Université de Montréal

Kinase C substrates and synaptic plasticity in *Aplysia.*

by

Gry Houeland

Département de physiologie

Faculté de médicine

Thesis submitted at Faculté des études supérieures

in accordance with the requirement for the degree of Philosophiae Doctor (Ph.D.)

in Sciences Neurologiques

Janvier 2007

© Gry Houeland, 2007

W
4
C58
2007
V. 063

Université de Montréal

Direction des bibliothèques

AVIS

L'auteur a autorisé l'Université de Montréal à reproduire et diffuser, en totalité ou en partie, par quelque moyen que ce soit et sur quelque support que ce soit, et exclusivement à des fins non lucratives d'enseignement et de recherche, des copies de ce mémoire ou de cette thèse.

L'auteur et les coauteurs le cas échéant conservent la propriété du droit d'auteur et des droits moraux qui protègent ce document. Ni la thèse ou le mémoire, ni des extraits substantiels de ce document, ne doivent être imprimés ou autrement reproduits sans l'autorisation de l'auteur.

Afin de se conformer à la Loi canadienne sur la protection des renseignements personnels, quelques formulaires secondaires, coordonnées ou signatures intégrées au texte ont pu être enlevés de ce document. Bien que cela ait pu affecter la pagination, il n'y a aucun contenu manquant.

NOTICE

The author of this thesis or dissertation has granted a nonexclusive license allowing Université de Montréal to reproduce and publish the document, in part or in whole, and in any format, solely for noncommercial educational and research purposes.

The author and co-authors if applicable retain copyright ownership and moral rights in this document. Neither the whole thesis or dissertation, nor substantial extracts from it, may be printed or otherwise reproduced without the author's permission.

In compliance with the Canadian Privacy Act some supporting forms, contact information or signatures may have been removed from the document. While this may affect the document page count, it does not represent any loss of content from the document.

Université de Montréal
Faculté des études supérieures

This thesis is entitled:

Kinase C substrates and snaptic plasticity in *Aplysia*.

Presented by:
Gry Houeland

Has been evaluated by a jury composed of the following people:

Richard Robitaille, reporting president
Vincent Castelucci, research director
Wayne Sossin, co-director
Luc Desgroseillers, jury member
John H. Byrne, external examinor

Résumé

Le but principal de ma recherche était d'étudier la plasticité synaptique chez l'*Aplysie.* J'ai étudié en particulier le rôle de la kinase C (PKC) dans la transmission synaptique. J'ai tenté d'élucider les modifications par la phosphorylation des protéines impliquées dans la relâche de neurotransmetteurs par la PKC. J'ai étudié ces questions dans le mollusque *Aplysia californica*, un modèle expérimental propice à l 'étude des relations qui existent entre l'apprentissage et la plasticité du système nerveux. Synaptotagmine-I (Syt-I) est une protéine vésiculaire qui fonctionne comme un détecteur de calcium et joue un rôle essentiel dans la relâche des neurotransmetteurs. Dans le processus de cloner Syt-I, nous avons trouvé une nouvelle isoforme avec deux acides aminés +VQ insérés dans le domaine N-terminal de C2-A ($Syt\text{-}I_{VQ}$). La surexpression de $Syt\text{-}I_{VQ}$ bloque la facilitation par la sérotonine. Ceci indique que la région VQ de la synaptotagmine joue un rôle important dans la facilitation des synapses déprimées. J'ai ensuite testé si $EGFP\text{-}Syt\text{-}I_{VQ}$ pouvait bloquer l'effet de PDBu, un phorbol ester qui active le PKC indépendamment de 5-HT. Le $EGFP\text{-}Syt\text{-}I_{VQ}$ n'était plus capable de bloquer le reversement de la facilitation, suggérant que le déficit est lié à la capacité de 5-HT d'activer PKC. Cet effet spécifique de $Syt\text{-}I_{VQ}$ sur la transmission s'explique si cette isoforme est la seule qui se trouve dans les vésicules synaptiques. Nous avons observé une différence d'expression dans les cônes de croissance et dans les axones entre les 2 isoformes. Les résultats indiquent que le site VQ est impliqué dans la régulation de la localisation des Syt-I. Nous avons aussi cloné une autre isoforme de synaptotagmine, Syt-I C2B-β avec 39 changements dans la région C2B. Une mesure de protéolyse avec la chymotrypsine démontre que cette isoforme peut, en absence de calcium supporter un changement de conformation normalement déclenché par celui-ci. Nous avons également montré que Syt-I C2B-β avait une plus grande affinité pour SNAP-25 que Syt-I C2B-α. En conclusion, ces résultats suggèrent que l'épissage change la structure du domaine C2B et pourrait ainsi affecter la fonction de la protéine.

SNAP-25 est une protéine impliquée dans la relâche de neurotransmetteur et membre du complexe SNARE. Cette protéine est aussi un substrat de PKC. J'ai cloné

l'Apl-SNAP-25. J'ai également exprimé les formes sauvages et mutées SNAP-25 de l'aplysie dans des neurones sensoriels de l'*Aplysie* afin de déterminer si la phosphorylation de cette protéine par PKC joue un rôle dans la transmission synaptique. La surexpression de SNAP-25 n'avait aucun effet sur les cinétiques de la dépression et de la facilitation synaptique. Cependant, nous suggérons que la phosphorylation de SNAP-25 par PKC joue un rôle dans la modulation synaptique. Notamment, nous avons trouvé qu'en présence de PDBu ou de protéines phosphomimétiques les cinétiques de la dépression ralentissaient. Cet effet a été bloqué dans les cellules exprimant un site de phosphorylation PKC non-phosphorylable ainsi qu'en présence de bisinolylmalemide-I (Bis), un bloqueur de PKC.

Mots-clés: Plasticité, *Aplysia*, synapse, synaptotagmine, épissage, SNAP-25, PKC, phosphorylation.

Abstract

The principal aim of my research was to study the synaptic plasticity at the sensori-motor synapse of *Aplysia*, and how it contributes to behavior, learning and memory. In my studies I focused on the role of protein kinase C (PKC) in synaptic transmission. I sought to define the step or steps in the transmitter release process that involve PKC phosphorylation and that contribute to synaptic plasticity, with the ultimate goal of identifying the PKC posttranslational (phosphorylation) modifications of specific proteins in the exocytotic pathway that regulate the number of vesicles available for release. I studied these questions in cultured *Aplysia* sensory-motor neurons using molecular biological and electrophysiological techniques. We discovered two novel splice forms in *Aplysia* synaptotagmin. We found a novel splice form with two amino acids VQ ($SytI_{VQ}$), inserted in the juxtamembrane domain of Syt I. The VQ and -VQ forms are expressed at equal levels in the *Aplysia* nervous system. Overexpression of the two forms have distinct effects, suggesting that the splice is of physiological importance. Overexpression of Syt I_{VQ} blocks the ability of serotonin to reverse synaptic depression, while overexpression of VQ does not. This effect is specific to the reversal of depression as there is no effect on the ability of serotonin to facilitate non-depressed synapses. The reversal of depression is mediated by PKC and synaptotagmin is a putative PKC substrate. However, despite the phosphorylation of *Aplysia* synaptotagmin at a conserved site adjoining the VQ, there is no change in phosphorylation between the two forms. Moreover, overexpression of synaptotagmin does not block the effect of phorbol esters, suggesting that the effect is due to a block of the ability of 5-HT to activate PKC in the cells overexpressing synaptotagmin. We discovered that while both VQ and -VQ forms are found in synaptic vesicles, they appear to be found in distinct vesicles in axons and growth cones, suggesting that we uncovered an important sorting signal for synaptotagmin.

A second splice in *Aplysia* synaptotagmin is an alternative exon used in the C2B domain. This splice form (C2B splice) is also expressed in the *Aplysia* nervous system. While all of

the known important amino acids in the C2B domain are conserved, the novel splice form shows a greater degree of calcium independent homo-dimerization and a greater degree of stability in the absence of calcium than the previously identified form.

SNAP-25 is a member of the SNARE protein complex implicated in synaptic vesicle docking and fusion and a substrate of PKC. We cloned *Aplysia* SNAP-25 and generated SNAP-25 constructs mutated in the PKC phosphorylation site Ser198, simulating either a phosphorylated or a non-phosphorylated form of SNAP-25. From our results, we suggest a role for SNAP-25 and its phosphorylation in some forms of synaptic modulation. Notably, we have found that phosphorylation of SNAP-25 by PKC in *Aplysia* neurons is required for PDBu mediated facilitation of depressed synapses.

Keywords: Plasticity, *Aplysia*, synapse, synaptotagmin, splicing, SNAP-25, PKC, phosphorylation.

Index

RÉSUMÉ iii
ABSTRACT v
INDEX ii
LIST OF TABLES xi
LIST OF FIGURES xii
LIST OF ABREVIATIONS xv
ACKNOWLEDGEMENTS xvii

I. INTRODUCTION..1

1.1 The dichotomy of memory functions...3
1.1.2 The brain must undergo changes that are maintained as long as the memory is stored. What are these changes?...5
1.1.3 What is the neuroanatomical support of our extraordinary capacity to store memory functions...6

1.2 The "engram"...7
1.2.1 Synaptic Plasticity...9

1.3 Vertebrates versus invertebrates...10

1.3.1 The ideal model...11
1.3.2 *Aplysia*...11
1.3.2.1 habituation...19
1.3.2.2 sensitization...20
1.3.2.3 dihabituation...20

1.3.2.4 classical conditioning........22
1.3.3 *Drosophila*........23
1.3.4 Mouse........25

1.4 The synapse........26
1.4.1 Signaling within neurons: the action potential........27
1.4.2 Synaptic transmission........27
1.4.3 The "calcium hypothesis"........29
1.4.4 The "quantal hypothesis"........29
1.4.4.1 What morphological feature might account for the quantum of transmitter?........30

1.5 The molecular biology of vesicle fusion and release........32
1.5.1 The initial trigger for neurotransmitter release is the activation of voltage-gated calcium channels and the influx of calcium........32
1.5.2 Calcium triggered exocytosis........33

1.6 The SNARE complex: structure assembly and disassembly........35
1.6.1 Key stages involved in synaptic vesicle fusion........39
1.6.2 The functional expression of engrams........39
1.6.3 SNAP-25........40
1.6.4 What is the Ca^{2+} sensor and how does it work to trigger release?........40

1.7 Protein phosphorylation........43
1.7.1 Protein Kinases........44
1.7.2 PKC........47
1.7.3 PKA........49

1.8 Personal contribution to the articles........50

II. ARTICLE 1 ...51

Differential regulation of transmitter release by alternatively spliced forms of synaptotagmin I. Nakhost A, Houeland G, Castellucci VF, Sossin WS (2003). ***J Neurosci*** **23:6238-44.**

ABSTRACT ...52
INTRODUCTION ...52
METHODS ...52
RESULTS ...53
DISCUSSION ...56
REFERENCES ...57

III. ARTICLE 2 ...59

Identification and characterization of a novel C2B splice variant of synaptotagmin I. Nakhost A, Houeland G, Blandford VE, Castellucci VF, Sossin WS (2004). ***J Neurochem*** **89:354-63.**

ABSTRACT ...60
INTRODUCTION ...60
METHODS ...61
RESULTS ...62
DISCUSSION ...65
REFERENCES ...69

IV. ARTICLE 3 70

PKC modulation of transmitter release by SNAP-25 at sensory to motor synapses in Aplysia. Houeland G, Nakhost A, Sossin WS, Castellucci VF (2007). *J Neurophysiol* 97:134-143.

