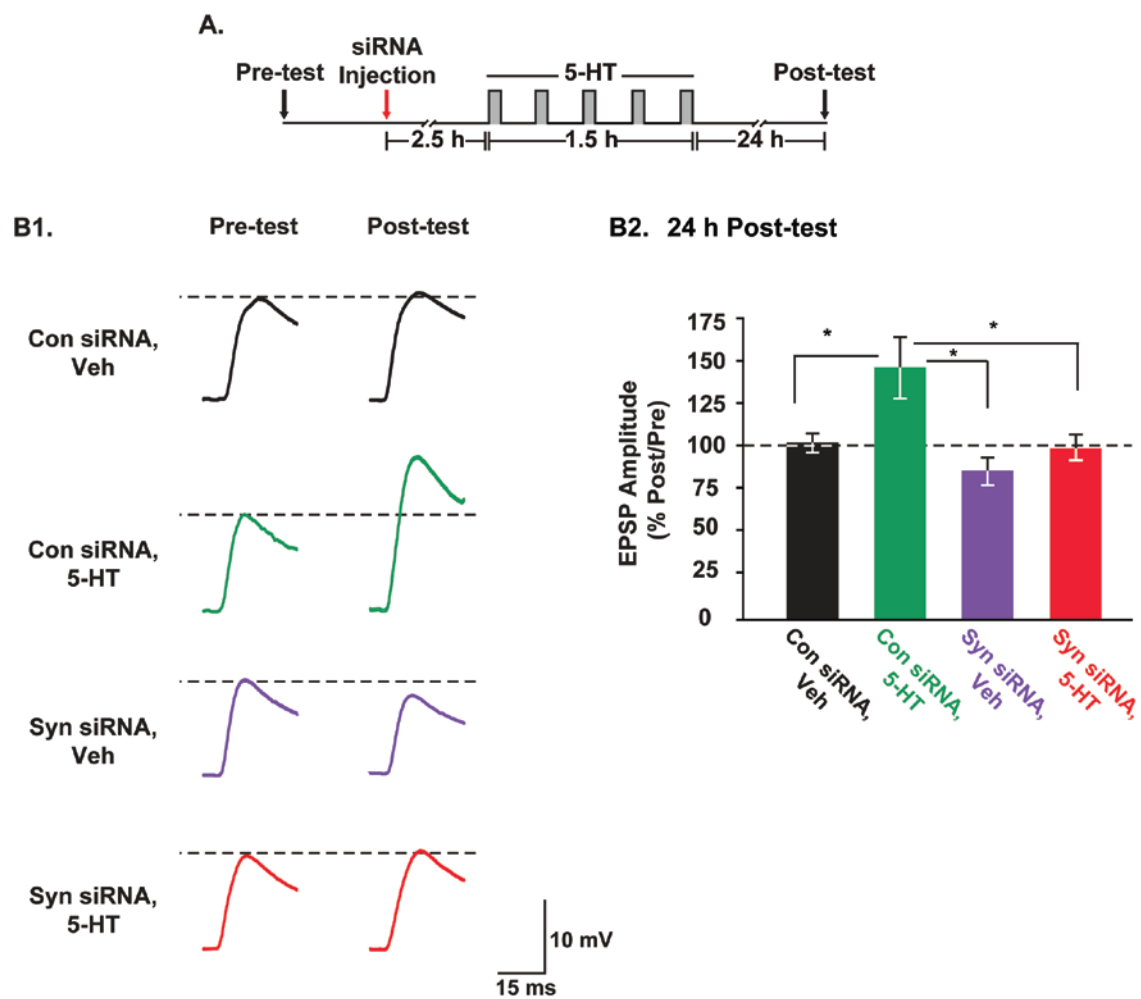


Figure 4.4. 5-HT-induced synapsin expression is necessary for LTF. **A**, Experimental protocol. Immediately following a pre-test to assess basal synaptic strength, sensory neurons were injected with either control or synapsin siRNA. Two and a half h after injection, cocultures were treated with either 5, 5-min pulses of vehicle or 5-HT with an ISI of 20 min (grey pulses). Synaptic strength was again assessed 24 h after the end of treatment (post-test). **B1**, Example EPSPs recorded from sensorimotor cocultures injected with control or synapsin siRNA before (pre-test) and 24 h after (post-test) treatment with 5-HT or vehicle. **B2**, Plot of average percent post/pre ratio (\pm SEM) of EPSP amplitudes. 5-HT induced significant facilitation in the control siRNA-injected cocultures ($*p < 0.05$), but not in the synapsin siRNA-injected cocultures ($p = 0.34$). The 5-HT-induced facilitation observed in control siRNA-injected cocultures was blocked in synapsin siRNA-injected cocultures ($*p < 0.005$). Injection of synapsin siRNA did not significantly affect synaptic transmission over a 28 h period ($p = 0.29$).