ABSTRACT 71
INTRODUCTION 71
METHODS 71
RESULTS 73
DISCUSSION 76
REFERENCES 79

V. DISCUSSION 81

5.1 Contribution of this thesis for the comprehension PKC modulation of transmitters release by PKC 81
5.1.1 Mutation of Syt I at phosphorylation sites does not alter transmitter release 81
5.1.2 Phorbol esters increase transmission at naïve synapses through phosphorylation of SNAP-25 82
5.1.3 Pharmacological tools that interfere with PKC function 85
5.1.4 PKC and short-term plasticity: rested versus depressed synapses 87
5.2 The role of calcium in neurotransmitter release in *Aplysia* 88
5.2.1 Does SNAP-25 modulation of calcium dynamics play a role in depression? 91
5.3 Localization of Syt Is in *Aplysia* 92
5.3.1 How does our work compare with studies of SNAP-25 in chromaffin cells? 93
5.3.2 *Aplysia* Syt I has been cloned previously 94

5.3.3 How does our work compare with studies of synaptotagmin in central neurons?.......95

5.4 Overexpression studies...96

5.4.1 The use of EGFP-fusion proteins in *Aplysia*...99

5.5 Pretranslational modifications...101

5.5.1 Alternative splicing during development..103

5.5.2 Possible roles of an alternative splice form in the Syt I juxtamembrane domain....105

5.6 Possible roles of an alternative splice form in the Syt I juxtamembrane domain.......106

VI. Conclusion..108

VII. References..110

VIII. Annex..161

8.1 Personal contribution to the articles...161

Liste of tables

II ARTICE 1

Table 1. Comparison of intrinsic and synaptic properties of sensory neurons expressing EGFP or various EGFP-Syt I constructs..57

LIST OF FIGURES

INTRODUCTION

Figure 1: *Aplysia* CNS..13

Figure 2: A dorsal view of *Aplysia* showing the gill-siphon withdrawal reflex and its modifications by experience through simple forms of learning...............................15

Figure. 3: A highly simplified scheme of a fragment of the circuit that subserves the GSW reflex...22

Figure. 4: The current models for synaptic vesicle exocytosis propose that fusion requires assembly and activation of the SNARE complex...36

Figure 5: the effect of the facilitating neurotransmitter serotonin (5-HT) on sensorimotor (SM) synapses in *Aplysia*..46

Figure 6: PKC isoforms..49

II ARTICLE 1

Figure 1: Cloning of a novel spliced isoform of Syt I...54

Figure 2: Localization of Syt I and Syt I_{VQ} in sensory neurons................................55

Figure 3: Syt I and Syt I_{VQ} are phosphorylated at serine 123 by PKC *in vitro*..................55

Figure 4: Syt I_{VQ}blocks the reversal of synaptic depression at a step upstream of PKC activation...56

II ARTICLE 2

Figure 1: Cloning of a novel splice isoform of Syt I..63

Figure 2: RT-PCR of Syt I C2B-a and Syt I C2B-b..64

Figure 3: Chymotrypsin proteolysis of the cytoplasmic domain of Syt I
C2B-a and Syt I C2B-b...64

Figure 4: Calcium dose–response experiments..65

Figure 5: Effect of splicing in C2B on binding to SNAP 25...................................66

Figure 6: Chymotrypsin proteolysis of the C2B domains of Syt I C2B-α
and Syt I C2B-ß...66

Figure 7: SDS–PAGE analysis of various soluble Syt I proteins...............................67

Figure 8: Localization of CFP-tagged Syt I C2B-ß in sensory neurons.......................67

Figure 9: Similar levels of bacterial contamination in the splice isoforms...................68

II ARTICLE 3

Figure 1: Cloning of *Aplysia* synaptosomal-associated protein of 25 KDa (SNAP-25)......73

Figure 2: Localization of SNAp-25 constructs in sensory neurons............................74

Figure 3: Effects of SNAP-25 Ser198 mutations on the rates of depression..................74

Figure 4: Rates of depression..76

Figure 5: Scatterplot of initial EPSPfor all constructs...77

Figure 6: Short-term facilitation of depressed synapses is partly blocked by overexpression of EGFP-SNAP-25 and EGFP-SNAP-25 S-D but not by any of the other mutants.........77

List of abreviations

ACh - Acetylcholine

AC - adenylyl cyclase

ADFP - activity dependent presynaptic facilitation

5-HT - 5-hydroxytryptamine

ANOVA - Analysis of Variance

cAMP- cyclic adenosine monophosphate

ATP - Adenosine 5'-triphosphate

Bis - bisindolylmaleimide

CA1 - Cornu Ammonis 1

CDR - cysteine-rich domain

CREB - cAMP-response element-binding protein

CS - conditioned stimulus

DAG - diacylglycerol

EM - Electron Microscopy

EPSP - excitatory postsynaptic potentials

GSW - gill-and siphon-withdrawal

GWSR - gill-siphon withdrawal reflex

HRP - horseradish peroxidase

Hz - Hertz

IP3 - inisitol 1,4,5 triphosphate

IPSP - inhibitory postsynaptic potentials

KO - Knock out

LTP - Long-term-potentiation

mEPP - miniature end plate potentials

NMJ - neuromuscular junction

PC12 - pheochromocytoma

PdBu - phorbol -12,13-dibutyrate

PE - Phorbolester

PIP_2 - phosphatidylinisitol 1,4,5-bisphosphate

PK - Protein Kinase

PKA - Protein Kinase A

PKC - Protein Kinase C

PLC - phospholipase C

RRP - Readily Releasable Pool

RT-PCR - reverse transcription-polymerase chain reaction

S-A - Serine-Alanine

S-C - Serine-Cysteine

S-D - Serine-Aspartate

S-E - Serine-Glutamate

SCP - small cardiopeptides

Ser - Serine

SM - Sensory-motor

SNARE - soluble NSF attachment receptor

SNAP - 25 - synaptosome-associated protein of 25 kD

SRP - Slow Releasable Pool

Syt - Synaptotagmin

US - unconditioned stimulus

VAMP - Vesicle Associated Membrane Protein

For my parents

Acknowledgements

To my thesis director Dr. Vincent Castellucci first of all for having accepted me in his laboratory, for always being available, even when he hundred administrative task to complete. For his eternal optimism, his endless patience, and for numerous stimulating discussions about science and everything else.

To my codirector, Dr. Wayne Sossin, for having integrated me in his lab and for his infinite knowledge in molecular biology

To Dr. Marc Klein for numerous stimulating discussions.

To all the former labmembers, especially Frederic Manseau, for his help with experimental setup and general advice. For his friendship and support.

To Annie Campbell for her technical assistance

Finally, I am forever indebted to my parents for their understanding, endless patience and encouragement.

I. Introduction

You have to begin to lose your memory, if only in bits and pieces, to realize that memory is what makes our lives. Life without memory is no life at all, just as an intelligence without the possibility of expression is not really an intelligence. Our memory is our coherence, our reason, our feeling, even our action. Without it, we are nothing.
Luis Buñuel (Sacks, 1985)

The question of how we learn, remember, and forget has long been the subject of much speculation. Considering how success and failures of our memory abilities affect our lives, this interest seems highly justified. Diseases that affect memories, like Alzheimer's clearly show that our personality does not survive the destruction of our capacity to form memories.

The brain is an astonishingly complex computational device that constructs our perception of the external world, fixes our attention, and controls our actions. What we perceive in the external world transform itself in our brain in sensations and impressions that constitute our memories, and modifies and reorganizes the ones we already have. They are the foundation of our personality, of our imagination and our creative spirit. Memory is the function in our brain that constitutes a link between what we have been and what we are. We depend on our memories for so much of what we do, such as whenever we are engaged in identifying, appreciating, and responding appropriately to the objects and persons we encounter in our environment and to the events in which we participate; in speaking, reading, writing, or communicating; in thinking, reasoning, and problem solving; and in reminiscing about our experiences. So much do we depend on our memory that we have become mostly unaware of its constant contributions, other than appreciate the pleasant memories that are sometimes triggered by an idle thought or to curse the occasional inability to retrieve some particular name or word on demand.
One key question that has been asked ever since the Antiquity is that of the localization of memories. In the ancient world, the function of the brain was not clear. Among the Greeks

controversy centred on whether the brain or the heart was the home of perception, intellect, emotion, imagination, memory and other functions of the mind. Homer was the first to compare the "heart of the soul" to a block of wax –"We assume that it exist in our soul an "impregnable piece of wax", a gift from Mnemosyne, the mother of all the mythological muses the goddess of memory. Descartes described it as a block of wax that can be engraved and that keeps this impression, which can be retrieved whenever needed for subsequent use (Tadié and Tadié, 1999).

Prior to the 19th century, when Freud and his followers wrestled with the mind, others devised methods to penetrate the brain's substance, the popular concept of memory as a private storehouse of images was acceptable. In fact it was not before 1885, when Herman Ebbinhaus, dissatisfied with treating memory as a philosophical problem described objective techniques for measuring memory (Ebbinhaus, 1913). He learned lists of non-sense syllables and at later times tested his recall by measuring the time necessary for relearning. A few years later, Edward Thorndike described similarly objective methods for measuring memory in cats and other laboratory animals. He placed an animal in a box fitted with an internal release and measured the effect of repeated exposures on the time elapsed before the animal triggered the release and escaped from the box (Thorndike, 1923). In fact, he discovered that memories were not stored in a specific centre where they were stockpiled, but that the entire brain participated in retrieving them. The development of these simple experimental methods for studying learning and memory started a rigorous and empirical school of psychology called behaviorism. James Watson and Burrhus Skinner argued that behavior could be studied with the precision achieved in the physical sciences, but only if students of behavior abandoned speculation about what goes on in the mind and focuses instead on observable aspects of behavior (Milner et al., 1998).

Before the introduction of functional neuroimaging, the study of amnesia was the only practical approach to the investigation of brain substrates of memory in humans. Whereas the application of functional neuroimaging could identify correlations between the activity

of distinct brain regions and the performance on memory tasks, the study of amnesiacs could potentially identify those structures that are obligatory for normal memory. The real hero in this research is the amnesic patient H.M., unfortunately unaware of his profound continual role in modern neuroscience. H.M developed severe memory deficits following an experimental operation (Scoville and Milner, 1957) to alleviate uncontrollable epilepsy. The operation surgically removed his medial temporal lobes, including the hippocampus (Mayford and Kandel, 1999).

1.1 The dichotomy of memory functions

Our brain is able to store different kinds of information and form different kinds of memories that fall into two different categories: implicit and declarative.

Declarative or explicit memory is the conscious recall of knowledge about people, places and things and is particular well developed in the vertebrate brain. This memory involves all our sensory perceptions, our feelings and motivations. When we remember an experience we recall what we have seen, heard, smelled, tasted, touched and felt. Implicit or non-declarative memory on the other hand is the non-conscious recall of motor skills and other tasks and includes simple associative forms, such as classical conditioning, and non-associative forms, such as sensitization and habituation. Whereas explicit memory is mostly studied in mammals, implicit forms of memory can be effectively studied in both non-mammalian vertebrates and invertebrates. To what degree do these two different forms of memory share common molecular components? Specifically, what parts of the nervous system are critical for learning? How is information about a learned event acquired and encoded in neural terms? How is the information stored, and once stored how is it retrieved?

Most neuroscientists believe that the answers to these questions lie in understanding how the properties of individual nerve cells in general, and how synapses in particular are changed when learning occur.

First I will consider the major scientific insights that have helped to identify and delineate signaling and communication in nerve cells and then I will place signaling in the broader context of modern cell and molecular biology.

Finding the underlying cellular basis for memory is like the Holy Grail for many neurobiologists. Of the various higher-cognitive abilities a human being possesses, such as reasoning and language, memory is the only one that can be studied effectively in simple experimental organisms. An average neuron forms and receives about 1000 to 20000 synaptic connections and the human brain contains about 10^{11} neurons. Thus at least 10^{14} synaptic connections are formed in the brain, making it the most complex structure, natural and artificial on earth (Kandel, 2000n). Although nerve cells can be classified into different types that share many common features, a key discovery in the organization of the brain is that nerve cells with basically similar properties are able to produce very different actions because of precise connection with each other. The complexity and relative inaccessibility of the nervous system, particularly in humans and other vertebrates limits the design and execution of effective memory research.

Most nineteenth century anatomists failed to appreciate that the cell body of the neuron, which housed the nucleus almost invariably gave rise to two types of extensions: to dendrites, that serves as input elements for neurons and that receive information from other cells, and to an axon which serves as the output element of the neuron and conveys information to other cells. Appreciation of the full extent of the neuron and its processes came ultimately with the histological studies of Ramón y Cajal. Cajal used silver salts to stain neurons, a specialized method developed by Camillo Golgi that revealed their entire stucture under the microscope. Cajal observed that neurons are in fact discrete cells, bounded by membranes, and suggested that nerve cells communicate with one another only at specialized points of appositions, contacts points that Charles Sherrington later

called synapses (Sherrington, 1897) (see section 1.4) As Cajal continued to examine neurons in almost every region of the nervous system, he showed an uncanny ability to conclude from static images remarkable functional insight into the dynamic properties of neurons. One of his most remarkable insights was the principle of dynamic polarization. According to this principle electrical signaling within neurons is unidirectional i.e., the signal propagate from the receiving pole of the neuron, the dendrites and the cell body, and then along the axon to the output of the pole of the neuron- the presynaptic axon terminal (Albright et al., 2000; Kandel, 2000)

1.1.2 The brain must undergo changes that are maintained as long as the memory is stored. What are these changes?

Because all transformation of neural information in the brain involve only neurons, glia, and their interconnections, neurobiologists since Ramón y Cajal (1911) believed that elementary aspects of learning and memory storage were likely to be resolved on a cellular level. In his lecture to the Royal Society in 1894, Cajal proposed that memory is stored in the growth of new connections. This prescient idea was neglected for almost half a century as students of learning fought over newer competing ideas. First, Lashley and a number of other gestalt psychologists proposed that learning leads to changes in the electric fields or chemical gradients, which they postulated surround neural populations and are produced by aggregate activity of cells recruited by the learning process. At about the same time Lorente de Nó (1938), proposed that memory is stored dynamically by a self-re-exciting chain of neurons (Kandel, 2000n). Finally Holger Hyden demonstrated that learning changes the base composition of nuclear RNA, and thus induces an alteration in gene expression. From these findings, Hyden concluded that establishment of memory is correlated with protein synthesis, and he demonstrated de novo synthesis of several high-molecular protein species after learning (Hertz et al., 2001).

The development of cellular techniques in the late 1940, early 1950s for the study of synaptic connections redefined Cajal's ideas. Intracellular microelectrode recording

methods were introduced to study the synaptic actions of individual neurons and electron microscopy was applied to visualize the fine structure of synapses. These methodological advances allowed central synapses to be studied directly and revived Cajal's idea that memory is stored in the growth of new connections. Prior to the last fourty years, evidence for this type of neuronal growth was restricted to embryonic development and regeneration of peripheral tissues.

1.1.3 What is the neuroanatomical support of our extraordinary capacity to store memories?

The question of where memory is stored emerged at the beginning of the 19th century as part of the larger question- to what degree can any mental processes be localized within the brain? The first person to address this question was Franz Joseph Gall, a German neuroanatomist who argued that the cerebral cortex is not homogenous but contains distinctive centers that control specific mental functions. Gall proposed that particular mental functions are discretely localized and believed that one can determine a mental profile from the shape of the skull. He asserted that the brain does not act as a unitary organ but is divided into at least 27 faculties, each corresponding to a specific mental faculty. He thought that even the most abstract and complex human traits, such as generosity and spirituality were assigned their spot in the brain. This anatomically oriented approach to personality became known as phrenology. Despite Gall's failure of his mental diagnostic methods, neuropsychological analysis of brain damaged patients with highly circumscribed behavioral deficits has been used as evidence for the existence of highly specific cognitive modules (Damasio, 1990; Baynes et al., 1998).

In the middle of the nineteenth century, Pierre Paul Broca, a French clinical neurobiologist, analyzed the behavioral consequences following selective lesions of the brain, much influenced by Gall's idea that functions could be localized. Using this approach he discovered that different regions of the cerebral cortex of the human brain are not functionally equivalent. Lesions to different brain regions produced defects in distinctively

different aspects of cognitive function. Some lesions interfered with the comprehension of language, others with its expression (Schiller, 1992). However, the relevance of the breakdown unveiled by pathology, did not reveal, Gall's clear-cut mental faculties.

1.2 The "engram".

100 years ago a German scholar, Richard Semon, termed the material recorded engraved by a stimulus in living tissues as the "engram" (Semon, 1904). The etymological roots of the term are Greek, and it means, "something converted into writing". Most of the popularity of the term "engram" stems from a paper by Karl Lashley (1950) entitled "In search of the engram". Lashley aimed at identifying "the habits of the conditioned reflex type" in the brain. His methodology was interference of function from dysfunction. He inflicted anatomical lesions on various parts of the rat or monkey brain, and tested the effect of the intervention on brightness discrimination and maze learning (Lashley, 1929; 1950). In spite his failure to locate the site of learned discriminations, his groundwork was influential for future models of memory research. His seminal contribution was not however in his ad hoc experimental conclusions: He reached the conclusion that the engram is widely distributed throughout the brain (Lashley, 1929). Rather, his contribution was in the methodology and the concepts. On the methodological side, he was a pioneer in combining careful lesion techniques with behavioral analysis. On the conceptual side, he found that the severity of the learning defect seemed to depend on the size of the lesion, not on their precise anatomical position, and he concluded that learning and other mental functions have no special locus in the brain and consequently cannot be pinned down to specific collections of neurons. However, the main objections were that his behavior tasks and the behavioral strategies required for successful performance were not well defined in terms of the sensory inputs. A subject with partial brain damage could still succeed in the task by using the undamaged areas, and furthermore, the lesions were not delicate enough to differentiate the contribution of distinct functional division in the brain.

At almost the same time that Lashley's research indicated that there was no memory engram, a Canadian neurosurgeon, Wilder Penfield produced evidence to the contrary. Penfield electrically stimulated the exposed brains of epileptic patients to determine the location of abnormal brain tissue and the functions its removal might endanger. When Penfield probed a region of the temporal lobe, the patients' responses took him by surprise. Stimulation anywhere on the cerebral cortex could bring responses of one kind or another, but he found that when he stimulated the temporal lobes, he could elicit meaningful, integrated responses such as memory, including sound, movement, and color. These memories were much more distinct than usual memory, and were often about things unremembered under ordinary circumstances. Yet, if Penfield stimulated the same area again, the exact same memory popped up. The temporal region he theorized, must be "part of an automatic mechanism which scans the record of the past," linking the cortex to deeper brain structures thought to be involved in memory. "In the vast circuitry of the human brain," Penfield confidently wrote, "the evidence of an engram is clear" (Penfield, 1951).

Later, Lashley's student, Donald Hebb developed his famous theoretical principles of memory formation at a cellular level (Hebb, 1949). At the synaptic level, Hebb coined a postulate of use-dependent synaptic plasticity. In its original version it states the following: "When an axon of cell A is near enough to excite a cell B and repeatedly and persistently takes place in firing it, some growth processes or metabolic changes takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased". In a Hebbian synapse, the increase in synaptic weight is thus a function of correlation of pre- and postsynaptic activity. Hebb proposed that the strength of the connection between the two neurons is increased for a long period of time when the firing of the presynaptic and postsynaptic neurons are closely correlated in time. Subsequently, this synaptic strengthening has been termed associative, because it associates the firing of a postsynaptic neuron with that of a presynaptic neuron. After such an event, when the first of the two neurons is activated, the chance for the postsynaptic neuron firing is increased. Hebb

implied that the synaptic strengthening is input-specific: when two neurons fire coincidentally the synapse between them is strengthen, but other synapses on either neuron remain unchanged (Bailey et al., 2000). Kandel and Tauc showed that synapses could be strengthened or weakened without requirement for an activity of either the pre- or the postsynaptic neurons as a result of the firing of a third, modulatory interneuron. They also suggested that this heterosynaptic modulation could have two forms, associative or non-associative. The associative form, combines features of both homosynaptic and heterosynaptic mechanisms, whereas the non-associative form is purely homosynaptic (Kandel and Tauc, 1965a; Kandel and Tauc, 1965b).

1.2.1 Synaptic Plasticity

Understanding how memories are stored and retrieved has been the goal of work in several scientific disciplines, including neuroscience, neuropsychology, psychology, and cognitive sciences. In neuroscience, research on learning and memory has been conducted largely within the context of plasticity i.e., the malleability of the synapses. Chemical synapses can be modified functionally and anatomically during development and regeneration, and most importantly, through experience and learning. Use dependent neuronal plasticity embodies stimulus induced regulation of intracellular signal transduction cascades, which culminates in either: 1) Functional alterations, typically short term, and involve changes in the effectiveness of existing synaptic connections and requires only covalent modification of pre-existing proteins, or 2) Anatomical alterations, typically long-term that comprise the modulation of gene expression, new protein synthesis, and the establishment of new synaptic connections (Goelet et al., 1986; Bailey et al., 1996). In homosynaptic plasticity, it is the intrinsic activity of the synapse itself that subsequently alters its own functional state. These functional changes can be triggered by biochemical processes localized within either the presynaptic terminal or the postsynaptic cell. In heterosynaptic plasticity, in contrast, a synapse between two neurons is modified by extrinsic action of a third neuron. This modulatory neuron can alter synaptic transmission either by direct synaptic action or

through diffuse release of a transmitter or hormone, to alter the function of the first synapse. An important question has been whether these different types of plasticity involve fundamentally different mechanism, or whether they share an alphabet of basic mechanisms that are combined in different ways.

Synaptic plasticity contributes to a variety of physiological and pathological processes in the adult brain- from learning and memory to amnesia and dementia, from drug dependence to epilepsy (Bliss and Collingridge, 1993; Martin and Kandel, 1996).

Different mechanisms underlie synaptic plasticity at different types of synapses. In theory, the efficacy of synaptic transmission could be modulated at any of the steps that lead to vesicle fusion, release of transmitters and activation of postsynaptic receptors. It is therefore important to thoroughly elucidate the basic mechanisms of synaptic transmission to ultimately understand the meanders of synaptic transmission.

1.3 Vertebrates versus invertebrates

As learning and memory have become accessible to study with the techniques of cellular and molecular biology, a variety of cellular mechanisms of neuronal plasticity have been identified that are thought to contribute to different forms of learning in invertebrates and vertebrates. Now, researchers can attempt to define the basic principles of learning by comparing these mechanisms and asking: what do they have in common, and how are they different?

Although the ultimate goal is to delineate the mechanisms by which memory in our own mind is formed, stored and retrieved, the favorite models are for obvious reasons species that are amenable for experimentation in laboratories. Unlike other mental process such as thought, language, and consciousness, learning is relatively accessible to cellular and molecular analysis.

Interest in the intelligence of simple organisms and their use to investigate problems of learning and memory, draws mainly from two conceptual frameworks: Darwinism (Boakes, 1984) and reductionism. The notion that evolution applies to the mind as well, combined with an interest in zoology and behavior, directed some investigators already a century ago to study the mental powers of organisms such as protozoa and insects (Peckham and Peckham, 1887; Jennings 1906; Day and Bentley, 1911). There are, for example few functional or biochemical differences between the nerve cells and synapses of humans and those of *Aplysia.* Since behavior and learning is an expression of nerve cell activity, it would be surprising if the learning capability of people did not have some elementary features in common with learning in simple organisms. And, if elementary learning are common to all animals with an evolved nervous system, there must be conserved features in the mechanisms of learning at a cellular –and molecular level that can be studied effectively even in simple invertebrates (Krasne, 1978).

1.3.1 The ideal model

The ideal model should have the following qualities: 1) The animal should be able to perform a simple task that can be modified by experience 2) the neuronal network responsible for the task should be characterized in detail. 3) the neurons and synapses in the network where the physiological changes associated with the learning or the modification of behavior take place should be identified, and finally 4) knowing all the important modifications we should be able to reveal the cellular and molecular processes that are involved (Castellucci and Trudeau, 1997).

1.3.2 Aplysia

The possibility of acquisition by repetition of a stimulus is found in all animal species, even the simplest ones: *Aplysia californica* has proven to be an ideal model for this task. The main asset of this marine mollusk is:

1) *Aplysia* has a relatively simple and accessible nervous system. Whereas the mammalian brain has a 10^{11} nerve cells, the central nervous system of *Aplysia* has only 20,000 arranged in widely spaced ganglia (Kandel, 2000n).

2) In *Aplysia*, certain elementary behaviors that can be modified by learning may use fewer than 100 cells. This neural simplicity makes it possible to delineate in detail the wiring diagram of the behavior and thus pinpoint the contribution of each individual nerve cell to the behavior in which they participate (Kandel, 1976).