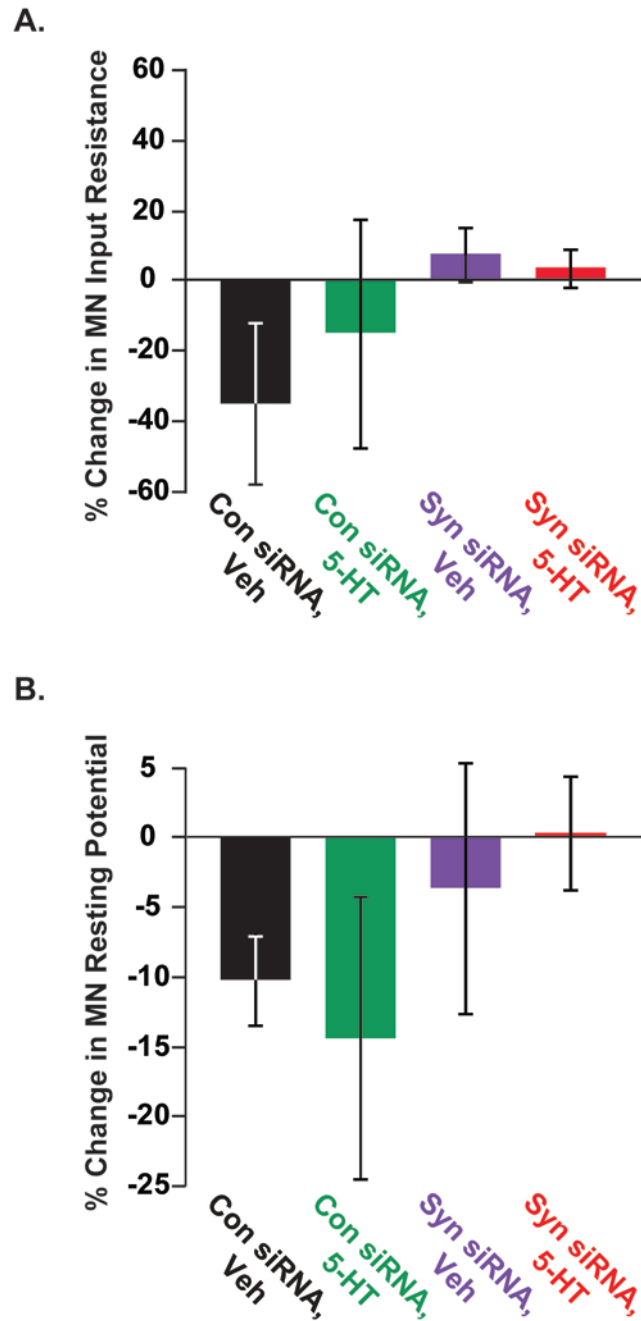


Figure 4.5. siRNA does not alter passive properties of the post-synaptic motor neurons. **A**, Plot of the changes in motor neuron (MN) input resistance. There was no significant effect of injection ($p = 0.13$), treatment ($p = 0.68$) or interaction ($p = 0.55$) for changes of MN input resistance. **B**, Plot of changes in MN resting membrane potential. There was no significant effect of injection ($p = 0.11$), treatment ($p = 0.96$) or interaction ($p = 0.56$) for changes in MN resting membrane potential.

siRNA does not affect short-term synaptic depression or facilitation of depressed synapse

Results from electrophysiology experiments indicated that synapsin siRNA alone does not affect basal synaptic efficacy, nor does it affect basal synapsin levels assessed using immunofluorescence (Fig. 4.3). However, an additional test of synaptic integrity was performed. The effects of synapsin siRNA on the short-term synaptic depression and 5-HT-induced facilitation of a depressed synapse were assessed (Castellucci and Kandel, 1976; Byrne, 1982; Emptage et al., 1996; Fioravante et al., 2007). Twenty-eight h after siRNA injection (which corresponds to 24 h after treatment when LTF is measured), a train of 8 stimuli at 0.05 Hz was delivered to induce short-term synaptic depression. 5-HT was applied to the culture dish immediately following the 8th EPSP, followed by 2 additional stimuli delivered at 0.05 Hz to test for the extent of facilitation of the depressed synapse.

Depression was assessed by forming the ratio of the amplitude of EPSP8 to that of EPSP1. There was no difference in depression between cocultures injected with control siRNA or synapsin siRNA (percent amplitude of EPSP8/EPSP1 \pm SEM: control siRNA: 31.9 \pm 1.5%; synapsin siRNA: 37.6 \pm 9.1%; $n = 10$, $t_9 = 0.57$; $p = 0.58$) (Fig. 4.6B2). Facilitation of a depressed synapse was assessed by forming the ratio of the amplitude of EPSP10 (40 s after 5-HT application) to that of EPSP8 (before 5-HT). There was no significant difference in facilitation between cocultures injected with control siRNA or synapsin siRNA (percent amplitude EPSP10/EPSP8 \pm SEM: control siRNA: 250.4 \pm 49.9%; synapsin siRNA: 242.7 \pm 46.7%; $n = 10$, $t_9 = 0.11$; $p = 0.91$) (Fig. 4.6B3). The lack of effect of synapsin siRNA on short-term

depression and subsequent facilitation of depressed synapses provides further evidence that the siRNA did not affect basic release mechanisms or short-term plasticity and supports the assertion that the effect of synapsin siRNA is specific to LTF.

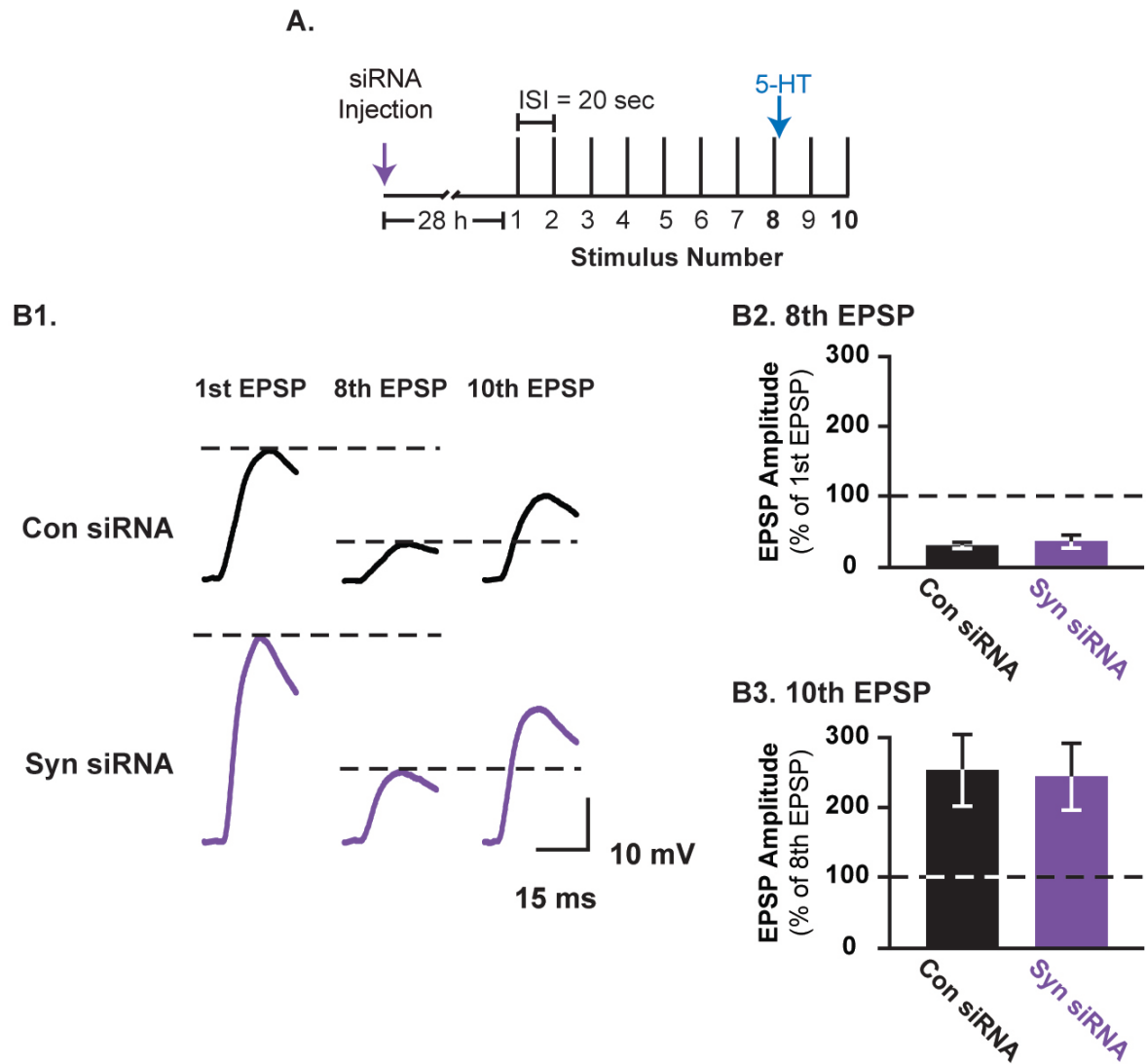


Figure 4.6. siRNA does not affect short-term synaptic depression or facilitation of a depressed synapse. **A**, Experimental protocol. Sensory neurons were injected with control or synapsin siRNA. Twenty-eight h later, a train of eight stimuli were delivered at 0.05 Hz. Immediately following the 8th stimulus, a bolus of 5-HT was delivered to the coculture followed by an additional 2 stimuli. **B1**, Example EPSPs recorded from sensorimotor cocultures injected with control or synapsin siRNA 28 h after injection. **B2**, Synaptic depression was assessed using the 8th/1st EPSP ratio (\pm SEM). No difference in the extent of depression between cocultures injected with control or synapsin siRNA was observed ($p = 0.58$). **B3**, Facilitation of a depressed synapse was assessed using the 10th/8th EPSP ratio (\pm SEM). Both groups exhibited similar 5-HT-induced facilitation ($p = 0.91$).