3) In addition to being few in numbers these cells are the largest nerve cells in the animal kingdom, reaching up to 1000 μm in diameter, as big as the entire brain of *Drosophila* and big enough to be seen with the naked eye. Because of their large size, distinctive pigmentation, location, shape, and firing pattern, it is possible to identify many of the cells as unique individuals. One can record from these cells for hours, and the same cell can be returned to and rerecorded over a period of days. The cells can easily be dissected out for biochemical studies, so that from a single cell one can obtain sufficient mRNA to make a cDNA library.

Finally these cells can readily be injected with labeled compounds or genetic constructs (Kandel, 2000n), and since both sides of the synapses are accessible to substances injected into the cell bodies, it is possible to manipulate presynaptic as well as postsynaptic mechanisms (Jin and Hawkins, 2003).

The *Aplysia* have attracted several cellular physiologists (Arvanitaki and Chalazonitis, 1958; Tauc and Gershenfeld, 1961; Kandel and Tauc, 1965). It is however, the research on plasticity and learning that is *Aplysia*, especially *Aplysia californica*'s claim to fame. It gained immortal fame from studies in the laboratory of Eric Kandel. He was a pioneer and has continued to be a major driving force in *Aplysia* research. In 2000 he shared the Nobel

Prize in medicine for 40 years of research in the cellular processes underlying learning and memory, first in *Aplysia*, and recently in mice.

Aplysia californica, a hermaphroditic marine snail with mottled purple skin, is found in shallow ocean waters, have small repertoire of stereotyped behaviors that includes feeding, egg-laying, and a variety of protective mechanisms. These responses include withdrawal of the tail, siphon and gill, and the release of defensive secretions such as ink and opaline (Walters et al., 1983)

The central nervous system consists of eight paired ganglia arranged around the eosophages, and a large fused ganglion in the abdomen.

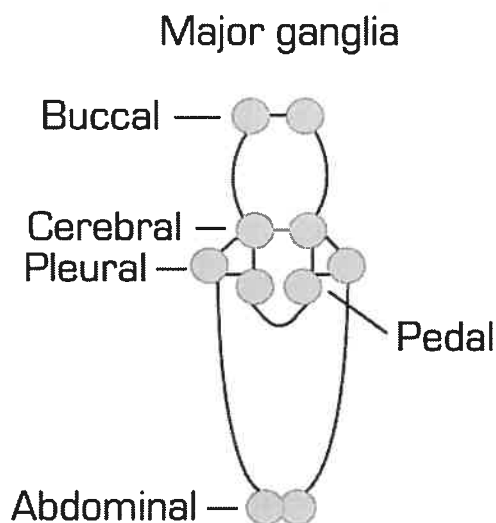


Fig. 1. *Aplysia* nerve cells are clustered together in five major bilateral pairs of ganglia, each containing about 2000 nerve cells.

Following a series of reductive and simplifying steps, the cellular and molecular mechanisms of learning in *Aplysia* has been pursued from the behaving animal via

preparations of isolated ganglia, to identified nerve cells and synapses in culture (Carew et al, 1971; Castellucci et al, 1970; Rayport and Schacher, 1986).

Not all synapses are equally adaptable. The strength of some synapses in *Aplysia* rarely changes, even with repeated activation. However, with synapses specially involved in learning and memory storage, such as the synaptic connections between the sensory neurons and their follower neurons in the gill-siphon -and tail withdrawal reflexes, a relatively small amount of training, especially if it is appropriately spaced, can produce large and long-lasting changes in synaptic strength. The study of these systems in *Aplysia* has provided an exceptionally favorable opportunity for relating synaptic changes in specific cells to behavior, because the monosynaptic pathway in the gill-and siphon-withdrawal (GSW) reflexes and the related pathway activated by tail stimuli belong to a reflex system that can be studied on the cellular level (Byrne et al., 1993; Hawkins et al., 1993).

The analysis of learning in *Aplysia* has focused mainly on the defensive reflexes, illustrated primarily by the gill-and siphon-withdrawal (GSW) reflex. The GSW is a very simple defensive reflex: the withdrawal of the gill upon stimulation of the siphon, an action that is like the quick withdrawal of a hand from a hot object (Pinsker et al., 1970). This reflex is advantageous because the neuronal circuit is partly monosynaptic, and it has been shown to undergo several simple forms of learning (Murphy and Glanzman, 1997).

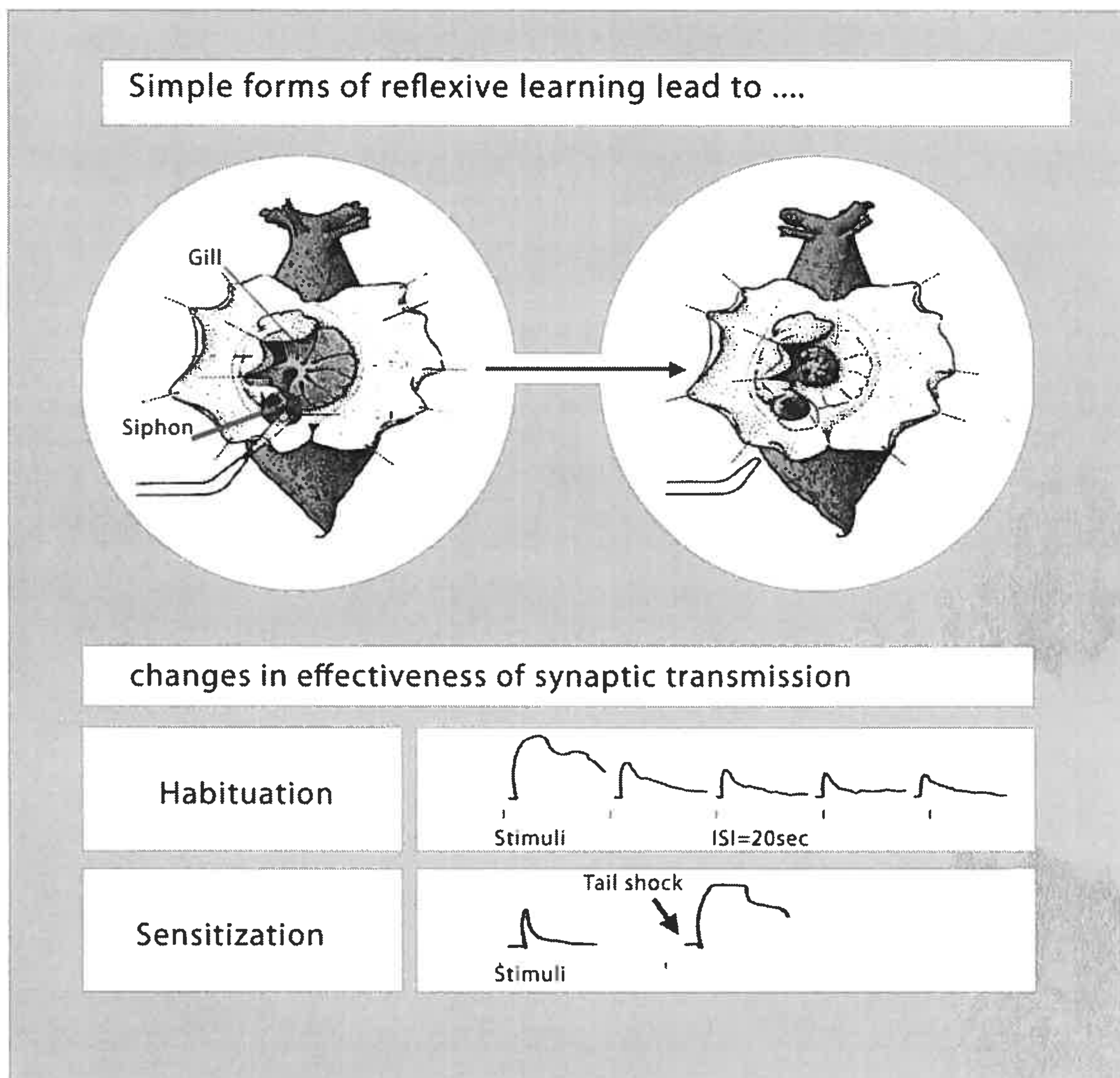


Fig. 2. A dorsal view of *Aplysia,* showing the gill-siphon withdrawal (GSW) reflex. The gill lies in the mantle cavity and is normally covered partially by the mantle shelf. A light touch to the siphon causes the siphon to contract and the gill to withdraw into the mantle cavity under the protection of the mantle shelf. Repetitive monotonous tactile stimuli result in habituation. A shock to the tail results in sensitization of the reflex.

In *Aplysia*, the gill is the external respiratory organ that is housed in the mantle cavity. The cavity is a respiratory chamber covered by the mantle shelf. When the animal is in a normal

relaxed state, the gill is partially covered by a sheet of skin (the mantle shelf), which ends in a fleshy spout, called the siphon. If a tactile stimulus is applied to the siphon, both the siphon and the gill are drawn into the mantle cavity. The GSW reflex can be habituated by repetitive monotonous tactile stimuli to the skin; sensitized by noxious or strong tactile stimuli to the tail or head; and it can also undergo classical conditioning.

The circuitry underlying the *Aplysia* siphon-elicited siphon-withdrawal reflex has been widely used to study the cellular substrates of simple forms of learning and memory. Although the basic form of the behavior is quite simple, the underlying circuitry is rather complex, consisting of monosynaptic and polysynaptic pathways, excitatory and inhibitory interneurons (Frost and Kandel, 1995). A groundbreaking finding that allowed biochemical and molecular investigation of this memory at the single cell level was provided by the work of Montarolo et al. (1986). These investigators showed that it is possible to reconstitute in vitro the main components of the neural circuit underlying sensitization and to reproduce synaptic responses *in vivo* by learning. The parallel use of both *in vitro* and *in vivo* models and the work of several groups in the last 20 years led to an understanding of the biological processes underlying sensitization of the GSW-reflex (Bailey et al., 1996; Byrne and Kandel, 1996, Carew, 1996). For the studies described in this thesis, we used the pleural ventrocaudal (VC) sensory neuron cluster of the pleural ganglion and the LFS siphon motor neurons of the abdominal ganglion reconstituted *in vitro* (Frost et al,, 1997; Walters and Cohen, 1997). These cells in particular, together with the gill motorneuron L7 have been widely used to study the cellular substrates of simple forms of learning and memory. The bilateral VC clusters are notable for their uniform appearance and the tight packing of their cells (Walters et al,, 1983) The VC cluster is comprised of mechanoafferent neurons that innervate a large part of the body surface of the animal. *In situ*, the LFS motor neurons receive monosynaptic afferents from the LE sensory neurons in the abdominal ganglion. The *Aplysia* siphon-elicited siphon-withdrawal reflex circuit consists of the LFS motor neurons and 10 interneurons (5 L29s, 3 L30s, 2 L34s) known to

convey excitatory input to them (Frost et al,, 1997). The full circuit contains three other central siphon motor neuron groups (e.g., LBS, LDS, RDS) (Frost and Kandel, 1995). *Aplysia* sensory-motor neuron synapses in isolated cell culture exhibit both short and long-lasting forms of plasticity characteristic of the intact animal (Rayport and Schacher, 1986; Montarolo et al., 1986; Eliot et al., 1994a,b; Lin and Glanzman, 1997; Bao et al., 1997; 1998). In addition, they have a number of different advantages for examining the mechanisms of plasticity. 1) The neurons are identified as individuals with known behavioral functions. 2) There are no other neurons in the dish and they do not form autapses. 3) One can unambiguously distinguish between homosynaptic and heterosynaptic effects and also know the source of spontaneous miniature EPSP with certainty (Jin and Hawkins, 2003). The neuronal networks causally related to these reflexes include two main components, a monosynaptic one, the sensory-to-motor neurone synaptic junctions, and a polysynaptic one made up of various excitatory and inhibitory interneuronal synapses (Frost et al., 1988; Trudeau and Castellucci, 1992; White et al., 1993). There are many sites that can be modified when the reflex is facilitated or depressed (Fischer and Carew, 1995; Trudeau and Castellucci, 1993a; Xu et al., 1995). Like many forms of learning-related synaptic plasticity, facilitation at *Aplysia* sensory-motor neuron synapses can involve different molecular mechanisms depending on experimental variables such as the history of activity and the duration of 5HT exposure (Byrne and Kandel, 1996).