Assessment of morphological alterations following 5-HT treatment

In addition to long-term changes in synaptic plasticity, treatment with 5-HT leads to structural changes in the sensory neurons, which are thought to represent the final step in the stabilization of the long-term process (Bailey et al., 2004). Indeed, when the 5-HT-induced morphological changes are blocked in culture, the extent and duration of facilitation is impaired (Hatada et al., 2000). Therefore, because synapsin has been implicated in processes of synaptogenesis and neurite outgrowth, the hypothesis that increased synapsin expression was contributing to enhanced plasticity through the formation of new connections (i.e. varicosities) between the sensory and motor neuron was tested.

Sensorimotor cocultures used to examine the effects of synapsin siRNA on LTF were also used to examine the effects of synapsin siRNA on 5-HT-induced morphological changes. Two strategies were employed for this analysis. The first type of analysis was modified from Angers et al. (2002) and the second type was similar to that previously used in Glanzman et al. (1990).

To begin the first type of analysis, a phase-contrast image was taken to capture the sensory neuron and motor neuron cell bodies and the majority of their processes in culture. Because the sensory and motor neuron processes are very extensive and cover a large area, it was not possible to image and analyze the entire coculture. Therefore, an 8 x 8 grid was superimposed over phase-contrast images and a random number generator indicated which 7 areas to image in a non-biased manner (Fig. 4.7). Once the confocal images of the 7 regions were captured, the total number of VAMP-positive sensory neuron varicosities was

counted. Two-way ANOVAs indicated that there was not a significant difference between the 4 groups for average number of VAMP-positive varicosities (main effect for injection ($F_{(1,24)} = 2.1$, $p = 0.17$), treatment ($F_{(1,24)} = 0.30$, $p = 0.59$) or interaction ($F_{(1,24)} = 1.17$, $p = 0.29$) (Fig. 4.9A; average number of sensory neuron varicosities per group \pm SEM: control siRNA+Vehicle, 27.4 ± 3.6 ; control siRNA+5HT, 30.5 ± 5.8 ; synapsin siRNA+Vehicle, 41.8 ± 11.5 ; synapsin siRNA+5HT, 32.5 ± 6.2). Because a significant difference between the number of varicosities within control siRNA-injected cocultures treated with vehicle or 5-HT was not observed as we expected from previously published research (Glanzman et al., 1990; Kim et al., 2003), a different type of analysis was employed.

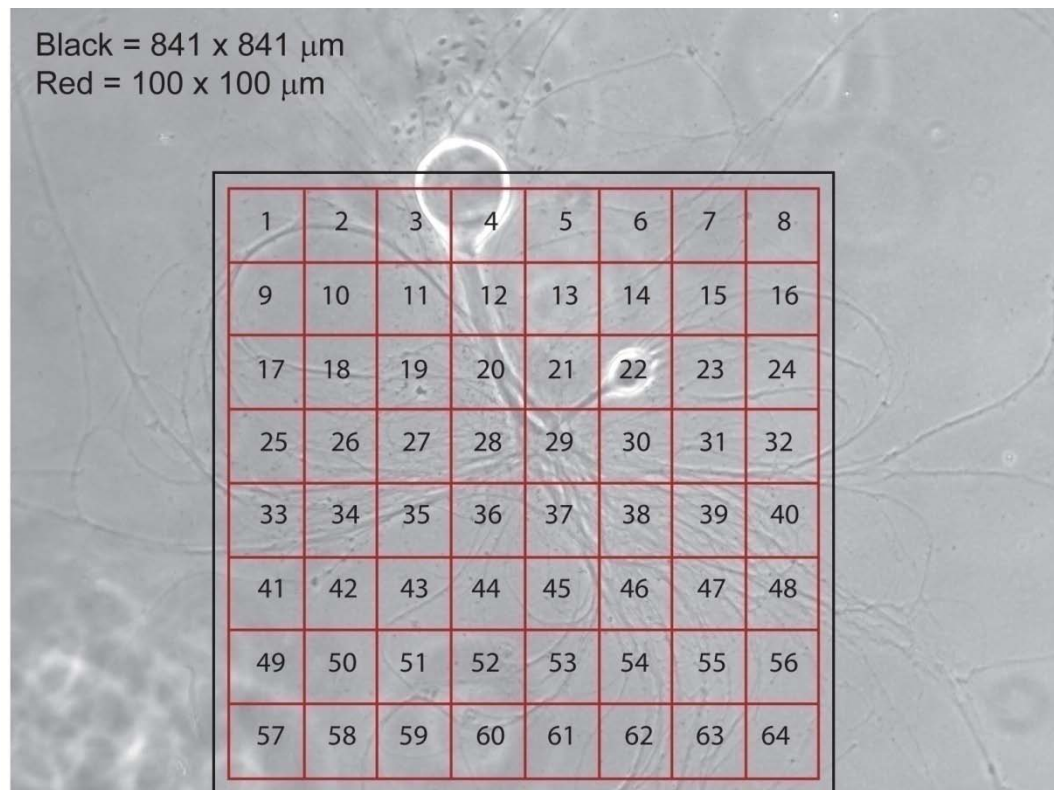


Figure 4.7. Morphological assessment using 8 x 8 grid method of control or synapsin siRNA-injected cocultures 24 h after treatment with vehicle or 5-HT. Example of a phase-contrast image of a fixed sensorimotor coculture from the LTF experiment. An 8 x 8 grid was superimposed over the region containing the most processes. Each individual red box is 100 x 100 μm . Random number generation from numbers 1-64 indicated which 7 regions (red boxes) would be imaged and analyzed.

The second strategy of morphology analysis examined sensory neuron processes along the main motor axon, the presume site of contact (Glanzman et al., 1990). For each coculture, a 250 μm region along the main motor axon was imaged (Fig. 4.8) and again the total number of sensory neuron varicosities was counted for each culture. A two-way ANOVA indicated that there was not a significant difference between the 4 groups for average number of VAMP-positive varicosities (main effect for injection ($F_{(1,24)} = 0.62$, $p = 0.44$), treatment ($F_{(1,24)} = 0.69$, $p = 0.42$) or interaction ($F_{(1,24)} = 0.99$, $p = 0.33$) (Fig. 4.9B; average number of sensory neuron varicosities per group \pm SEM: control siRNA+Vehicle, 15.1 ± 2.4 ; control siRNA+5HT, 20.3 ± 5.8 ; synapsin siRNA+Vehicle, 15.7 ± 2.6 ; synapsin siRNA+5HT, 15.3 ± 3.4). Although a trend appeared with this method of assessment, a power analysis indicated that it would take a considerable amount of work to reach significance. Therefore, further analysis of this data was discontinued.

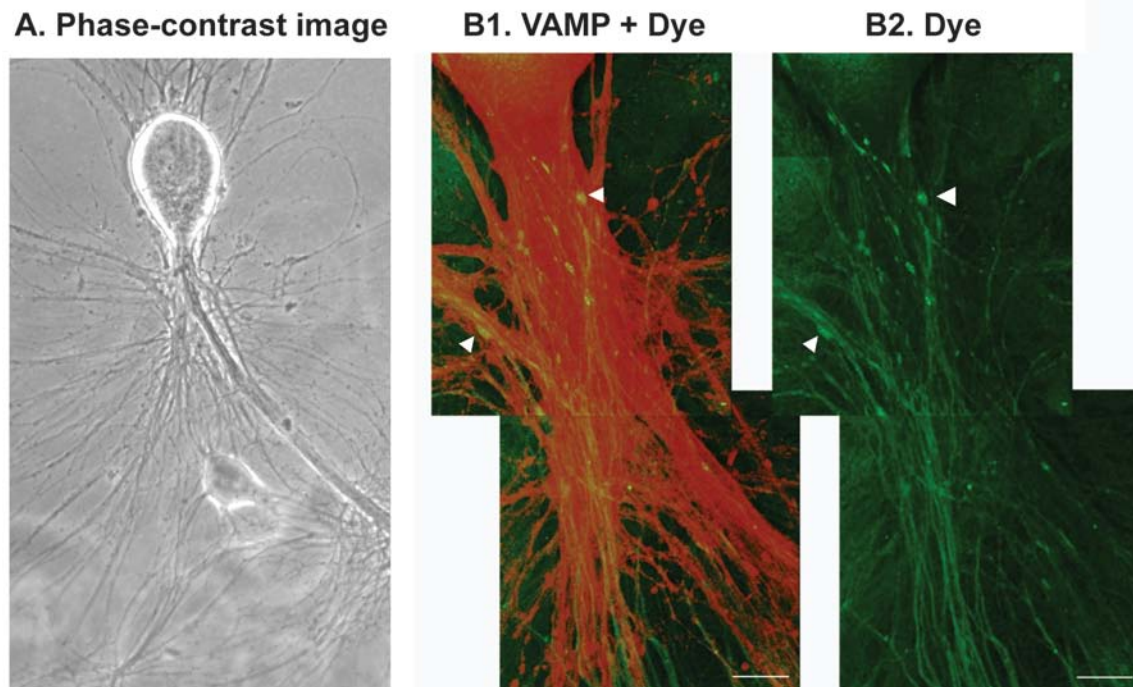
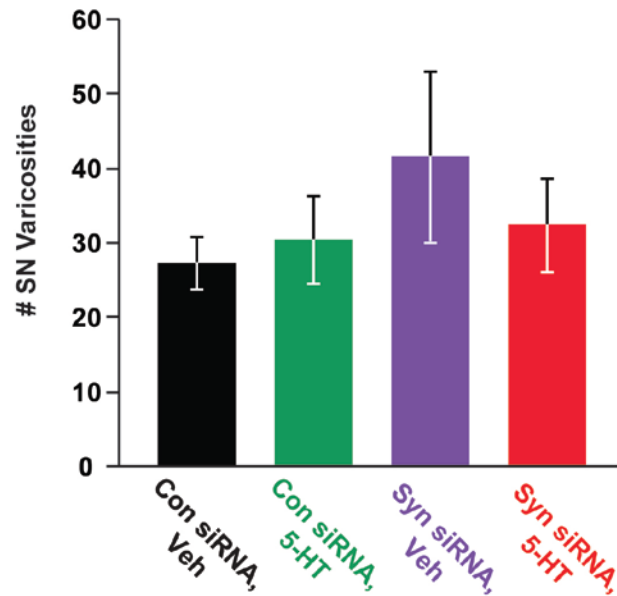


Figure 4.8. Morphological assessment along the main motor axon of control or synapsin siRNA-injected cocultures 24 h after treatment with vehicle or 5-HT. **A**, Phase contrast images were taken of sensorimotor cocultures prior to immunostaining to visualize processes. **B1**, Example coculture exhibiting colocalization of VAMP and injection dye. **B2**, Example coculture exhibiting sensory neuron processes filled with injection dye. Scale bar, 25 μ m.