The transmitter at the sensory to motor junction is most likely glutamate (Trudeau and Castellucci, 1993; Lechner and Byrne, 1998; Storozhuk and Castellucci, 1999; Levenson et al, 2000; Cohen et al., 2003; Antzoulatos and Byrne, 2004). The sensitizing or facilitating stimulation activates several types of facilitator interneurons, some of which have been identified (Hawkins et al., 1981; Goelet et al., 1986; Hawkins and Schacher, 1989; Trudeau and Castellucci, 1993; Liu et al., 2004;). These neurons use at least four different transmitters: 5-hydroxy-tryptamine (serotonin or 5-HT), the small cardiopeptides SCP_A and SCP_B and a fourth one presently unidentified (Abrams et al., 1984; Ghirardi et al., 1995).

Most of the work on synaptic facilitation has focused on the action of 5-HT. Serotonin induces changes in ionic conductances leading to spike broadening and enhancement of excitability in the sensory neurons and in some motor neurons. Serotonin leads to an increase in synaptic release in two ways, one that is dependent on spike broadening and the other that is independent of spike broadening. These two facilitatory actions of 5-HT are mediated by at least two second messenger activated protein kinase systems, protein kinase A (PKA) and protein kinase C (PKC). The two biochemical cascades overlap in their contributions to synaptic facilitation; their contributions are not simply synergistic but are state- and time-dependent (Burrell and Sahley 2001; Kandel, 2001; Leal et al., 2005). Recent observations by Glanzman and colleagues (Li et al., 2005) have indicated that facilitation of the sensori-motor synapses during and after 5-HT exposure depends also on a rise in postsynaptic intracellular calcium and release of calcium from postsynaptic stores, which then signals back to the presynaptic terminal by some unknown mechanism. In cell culture, application of 5-HT can be substituted for a harmful modulatory stimulus. A single application of 5-HT produces short-term facilitation, same as does single tail shock, whereas four more repeated applications of 5-HT produce long-term synaptic facilitation (Montarolo et al., 1986 from Castellucci et al., 1988). Blocking the action of these serotonergic cells blocks the effect of sensitizing stimuli (Glanzman et al., 1989).

The sensory to motor connection can undergo homosynaptic depression (Armitage and Siegelbaum, 1998; Byrne and Kandel, 1996), homosynaptic facilitation (Bao et al., 1997; Li et al., 2005; Lin and Glanzman, 1994b; Muller and Carew 1998) and heterosynaptic depression involving FMRFamide and dopamine (Abrams et al., 1984; Montarolo et al., 1986). There are also unidentified sensory neurons, probably at the periphery that can contribute to the reflex; they have lower threshold of activation than those of the other groups but their modulation seems to be similar (Frost et al., 1988). Anatomical studies conducted by Bailey and Chen (Bailey and Chen, 1988) indicated that long-term memory (lasting several weeks) is accompanied by a plethora of alterations at identified sensory neuron synapses. Their results indicate that the size of the active zones and the total number

of varicosities are larger in sensitized animals compared to controls and smaller in habituated animals. Castellucci and Kandel attempted to localize the change produced by habituation and sensitization to either the presynaptic or postsynaptic component of the synapse. They applied a quantal analysis to the synaptic connections between the sensory and motor cells, and found that the short-term homosynaptic depression that accompanies habituation involves a reduction in the amount of transmitter released from the presynaptic neuron (Castellucci and Kandel, 1974).

The *Aplysia* GSW reflex can be modified by four simple forms of learning paradigms: 1) habituation, 2) sensitization, 3) dishabituation, and 4) classical conditioning

1.3.2.1 Habituation is the simplest form of implicit learning, where the animal show a progressive decrease in reflex strength with repeated innocuous stimulation. If the stimulus is neither beneficial nor harmful, the animal learns after repeated exposure, to ignore it. Habituation prevents recurrent non-threatening environmental stimuli from endlessly distracting the animal from potentially meaningful stimuli of behavioral significance. Habituation is caused by a homosynaptic depression in the activity of a sensory-motor connection (Castellucci et al., 1970, 1974) and is associated with depression of the synapses from sensory neurons onto gill and siphon or tail motor neurons produced by a reduction in transmitter release from the presynaptic terminals following each action potential. Habituation can last from a few minutes to up to several weeks if repeated training sessions are administrated over several days (Carew et al., 1973). Release of neurotransmitter typically occurs at axonal swellings, termed varicosities, in contact with the processes of a postsynaptic neuron. Anatomical studies show that the number of varicosities (Bailey and Chen, 1988) as well as the number and size of the vesicle pool in the active zone are significantly decreased in long-term habituated synapses (Bailey and Chen, 1983), indicating that morphological changes accompany the changes in behavior. At *Aplysia* synapses, short-term depression does not appear to involve a depletion of releasable

vesicles or a decrease in presynaptic Ca^{2+} influx, but may involve an inactivation of the release process itself (Eliot et al., 1994b; Armitage and Siegelbaum, 1998). However, Bailey and Chen (1988) and study by the group of Marc Klein (2002) suggest depletion as a possible mechanism. Although considered the simplest of all types of learning, the cellular and molecular analysis of habituation in *Aplysia* has so far yielded less elaborate mechanistic models than the analysis of sensitization or classical conditioning.

1.3.2.2 Sensitization is the enhancement of reflex responses. When a harmful stimulus is applied to the neck or tail of *Aplysia*, facilitating neurons are activated that in turn act on the sensory neurons to enhance transmitter release (Castellucci and Kandel, 1976). Defensive reflexes for withdrawal and escape becomes amplified, the animal learns to respond more vigorously, not only to that stimulus but also to other stimuli, even harmless ones. The phylogenic value of sensitization is rather straightforward: An *Aplysia* that has just avoided being eaten by a lobster that has pinched its tail, is indeed well advised to respond with a lower threshold to a similar stimulus, because it is almost certain that the lobster will attack again. During short-term sensitization or dishabituation of the withdrawal reflex, the monosynaptic connections between sensory neurons and motor neurons are enhanced by heterosynaptic facilitation (Byrne and Kandel, 1996). Facilitation or dishabituation is the enhancement of a habituated reflex response by a noxious stimulus, whereas sensitization is an enhancement of a non-habituated reflex response by that stimulus (Carew et al., 1971).

1.3.2.3 Dishabituation has been thought to be due either to reversal of the process of habituation or to a second process equivalent to sensitization superimposed on habituation (Hawkins et al., 2006). Hochner and colleagues (1986 a, b) suggested that dishabituation and sensitization in adult *Aplysia* are produced, at least in part, by different cellular mechanisms. This reversal of synaptic depression parallels the behavioral process of dishabituation. They also proposed that this facilitatory mechanism might represent a direct

modulation of transmitter release, either an increase in the availability of transmitters for release, or a modulation of the release mechanism itself. In contrast to a unitary process view, dishabituation and sensitization emerge as separate behavioral processes according to very different developmental timetables in *Aplysia*. The magnitude of dishabituation appears to be determined by the interaction of 3 underlying processes: (1) the dishabituation process itself, (2) an inhibitory process that competes with dishabituation and, (3) a facilitatory process (sensitization) that augments dishabituation (Rankin and Carew, 1988). At depressed *Aplysia* sensory to motor synapses, 5-HT facilitates transmitter release by dishabituating the synapse primarily through activation of a Ca^{2+}-independent form of PKC, PKC Apl II (Ghirardi et al., 1992; Manseau et al., 2001). While activation of PKC by 5-HT increases transmitter release only at depressed synapses, activation of PKC by phorbol esters (PE) increases transmitter release at both naïve and depressed synapses (Braha et al., 1990; Ghirardi et al., 1992; Nakhost et al., 2003). At naïve synapses 5-HT facilitates transmitter release primarily through a protein kinase A (PKA) pathway. In the prescence of a PE, the Ca^{2+}-dependent PKC, Apl I is also recruited. It is also possible that the initial increase in synaptic strength caused by activation of PKA may make further modifications induced by 5-HT irrelevant. However, recently Jin et al. (2005) showed that PKC-mediated increases in synaptic strength at naïve synapses could be activated through prolonged applications of 5-HT.

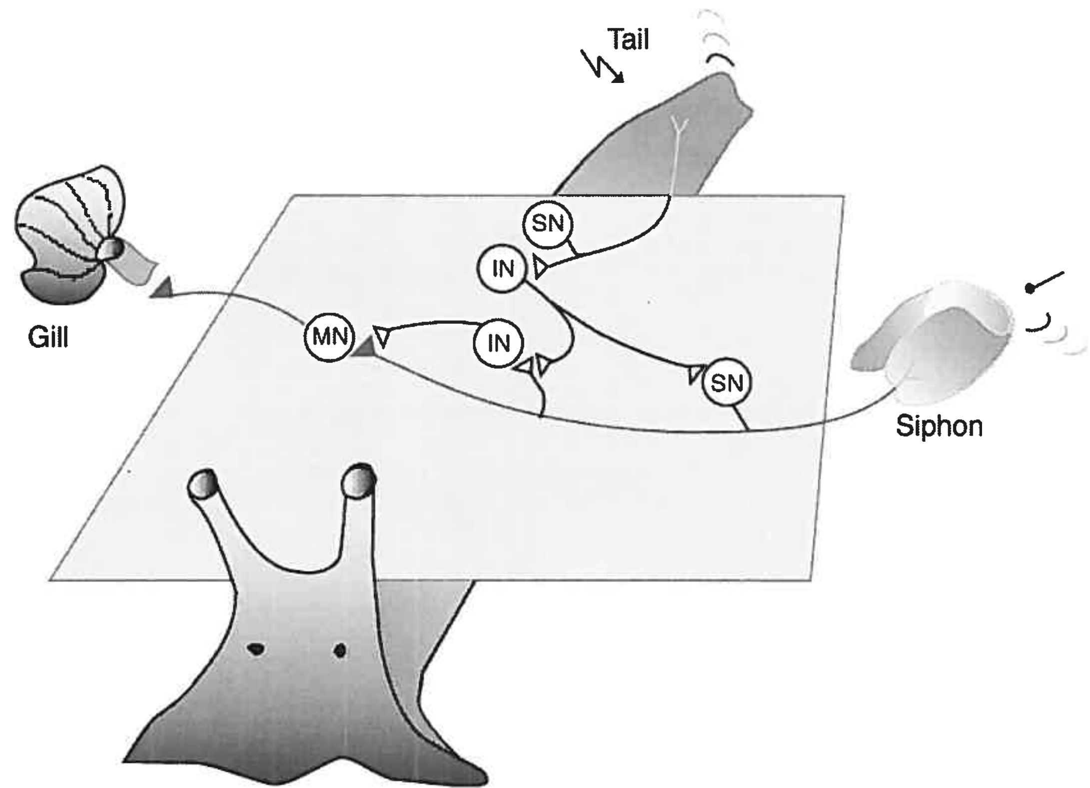


Fig. 3. A highly simplified scheme of a fragment of the circuit that subserves the GSW reflex. The neuronal networks causally related to these reflexes include two main components, a monosynaptic one (marked in red), the sensory-to-motor neurone synaptic junctions, and a polysynaptic one made up of various excitatory and inhibitory interneuronal synapses.

1.3.2.4 Classical conditioning is a more complex form of learning than sensitization. Rather than learning only about one stimulus, the organism learns to associate one type of stimulus with another. This form of learning, originally described in dogs by the Russian physiologist and psychologist Ivan Pavlov (Pavlov, 1927), occurs when a behaviorally neutral stimulus, usually a light tone or a tactile stimulus (the conditioned stimulus or CS) is presented to an animal together with a reinforcing stimulus (the unconditioned stimulus

or US). Classical conditioning has been portrayed as sharing cellular mechanisms with sensitization, however, in contrast to sensitization, which enhances the responses of subsequent stimulations of the skin at any location; the facilitation in classical conditioning is specific to the pathway that has mediated the conditioned input. The GSW-reflex of *Aplysia*, can be enhanced by classical conditioning, however, the timing of the CS and US is critical. It depends on close forward pairing, (about 0.5 s) of sensory neuron activity, the CS (siphon touch) and the excitatory interneuron activity, the US, (a strong shock to the tail). The sequential activation of the sensory neuron during a critical interval by the CS and the US leads to greater presynaptic facilitation than when the two stimuli are not accurately paired. Classical conditioning in *Aplysia* was first described by Kandel and colleagues in 1981 (Carew et al., 1981). This form of invertebrate learning was originally hypothesized to be due to an exclusively presynaptic mechanism, known as activity dependent presynaptic facilitation (ADFP). A 1984 study (Carew et al. 1984) where postsynaptic hyperpolarization during the US did not block the associative enhancement of EPSP, concluded that ADFP does not involve a Hebbian mechanism. A decade later, the discovery that sensory-motor synapses of *Aplysia* possessed the capacity for NMDA receptor dependent LTP (Lin and Glanzman, 1997; Lin and Glanzman, 1994a; Lin and Glanzman, 1994b) indicated that classical conditioning also depends in part, on Hebbian LTP (Glanzman, 1995).