A. Total number of varicosities in 7 designated regions



B. Total number of varicosities in images within 250 μm of the main motor axon

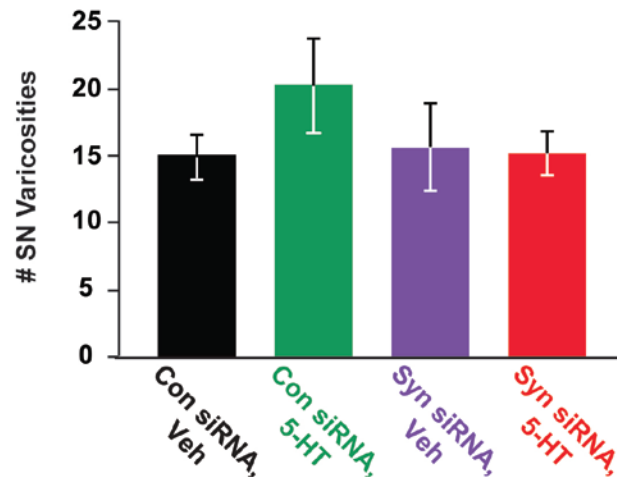


Figure 4.9. Total number of sensory neuron varicosities in control or synapsin siRNA-injected cocultures 24 h after treatment with vehicle or 5-HT. **A**, Summary data of the total number of sensory neuron (SN) varicosities from cocultures assessed by analyzing 7 previously identified regions of a sensorimotor coculture. A two-way ANOVA indicated that there was not a significant difference between the groups (injection, $p = 0.71$; treatment, $p = 0.59$, interaction, $p = 0.29$). **B**, Summary data from cocultures assessed by analyzing sensory neuron processes within confocal images of the first 250 μm of the main motor axon. A two-way ANOVA indicated that there was not a significant difference between the groups (injection, $p = 0.44$; treatment, $p = 0.42$, interaction, $p = 0.33$).

DISCUSSION

The role for synapsin in long-term plasticity, learning and memory has previously been investigated through the use of various synapsin knockout models or through correlations of synapsin levels with behavior or enhanced plasticity. This study of synapsin is primarily different in that RNAi was used to acutely target the 5-HT-induced elevated synapsin levels. This method did not alter basal protein levels or synaptic transmission as knockout models do. Therefore, to expand on results suggesting that synapsin levels are correlated with enhanced synaptic plasticity and memory, the functional significance of elevated synapsin levels in sensory neurons was addressed. Through RNAi, this study provides the first evidence for a requirement for regulated synapsin expression during the consolidation of long-term synaptic plasticity.

Off-target effects of RNAi

Because EPSP measurements are a sensitive indicator of cellular function and integrity any potential off-target effects of both the control and synapsin siRNA would have been realized with the post-test vs. pre-test comparison because siRNA was injected after the initial EPSP pre-test. There was no change in synaptic strength in both control siRNA-injected and synapsin siRNA-injected cultures treated with vehicle 28 h after injection. These results are in line with other experiments in which dsRNA or siRNA is injected into *Aplysia* sensory neurons within 24 h of treatment and does not appear to affect basal synaptic transmission (Lee et al., 2001).

Dharmacon, the company that designed the synapsin siRNA used in these experiments, designs siRNA sequences in order to limit off-target effects. For example, increasing the concentration of individual siRNAs for RNAi can lead to increased off-target effects. Therefore, Dharmacon designed 4 individual siRNAs to be used together to achieve knock-down without jeopardizing specificity to the target siRNA. In addition, the sense strand of the siRNA is chemically modified to impede entry into the RISC complex (Jackson et al., 2006) and the seed region (positions 2-7) is carefully examined because this region is associated with off-target effects when this region is a match for the 3' untranslated region (UTR) of mRNAs (Birmingham et al., 2006).

Basal synapsin expression and plasticity in the presence of siRNA

A critical aspect of these experiments was to determine the appropriate time to inject the siRNA relative to the time of treatment and testing as RNAi experimental design and methods must be independently tailored to the target mRNA (Ryther et al., 2005). Because the aim was to only block the 5-HT-induced increase in synapsin protein and not affect basal protein levels, additional experiments and measurements were performed to assess this goal. Using immunofluorescence techniques to examine relative changes in synapsin levels, results indicated that, compared to control siRNA, synapsin siRNA injection does not affect basal protein levels at 28 h after injection, the time point that corresponds to the 24 h post-test (Fig. 4.3). In addition, results indicated that synapsin siRNA did not affect basal synaptic transmission or short-term synaptic plasticity

suggesting that synapsin siRNA injection within this time frame only affected the enhancement of long-term synaptic strength.

Previously, the Byrne lab found that overexpression of synapsin in sensory neurons increased synaptic depression whereas 5-HT-induced facilitation of a depressed synapse was significantly enhanced (Fioravante et al., 2007). If an off-target effect of synapsin siRNA was to increase synapsin levels, we would hypothesize that both of these measures would have been affected in our experiment.

Synapsin and morphology

As seen in other model systems, synapsin protein levels tend to increase during prolonged enhancement of synaptic plasticity (Hicks et al., 1997; Morimoto et al., 1998; Sato et al., 2000). This was also the case during the consolidation phase of LTF and it is possible that elevated synapsin levels could act as building blocks necessary for the establishment of new synaptic connections. According to this hypothesis, blocking the increase in synapsin would impair formation of new varicosities and abolish LTF. In cultured hippocampal neurons, synapsin I is expressed before the establishment of synaptic contacts (Fletcher et al., 1991) and cells lacking synapsin I or II exhibit both retarded neurite outgrowth and synaptogenesis (Ferreira et al., 1994; Ferreira et al., 1998). In addition the introduction of synapsin I or II accelerates the rate of functional and structural synaptogenesis (Han et al., 1991; Lu et al., 1992).

The morphological changes observed after 5-HT treatment in sensorimotor coculture appears to be driven by actin dynamics as indicated by experiments performed by Hatada et al. (2000) which found that inhibiting actin polymerization blocks the 5-HT-associated increase in number of sensory neuron varicosities as well as neurite outgrowth. Therefore, proteins that regulate actin dynamics are of particular interest as these proteins may be part of the 5-HT-induced cascade that leads to enhanced synaptic strength.

How could synapsin contribute to these morphological changes? Synapsin is interacts with cytoskeletal elements such as actin. Elevated exogenous levels of synapsin led to the bundling and reorganization of filamentous actin (F-actin) and the formation of elongated processes in nonneuronal cells (Han and Greengard, 1994) whereas depletion of synapsin in hippocampal neurons results in an abnormal distribution of F-actin (Ferreira et al., 1994). Therefore, the 5-HT-induced morphological changes that are dependent upon actin polymerization (Hatada et al., 2000) may be triggered by the 5-HT-induced elevation of synapsin levels. However, as the lab previously reported, overexpression of synapsin alone does not lead to an increase in VAMP-positive varicosities in cultured sensory neurons (Fioravante et al., 2007). Therefore, other 5-HT-induced mechanisms are needed to initiate this process. For example, 5-HT treatment results in the activation of multiple kinase pathways and the synapsin-actin interaction is highly dependent on the phosphorylation state of synapsin (Valtorta et al., 1992; Nielander et al., 1997; Kao et al., 2002). It is possible then that the morphological changes that occur after 5-HT are dependent on both increased synapsin expression as well as the

phosphorylation state of synapsin. Synapsin phosphorylation is further discussed in Chapter 5.

Alternatively, cases exist where morphological changes are not associated with enhanced synaptic strength in *Aplysia* (Casadio et al., 1999; Hatada et al., 2000; Wainwright et al., 2002) and a positive correlation depends on the method of analysis (Fig. 4.9). Even though elevated synapsin levels are important for enhanced synaptic strength, morphological changes may not be correlated or dependent upon synapsin expression.

It is also possible that synapsin could have a novel function, unrelated to 5-HT-induced synaptogenesis, but still necessary for LTF. To distinguish between these potential mechanisms, as well as others, and to examine the role of synapsin in LTF-associated morphology, it would first be necessary to establish a consistent method in which to examine sensorimotor coculture morphology. Regardless, the observation that elevated synapsin levels are necessary for LTF is an intriguing finding that further advances the understanding of synapsin, building upon previous work correlating elevated levels with plasticity and behavior.

CHAPTER V. CONCLUDING REMARKS

Potential function of elevated synapsin levels during the consolidation of LTF

Although this study provided the first evidence that stimulus-induced elevated synapsin levels are necessary for long-term enhancement of synaptic plasticity, the functional mechanism of action of synapsin in LTF remains unclear. LTF requires both transcription and translation (Montarolo et al., 1986) and 5-HT results in the activation of multiple pathways (Barbas et al., 2003). Aside from the potential transcriptional regulation of *synapsin* through the cAMP-PKA-CREB1 pathway described in Chapter 3, post-translational modifications of synapsin (i.e. phosphorylation of synapsin) may also be important for LTF. The regulation of synapsin phosphorylation has been implemented in many forms of short-term synaptic plasticity (Hilfiker et al., 1999) and neurite outgrowth (Kao et al., 2002). Therefore it is possible that once synapsin levels are elevated, the functional significance of synapsin depends on post-translational mechanism. Examples of how synapsin phosphorylation could be involved an increase in neurotransmitter release and neurite outgrowth are summarized in Figure 5.1.