1.3.3 Drosophila

The fruitfly, *Drosophila melanogaster* has long been an organism of choice for molecular geneticists. A tiny creature, 3mm in length, equipped with complex body structures, including a brain of 250,000 neurons. *Drosophilas* are conveniently small, and remarkably inexpensive. Their generation time is about 10 days at room temperature, and their life cycle includes easily identifiable phases (Ashburner, 1989). The small number of chromosomes, the convenient chromosomal cytology, the availability of spontaneous mutants, the short generation time, and ease of breeding, initiated a meticulous, systematic

analysis of *Drosophila* genetics. The fact that flies can learn to remember a variety of associative tasks makes *Drosophila* an excellent system to characterize genes involved in learning and memory (Davis, 1993). Studies of learning in flies frequently use a classical conditioning paradigm that involves the temporal coupling of an electric shock with a particular odor; flies learn to avoid the shock-associated odor after training. Behavioral screening of mutagenized flies using such learning paradigms have led to the isolation of genes involved in learning and memory. Over the years, "learning mutants" of *Drosophila* have contributed significantly to our current knowledge about the molecular mechanisms of acquisition and consolidation of simple memory. It has created remarkable evidence in support of the cAMP signal transduction cascade and of the cAMP-response element-binding protein (CREB), a type of protein that regulates the expression of genes, and plays a key role in neuronal plasticity.

In *Drosophila* synaptic transmission is usually analyzed at the neuromuscular junction (NMJ) during the embryonic, third instar larval, and adult stages of development. Although this methodology does not involve interneuronal connections, the basic release mechanisms is unlikely to be substantially different, as the molecular component of these two glutamatergic synapses are highly conserved (Littleton and Bellen, 1995). In the *Drosophila* NMJ identified motor neurons innervate specific muscle targets in a highly stereotypic manner, allowing consistent and reproducible analysis of synaptic parameters between animals (Yoshihara and Montana, 2004). Yet *Drosophila*, in spite of offering unique advantages to the geneticist, is not the dream machine of the neurophysiologist, central neurons in the fruit fly are not easily available to electrode recordings in the same way as central mammalian neurons. Furthermore, being invertebrate, *Drosophila* is incapable of providing clues of the mammalian brain at the circuit and system level.

In recent years, some of the enthusiasm for using simple organisms to analyze the neurological bases of behavior has declined, because state of art molecular biology can now be used to approach problems in higher organisms that previously were only approachable

in lower ones. Powerful neurogenetics, for example, is already practiced in mice, depriving *Drosophila* of its monopoly in the neurogenetical analysis of memory.

1.3.4 Mouse

Mus musculus, the common mouse is a pest for householders, a pet for animal lovers, and a blessing for molecular biologists. Mice are the mammalian counterparts of *Drosophila* for learning and behavioral studies. Although they lack *Drosophila* advantages for genetics studies, the ability to generate mice with specific gene "knock outs", and possibility of assessing the effect of the targeted mutations in classical behavioral tests, made mice attractive animals for memory studies. Unlike *Drosophila*, where specific genes required for memory can be identified in specific screens, studies in mice are somewhat limited to guessing the players. With mice, it is now possible to add engineered genes to the mouse genome, or remove other genes at will, and to generate mouse lines that will express the mutation and propagate it to their progeny. Knock out (KO) mice, where a gene is ablated *in situ*, have been used to identify the roles of a variety of protein kinases, of subtypes of glutamate receptors and of a variety of transcription factors involved in long-term potentiation (LTP). They have also proved useful in particular for probing the relations between LTP and learning, and the role of hippocampus in learning and memory (Grant et al., 1992; Mayford et al., 1996; Tsien et al., 1996a). Furthermore, novel techniques now permit the generation of tissue, -cell-type, -and temporarily restricted gene knockouts (Tsien et al., 1996b; Shimizu et al., 2000). These conditional mice techniques offer considerable advantages for the study of learning and memory, because they could be used to dissociate the effect of a mutation on development from those on behavioral plasticity, and furthermore, localize the defect to specific brain regions and circuits. In parallel with the aforementioned trend, the use of brain slices (e.g. Dobrunz and Stevens, 1999) and of neuronal cell cultures (e.g. Tardin et al., 2003; Jaskolski and Mulle, 2004; Abel et al., 1997) from complex nervous system has gained much popularity, because such simplified preparations permit exploitation of highly advanced molecular and computational

techniques e.g. the investigation of LTP in the hippocampus and fear-conditioning in the amygdala. Among mammals the mouse is still unique in this respect; appropriate neurogenetic techniques are not yet available, for example in rats. In addition, they are small (20-35g), the size of their brain is manageable, and the generation time is 3-4 months.

1.4 The synapse

The neurons in our brain communicate with one another through specialized structures called synapses. The term synapse (Greek for syn-haptein "to make contact") is commonly attributed to Sherrington. Sherrington was a neurophysiologist who believed that nerves terminate in free endings and that the transfer of information from these endings to their targets differs markedly from the propagation of information along neuronal branches. When requested to revise his contribution to a textbook of physiology (Foster and Sherrington, 1897), he reasoned that since the research on this functional junction between nerve cells had already matured to become an important topic in physiology, this type of junction deserved a special term. Hence, the synapse was born (Sherrington, 1941).
Synapses come in many flavors. They can be classified by their morphology, location, function (e.g. inhibitory vs. excitatory), types of neurotransmitters and their receptors. A major taxonomy distinguishes chemical from electrical synapses. John Eccles, (Sherington's student), believed all synaptic transmission was electrical, that the action potential in the presynaptic neuron generates a current that flows passively into the postsynaptic cell. He resisted initially the idea of a chemical transmission proposed by Henry Dale and his followers, but later he became a major proponent of it (Kandel, 2000). It is now accepted that although most synapses use a chemical transmitter, some operate purely by electrical means.

Neurobiologists now accept the existence of two major modes of synaptic transmission: electrical, which depends on current, through gap-junctions that bridge the cytoplasm of pre- and postsynaptic cells. Electrical transmission plays a role in synchronizing neural

activity. In chemical transmission, pre- and postsynaptic cells have no structural continuity they are separated by a discrete extracellular space, the synaptic cleft (Bennett, 2000).
The most common transmitters of the CNS are glutamate, GABA, dopamine and serotonin. Eccles and his collaborators (Eccles, 1963) showed that synaptic communication in the CNS, as in the periphery, is mediated by ionic currents that flow across the postsynaptic membrane, generating excitatory or inhibitory postsynaptic potentials (EPSPs and IPSPs). Depending on which ions carry the postsynaptic currents, the respective transmitters are classified as excitatory or inhibitory.

1.4.1 Signaling within neurons: the action potential

Nerve cells are able to carry signals over long distances because of their ability to generate an action potential. An action potential generates a local flow of current that is sufficient to depolarize the adjacent region of the axonal membrane and is propagated without failure along the axon to the nerve terminal. In 1939 while recording from the giant axon of the squid Kenneth Cole and Howard Curtis found that the ionic conductance across the membrane increases dramatically during the action potential, suggesting that the action potential reflects the flow of the ionic current. Hodgkin, Huxley and Katz extended these observations in a series of papers in the early 1950s. They found that the amplitude of the action potential is reduced when external Na^+ concentration is lowered, indicating that Na^+ influx is responsible for the rising phase of the action potential (Hodgkin and Katz, 1949). Their data also suggested that the falling phase of the action potential was caused by a later increase in K^+ permeability (Hodgkin et al., 1952).

1.4.2 Synaptic transmission

Synaptic transmission is initiated when an action potential triggers neurotransmitter release from a presynaptic nerve terminal (Katz, 1969) and is determined by the amount of transmitter release from the presynaptic neurons and by the transduction of the chemical signal into an electrical response by the target cell. An action potential induces the opening

of Ca^{2+}-channels and the resulting Ca^{2+} transient stimulates synaptic vesicle exocytosis. Transmitter release depends on the size of the readily releasable pool (RRP) of transmitter (Rosemund and Stevens, 1996; Gillis et al., 1996), thought to represent the release-ready synaptic vesicles docked at the active zone (Schikorski and Stevens, 2001), and the efficacy of the release process.

Synaptic transmission includes a chemical step, where the signaling substance, called a transmitter, is released very locally from the sending, presynaptic cell and then acts transiently on receptors of the receiving, postsynaptic cell. The receptor is part of an ion channel and mediates, upon occupation by the transmitter, a brief flux of ions across the postsynaptic membrane generating a change in the postsynaptic membrane potential (Sakmann, 1991).

The signal that actually initiates the cellular response of the postsynaptic cell is the flux of ions across the postsynaptic membrane. The size, duration and direction of this ion flux, as well as the nature of the ions traversing the postsynaptic membrane, determines whether this response will either activate voltage sensitive membrane conductance and initiate action potentials, or instead reduce the cells electrical activity. The cellular response may also be determined by a change in intracellular ion concentrations, in particular the concentration of calcium ions, which act as a second messenger for many cellular responses. Receptors can also gate ion channels indirectly. These receptors often referred to as metabotropic receptors, produce slow synaptic responses, which persist for seconds or minutes. They are coupled via a detachable transducer, called G-protein and act by altering intracellular metabolic reactions. Activation of these receptors stimulates the production of second messengers, small freely diffusible intracellular metabolites such as cAMP and diacylglycerol (DAG). Many such second messengers activate protein kinases (PKs), an ubiquitous type of enzyme that modifies proteins and regulates their function by catalyzing the addition of a phosphate group. Some of the modified proteins are other enzymes, others are signaling and regulatory molecules, still others translocate to the nucleus and modify transcriptional regulatory proteins, in this way controlling gene expression. Second

messengers can covalently modify preexisting proteins as well as regulating the synthesis of new proteins. Whereas fast synaptic transmission is critical for routine behavior, slow synaptic transmission is often modulatory and act upon neural circuit to regulate the intensity, form, and duration of a given behavior (Kandel et al., 2000).

1.4.3 The "calcium hypothesis".

The classical calcium hypothesis describes the quantitative relationship between release of neurotransmitter and extracellular Ca^{2+}-concentration (Katz and Miledi, 1965). This hypothesis emerged from a series of experiments of the frog neuromuscular junction and the squid giant synapse. During the 1960s, Katz, together with Miledi undertook a series of elegant studies using a focal calcium pipette to demonstrate that in the absence of extracellular calcium, a nerve impulse still enters the nerve terminal but fails to trigger the release of transmitters. His work provided some of the earliest definitive evidence for the idea that calcium entry into the terminal was required for neurotransmitter release.

According to the calcium hypothesis, a nerve impulse causes calcium ions to enter the presynaptic terminal where they bind a molecular calcium sensor that cooperatively (Dodge and Rahamimoff, 1967) control vesicular release and causes an increase in the probability of release p so that vesicle fusion occurs. Dodge and Rahamimoff first described the phenomenon of calcium cooperativity of synaptic transmission for frog neuromuscular junctions, reporting that the amplitude of excitatory junctional potentials is related to the fourth power of extracellular Ca^{2+}.

1.4.4 The "quantal hypothesis".

Katz discovered that neurotransmitters, such as acetylcholine (ACh), are released not as a single molecule, but as multimolecular packets called quanta. At the neuromuscular junction each quantum comprises about 5000 molecules of transmitter (Fatt and Katz, 1951; Castillo and Katz, 1954). Each quantum of ACh is packaged in a single small

organelle, the synaptic vesicle, and is released by exocytosis at specialized release sites within the presynaptic terminal called the active zone.