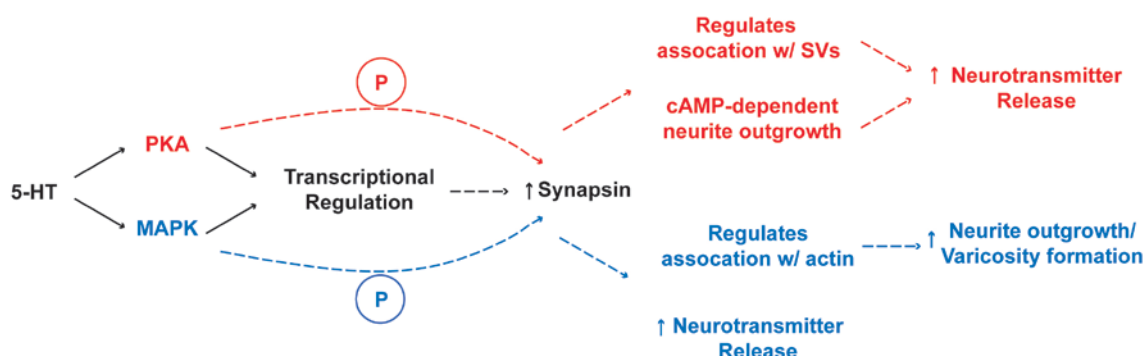


Figure 5.1. Potential mechanisms by which synapsin phosphorylation could regulate the 5-HT-induced increase in neurotransmitter release and neuronal growth. 5-HT treatment leads to an increase in both PKA and MAPK activity. Both PKA and MAPK regulate transcription of genes that are necessary for LTF. In addition to transcription factors, PKA and MAPK may also phosphorylate elevated synapsin levels, which are necessary for LTF (Fig. 4.4). Based on results obtained in other systems, it is possible that PKA and MAPK phosphorylation of newly synthesized synapsin may contribute to LTF. PKA phosphorylation of synapsin is important for synaptic vesicle pool regulation as well as cAMP-dependent neurite outgrowth. Therefore it is possible that PKA-dependent phosphorylation of synapsin may be important for an increase in neurotransmitter release as well as the growth of new synaptic connections (red). In addition, MAPK phosphorylation regulates synapsin interaction with actin which is implemented in changes in neuronal morphology as well as neurotrophin-enhanced neurotransmitter release (blue). The role of 5-HT-induced synapsin expression is of particular interest and may be through the phosphorylation of the protein.

Synapsin and the cAMP/PKA pathway

The relevance of the cAMP/PKA pathway in *Aplysia* has also been identified in various other forms of memory in different model systems (Drain et al., 1991; Frey et al., 1993). Aside from the potential synapsin transcriptional regulation through the cAMP-PKA-CREB1 pathway described in Chapter 3, post-translational modifications of synapsin (i.e. phosphorylation of synapsin by PKA) may also be important for LTF. Five pulses of 5-HT leads to a biphasic profile of PKA activity which is increased initially, goes back to baseline and increases again at 20 h after treatment (Muller and Carew, 1998). This temporal profile is similar to the expression pattern of synapsin following 5-HT treatment (Figs. 1.4, 2.2). PKA phosphorylation of mammalian synapsin Ia decreases its association with actin and synaptic vesicles resulting in an increase in neurotransmitter release (Hosaka et al., 1999; Cesca et al., 2010). PKA phosphorylation is also necessary for synapsin-induced enhancement of neurotransmitter release in invertebrate neurons (Fiumara et al., 2004), cAMP-induced neurite outgrowth in intact *Xenopus laevis* embryos (Kao et al., 2002) and the modulation of synaptic vesicle recycling during synapse maturation in cultured hippocampal neurons (Bonanomi et al., 2005). PKA phosphorylation of basal levels of synapsin, as well as newly synthesized synapsin, during LTF is of interest and may reveal a potential mechanism by which synapsin contributes to LTF.

Synapsin and MAPK pathway

In *Aplysia* sensory neurons, PKA appears to activate MAPK (Michael et al., 1998). Synapsin could also be part of the 5-HT-induced pathway involving the neurotrophin, Transforming Growth Factor β 1 (TGF- β) (Zhang et al., 1997), and MAPK activation (Martin et al., 1997; Michael et al., 1998) both of which are necessary for LTF. The lab previously found that TGF- β leads to the phosphorylation of synapsin in an ERK-dependent manner (Chin et al., 2002b). In synaptosomes, neurotrophin-enhanced glutamate release is mediated through ERK phosphorylation of synapsin I (Jovanovic et al., 1996; Jovanovic et al., 2000) and constitutive activation of the ERK pathway in mice enhances learning and LTP but these effects are blocked in synapsin I KO mice (Kushner et al., 2005) suggesting that basal levels of synapsin are a necessary but downstream component of the neurotrophin-ERK pathway.

The phosphorylation of synapsin I by MAPK may also be important for proper synapsin-actin interactions as MAPK phosphorylation attenuates synapsin binding to actin filaments (Matsubara et al., 1996). Therefore it is possible that synapsin could function to regulate vesicle pools in a MAPK-dependent manner, as is suggested in short-term facilitation in *Aplysia* (Angers et al., 2002). Further experiments would be necessary to study these hypotheses as well as to determine the functional role of basal synapsin levels on LTF.

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