Together with Paul Fatt, Katz discovered that in the absence of any form of stimulation, the end plate region of the muscle fiber is not completely at rest, but displays electric activity in the form of discrete, randomly recurring miniature end plate potentials (mEPP), each in the order of about 0,5 mV. What accounts for the fixed size of mEPPs? In 1954 Castillo and Katz discovered the unitary nature of mEPP arises from the synchronous impact of current through about 2000 acetylcholine (Ach) receptor-channels, where each ACh receptor channel is responsible for only about 0.3 μV. Thus, synaptic potentials seem smoothly graded in recordings only because each quantal potential is small relative to the total potential (Katz, 1970; Kandel, 2000).

1.4.4.1 What morphological feature might account for the quantum of transmitter?

Castillo and Katz postulated that synaptic vesicles discharge their contents by fusing with the presynaptic membrane in a process known as exocytosis (Castillo and Katz, 1954). Direct evidence that exocytosis of a single synaptic vesicle is responsible for the release of one quantum of transmitter was difficult to obtain, because the chance of finding a vesicle in the act of being discharged. Moreover, there was wide speculation on how axon endings could sustain high rates of secretion for prolonged periods of time without exhausting their supply of synaptic vesicles. Considering the great distance of nerve terminals from perikarya in the majority of neurons, some form of local membrane recycling appeared to be necessary (Gray and Willis, 1970). Conclusive evidence for recycling was provided in the early 1970s when two groups used electron microscopy to examine how stimulation altered the ultrastructure of the frog neuromuscular junction (NMJ). Thomas Heuser, one of Bernard Katz' postdoctoral fellows, attempted to capture a picture of neurotransmitter "quanta" being released. The only approach available to him at the time was to stimulate the nerve intensively and subsequently throw it into a fixative. Fellow scientists who saw the images said the overstimulated nerves were "just destroyed," and "not relevant to anything."

Heuser had a suspicion however, that the internal structures were not just signs of degradation, but instead were products of endocytosis.

Together with Tom Reese at the NIH he decided to investigate his idea. He stimulated the frog NMJ at 10 Hz for 1 min, in the presence of an enzyme used as an EM marker, horseradish peroxidase (HRP), which catalyzes the formation of an electron dense reaction product in the tissue after it is soaked in the precursor (diaminobenzidine). The tissue was rapidly fixed after stimulation (within msecs). They found HRP reaction product in clathrin-coated vesicles near the membrane. If they fixed slightly later, they saw reaction product in synaptic vesicles. They could then deplete the vesicles of HRP when they restimulated in the absence of HRP. From these and many similar experiments, it has been established that vesicle membrane is retrieved from the plasma membrane and recycled into new synaptic vesicles. Retrieved vesicle membrane is recycled through clathrin-coated vesicles into endosomes near the terminal, then sorted and budded off again from membrane cisternae to form new synaptic vesicles (Powell, 2005; Heuser and Reese, 1973). In contrast, Ceccarelli et al. (1973) observed little change in the ultrastructure of the terminal following stimulation at lower frequency (2 Hz for up to 4h). They reached an altogether different conclusion: that vesicles reformed directly from the plasma membrane at the site of their release (Ceccarelli et al., 1973). This laid the groundwork for what would be called the "kiss-and-run" hypothesis: that synaptic vesicles could deliver their cargo by fusing slightly with the membrane and then reform by pinching back off (Fesce et al., 1994). This model has attracted new interest with the demonstration that peptide-containing secretory granules can indeed secrete part of their content through a transient fusion pore (Chow et al., 1992; Cochilla et al., 1999; Fesce and Meldolesi, 1999) as well as cathecholamine containing PC12 cells (Wang et al., 2003).

These different views led to a decade of competition between the two groups. For synaptic vesicle exocytosis, the question of kiss-and-run versus full fusion plus recycling is still very much up in the air. Some researchers now believe that both forms of exocytosis occur, but

that cells only use kiss-and-run when vesicles are in short supply (Wightman and Haynes, 2004), others report that calcium promotes kiss-and-run (Klingauf et al., 1998; Neves et al., 2001; Ales et al., 1999; Cousin and Robinson, 2000), whereas others find no effect (Stevens and Williams, 2000), or calcium provoked inhibition (Hsu and Jackson, 1996). The Heuser and Reese experiments support a model in which synaptic vesicle recycling is mediated by the formation of coated vesicles, is relatively slow, and occurs distally from active zones. Because heavy levels of stimulation were needed to visualize the coated vesicles, Ceccarelli's experiments argued that synaptic vesicle recycling does not require the formation of coated vesicles. On the contrary, he claimed: it is relatively fast, and occurs directly at the active zone in a "kiss-and-run" reversal of exocytosis under more physiological conditions. For the next thirty years, these models have provided the foundation for studies of the rates, locations, and molecular elements involved in synaptic vesicle endocytosis.

1.5 The molecular biology of vesicle fusion and release.

Kinetic analysis suggests that the exocytotic release of neurotransmitter from synaptic vesicles involves a cycle composed of at least four distinct steps: (1) the transport of synaptic vesicles from a reserve pool to a releasable pool at the active zone; (2) the docking of vesicles to their release sites at the active zone; (3) the fusion of synaptic vesicle membrane with the plasma membrane during exocytosis, in response to a local increase in intracellular Ca^{2+}; and (4) endocytosis, the retrieval and recycling of vesicles.

1.5.1 The initial trigger for neurotransmitter release

Neither Na^{+} influx nor K^{+} efflux is required to release neurotransmitters at the synapse, only Ca^{2+}. Synaptic transmission is initiated by a rapid rise in the concentration of calcium in the nerve terminal. This increase is detected by the secretory machinery, leading to transmitter release via fusion of synaptic vesicles with the presynaptic plasma membrane. When an action potential invades the terminal, it opens voltage gated-gated Ca^{2+} channels

near the active zone of the release site. Physiological evidence, such as the speed of transmission and the effect of the fast (BAPTA) and slow (EGTA) -calcium buffers on neurotransmitter release, supports the idea that these Ca^{2+} channels must be located very close to the release sites, presumably in the active zones (Kawasaki et al., 2004). Linás et al. showed, at the squid giant synapse, that the delay between the measured presynaptic Ca^{2+} and the corresponding postsynaptic responses is less than 200 µsec (Llinás, 1981).

1.5.2 Calcium triggered exocytosis

The local increase in Ca^{2+} concentration greatly enhances the probability of vesicle fusion and transmitter release. Although calcium is present in abundance within cells, it is well sequestered and is available only on demand. Upon certain cellular stimulus, Ca^{2+} concentration at specific nano environments in a cell becomes elevated by several orders of magnitude within a brief period (<1 ms). Not surprisingly calcium ion channels have been found in direct association with t-SNAREs (Rettig et al., 1995). An elegant study by Robitaille et al. (1990) at the frog neuromuscular junction demonstrated that calcium channels are highly concentrated in the active zones of the presynaptic nerve terminals and in perfect alignment with the clusters of postsynaptic ACh receptors. Synaptic vesicles fuse with the plasma membrane constitutively under resting conditions, but the probability of vesicle fusion is increased dramatically by elevations in cytosolic Ca^{2+}. The rapid effect of Ca^{2+}, (a fraction of a millisecond) (Sabatini and Regehr, 1996), suggests that Ca^{2+} does not induce release by a complex reaction, for example by initiating assembly of new SNARE complexes (see section 1.6) or causing large conformational changes. Instead it is likely that the reaction is largely completed before the arrival of the Ca^{2+} trigger (Sudhof, 1995).

In recent years, Katz' Ca^{2+} hypothesis has been refined by several authors (Simon and Llinás, 1985; Yamada and Zucker, 1992; Naraghi and Neher, 1997) into the "Ca^{2+} microdomain hypothesis". According to this hypothesis, the depolarization-triggered opening of Ca^{2+}-channels results in a very high Ca^{2+} concentration (a few hundred µM) in

tiny microdomains below each Ca^{2+}-channel and in their close proximity. Such a high concentration is required to activate the release system, and it is assumed that this is because the putative Ca^{2+} receptor has a low affinity for Ca^{2+}. Many presynaptic terminals also have ionotropic and metabotrobic receptors for transmitters, and these, in turn, modulate Ca^{2+} influx during an action potential an thus modify release. Although the notion of Ca^{2+} as the key intracellular trigger of neurotransmitter release was established around 50 years ago, the downstream mechanism responsible for membrane fusion was unknown until relatively recently (Morgan, 2005).

The speed of exocytosis is critical for neural function and relies on a cascade of protein-protein interactions that mediate docking of synaptic vesicles to the plasma membrane, a priming step that prepares the vesicles for release upon Ca^{2+} influx (Sudhof, 1995; Sudhof, 2002). However, the molecular mechanism by which calcium triggers fusion remains largely unknown (Hilfiker and Augustine, 1999). Precise timing of release is also important for the information encoding in neuronal networks, to allow for temporal coordination of synaptic input from heterogenous sources. Such fast kinetics imposes major demands on the molecular machinery that coordinates and mediates stimulus-secretion coupling in synapses (Rosenmund et al., 2003).

More than 1000 proteins functions in the presynaptic nerve terminal and hundreds are thought to participate in exocytosis. In this myriad of proteins, which are the performers and which are only strategic assistants? How do these proteins collaborate, and how is it possible to understand the function of such a multitude of proteins?

In the late eighties, two molecular biologists, Richard Scheller and Thomas Sudhof believed that the key to understanding synaptic transmission was to clone all the proteins associated with synaptic vesicles and all of the proteins associated with synaptic vesicle proteins. This program turned out to be a turning point in the general understanding of neurotransmitter release.

1.6 The SNARE complex: structure assembly and disassembly

Intracellular membrane fusion involves similar machinery in all vesicular traffic steps and is conserved throughout evolution (Sudhof, 1995; Graham et al., 2002). The core of this machinery is formed by a set of proteins, termed soluble NSF (N-ethylmaleimide-sensitive fusion factor) attachment protein receptors (SNARE). SNAREs play an essential role in most intracellular membrane fusion events (Ferro-Novick and Jahn, 1994; Rothman, 1994). About 15 years ago the SNARE proteins VAMP (vesicle associated membrane protein) (Sudhof et al., 1989; Trimble et al., 1988), SNAP-25 (synaptosome–associated protein of 25 kDa) (Oyler et al., 1989), and syntaxin (Bennett et al., 1992) were cloned. These proteins can be divided into two categories: target membrane SNARES (t-SNARES) including syntaxin and SNAP-25 and the vesicle SNARE (v-SNARE) VAMP also known as synaptobrevin (Sollner et al., 1993). During the fusion of a transport vesicle with a target membrane compartment, the formation of the tetrameric SNARE complex brings the two membranes together and allows the specific fusion of the vesicle with the compartment exhibiting the appropriate t-SNAREs (Weber et al., 1998) (Figure 4). When t- and v-SNAREs are allowed to mix in a calcium free buffer, prior to the addition of calcium, no fusion occurs. On the contrary when t- and v-SNAREs vesicles are allowed to mix in a calcium-buffer, vesicles aggregate and fuse (Jeremic at al., 2004). The SNARE proteins are characterized by a sequence called SNARE motifs of approximately 60-70 residues in length (Weimbs et al., 1997). The SNARE motif is most remarkable for its repeating pattern of hydrophobic residues, spaced such that the adoption of a α–helical structure places all the hydrophobic side chains on the same face of the helix (Antonin et al., 2002; Poirier et al., 1998; Sutton et al., 1998).

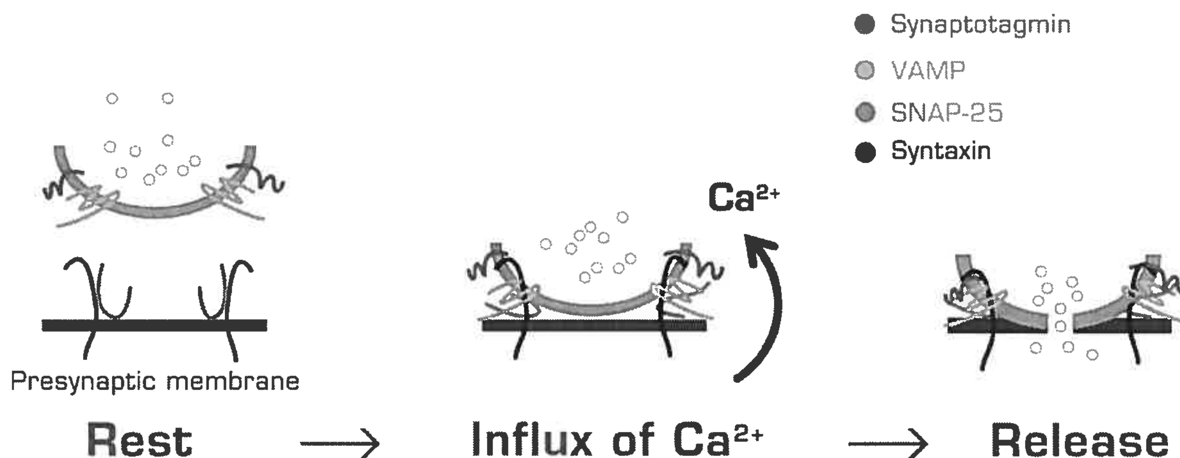


Fig. 4. The current models for synaptic vesicle exocytosis propose that fusion requires assembly and activation of the SNARE complex.

The SNARE complex is stabilized by hydrophobic interactions between the 4 helices in a series of layers with one helix donated by syntaxin, one by VAMP and two by SNAP-25 (Sutton et al., 1998). Mutations in these layers disrupt SNARE-SNARE interactions in vitro and compromise the SNARE complex stability (Fasshauer et al., 1998; Chen et al., 1999; Washbourne et al., 1999). Most SNAREs contains a single, C-terminal transmembrane domain adjacent to the SNARE motif, and others feature hydrophobic posttranslational modifications instead of a transmembrane domaine (such as neuronal SNAP-25) (Jahn et al., 2003). Whether the SNARES are sufficient for fusion is still under debate (Rizo, 2003), but there is little doubt that the core complex formation is crucial for exocytosis.

The discovery that these proteins are specific substrates for the protolytic actions of various Clostridial neurotoxins (Sudhof et al., 1993; Schiavo, 2000) provided the first evidence for their involvement in exocytosis. These neurotoxins specifically cleave a SNARE protein within a single site within its cytosolic portion. Such specific cleavage leads to a prolonged, but eventually reversible inhibition of exocytosis, which in vivo results in paralytic syndromes called botulism and tetanus (Montecucco, 2005). Cracked pheochromocytoma

(PC12) cells can be made permeable to small particles such as protein toxins or antibodies while retaining the integrity of their intracellular structures (Hay and Martin, 1992). When introduced into PC12 cells botulinum neurotoxin E, which cleaves SNAP-25 26 amino acids from the C terminus of the protein (Schiavo et al., 1993), inhibits Ca^{2+}evoked neurotransmitter release and consequently vesicle fusion. Fusion could be rescued by adding a 65 amino acid C-terminal fragment of SNAP-25 (Chen et al., 1999) and depended on the continued presence of both C-terminal fragment of SNAP-25 and Ca^{2+}. Once "zipped up" into a tight complex, with a high chemical and thermal stability, the core complex is resistant to cleavage by these toxins, and resists denaturation by sodium dodecyl sulfate (SDS) at temperatures up to 80C° (Chen et al., 1999; Xu et al., 1999). The energy that is released by this on the formation of this highly stable helix bundle can indeed be used to overcome the energy barrier for fusion. A similar mechanism to that one is proposed for viral membrane fusion (Hanson, 1997; Lin and Scheller, 1997).
Studies using these neurotoxins as well as genetic ablation studies demonstrate that SNARE proteins are not required for fusion per se (Schoch et al., 2001; Washboune et al., 2002) but rather serve to provide a high Ca^{2+} sensitivity of the fusion process (Gerona et al., 2000; Stewart et al., 2000; Reim et al., 2001).

The orientation of the four helices is parallel, so that the membrane anchors of syntaxin and synaptobrevin are located on the same side of the complex. This structure led to the suggestion that the SNARE complex when formed in trans would act as a molecular zipper, such that the formation toward the transmembrane anchors brings the membranes into contact and eventually leads to fusion (Hanson et al., 1997). This model for SNARE action would predict that assembly of the SNARE complex might be rate limiting for secretion. Fast chemical neurotransmission depends on an extremely tight temporal coupling (<0.1 ms) between the calcium trigger for exocytosis and neurotransmitter release (Sabatini and Regehr, 1999), which is faster than many enzyme reactions. This suggest that calcium only induces completion of a fusion reaction that is largely performed before calcium comes into

play and also raises the question whether residues in the SNARE domains are modified to enable physiological regulation of synaptic transmission or whether they are conserved so as not to compromise the basic fusogenic function of SNARE complexes. However, it is likely that assembly is tightly regulated, because purified reconstituted cognate v- and t-SNAREs form trans SNARE complexes that catalyze membrane fusion in a Ca^{2+} -independent manner (Weber et al, 1998; McNew et al 2000). Although this model has been widely accepted, the relevance of these results has been questioned based on the limitations of the assays used, the high protein densities, the reconstitution method, the slow speed of lipid mixing and the lack of correlation with in vivo data (Dennison et al., 2006). Recent data also suggest that Ca^{2+}-dependent SNARE binding may not be absolutely essential for triggering exocytosis, although it may contribute to the regulation of exocytosis (Shin et al, 2003), which indicates that additional proteins are needed to confer Ca^{2+} regulation on SNARE-mediated fusion.

For several proteins a point of action has been identified, but it is unclear what exactly these proteins do at that point. A further complicating factor is that the relative simplicity of the protein composition of synaptic vesicles suggests that many vesicle proteins must have multiple functions in order to account for all the steps in the vesicle cycle. The best example of this is synaptotagmin (Syt), which is thought to function in triggering exocytosis (Geppert et al., 1994) and has also been hypothesized to both stabilize the docked state of vesicles (Bennett et al, 1992; Broadie et al., 1994; DiAntonio and Schwartz, 1994; Schiavo et al., 1997; Reist et al., 1998) and to may also play a role in recycling of synaptic vesicles (Zhang et al., 1994, Jorgensen et al 1995; Fukuda et al., 1995; Reist et al., 1998; Poskanzer et al., 2003; Poskanzer et al., 2006), possibly by virtue of its interactions with clathrin associated adapter molecules (Zhang et al., 1994; Haucke et al., 2000).

1.6.1 Key stages involved in synaptic vesicle fusion.

Briefly, initially syntaxin is bound to Munc-18, and VAMP is probably bound to synaptophysin. At the docking stage the syntaxin-Munc-18 complex is dissociated. VAMP then binds to Syntaxin and SNAP-25. At the priming stage, the system becomes competent to undergo fusion following a raise in calcium concentration, most probably involving a calcium binding protein such as synaptotagmin (Syt). At the recycling stage SNAP (soluble NSF-attachment protein) and NSF bind to the SNARE complex. A cytosolic ATPase dissociates this complex during priming of the exocytotic apparatus (Brunger, 2000). Existence of a prefusion reaction preceding the point of calcium action is suggested not only by the speed of the calcium action but also by the finding that synaptic vesicle exocytosis can be non-physiologically elicited by hypertonic sucrose in the absence of calcium (Rosemund and Stevens, 1996). Thus, calcium is not required for fusion as such, but rather for an added regulatory event.

1.6.2 The functional expression of engrams.

At any given time, 5-10 vesicles are attached to most active zones. All of the attached vesicles are apparently "ready" for release, since they can all be stimulated by hypertonic sucrose to undergo exocytosis (Rosenmund and Stevens, 1996). Nevertheless, a calcium signal during an action potential does not always trigger exocytosis. At most synapses, release is observed with a relatively low probability. Furthermore, when calcium is successful in triggering exocytosis at an active zone, it usually triggers the fusion of a single vesicle, although multiple vesicles are ready to be released at the active zone. Since every action potential normally leads to a fairly uniform flooding of the active zone with calcium, there must be negative regulatory elements involved in addition to the positive regulatory elements, i.e.; there must be mechanisms that inhibit fusion just as there are mechanisms to trigger fusion.

In terms of overall synaptic signaling in the central nervous system, the "unreliability" of synaptic exocytosis is advantageous. The low release probability gives the synapse

considerable leeway for regulation. By changing release probability at individual synapses, the properties of synaptic networks can be finely tuned. Previous activity and the reception of neurotransmitter signals, neuropeptide signals, or both from other synaptic terminals can dramatically change the pattern of exocytosis.

1.6.3 SNAP-25

Mice with a genetic ablation of SNAP-25 develop normally *in utero*. These mice also proved that SNAP-25 is essential for evoked, but not spontaneous release at the NMJ (Washbourne et al., 2002). SNAP-25 is bound to the plasma membrane by palmitolation of four cysteine residues in the linker region between the two α–helices (Hess et al., 1992; Washbourne et al., 2001). In addition to its essential role in neurotransmitter release, the possible involvement of SNAP-25 in neurite extension and sprouting (Bonner et al., 1994; Osen-Sand et al., 1993), regulation of ion channel functions (Ji et al., 2002; MacDonald et al., 2002; Wiser et al., 1996; Yao et al., 1999), and neurotransmitter receptor incorporation into the plasma membrane (Lan et al., 2001) has been suggested.

1.6.4 What is the Ca^{2+} sensor and how does it work to trigger release?

Since the work of Dodge and Rahaminoff (1967), neurobiologists have talked about the calcium sensor for release. To function as a calcium sensor certain features are necessary. These include 1) an ability to bind to Ca^{2+} 2) an integral relationship with the fusion machinery, and 3) an ability to couple Ca^{2+} influx with the process of fusing two phospholipids bilayers into one. The initial hint came with the cloning of p65 (Perin et al., 1990). This molecule, soon renamed synaptotagmin is an integral vesicle membrane protein. There is at least 13 Syt isoforms (in mammals) with differential intracellular location (reviewed in Sudhof, 2002). Syt I accounts for 7% of total vesicle proteins (Perin et al., 1990; Chapman and Jahn, 1994) and is selectively enriched in synaptic vesicles (Geppert et al., 1994). Much less is known about the other synaptotagmins, although many of them are abundantly co-expressed with Syts I and II in brain, and are evolutionarily

conserved. Apart from Syt I, a number of other synaptotagmin isoforms are expressed in neuroendocrine cells, in which they participate in release (e.g., SytIII, SytVII, and SytVIIII) (Sugita et al., 2001, 2002; Fukuda et al., 2002; Tucker et al., 2003). These isoforms have not been implicated as calcium sensors for synchronized release as measured using electrophysiological techniques; however, they may act during different phases of asynchronous release. The most abundant of these "other" synaptotagmins, Syts III and VII, are localized on the plasma membrane opposite to synaptic vesicles and exhibit distinct Ca^{2+} affinities, suggesting that plasma membrane and vesicular synaptotagmins may function as complementary Ca^{2+} sensors in exocytosis with a hierarchy of Ca^{2+} affinities (Butz et al., 1999; Sugita et al., 2002). Although early studies questioned the role of synaptotagmin as a calcium sensor (Broadie et al., 1994: Di Antonio et al., 1993), a consensus has developed that this protein probably does detect calcium ions and couples transient increases in calcium concentration to the exocytotic machinery (Augustine, 2001; Geppert and Sudhof, 1998). Consistent with this consensus, Syt I KO mice lack the fast calcium dependent phase of neurotransmitter release even though wild-type levels of vesicles are docked at the membrane (Geppert et al., 1994). Still, these mice exhibit no change in the slow asynchronous component of release or in calcium independent forms of exocytosis, suggesting that Syt I functions selectively for the fast component. *Drosophila* Syt I mutants also show reduced evoked neurotransmitter release (Littleton et al., 1993, 1994; Mackler et al., 2002). Further evidence for this notion includes the observation that transmitter release evoked by presynaptic action potentials is greatly decreased by presynaptic injection of Syt I fragments (Bommert et al., 1993) or by mutation or deletion of the synaptotagmin gene (Broadie et al., 1994; Geppert et al., 1994). The question now is how synaptotagmin performs its calcium sensing and coupling functions.

Syt I contains an N-terminal intraluminal sequence, a single transmembrane region, a central linker, and two C-terminal C2 domains (Perin et al., 1991; Fernandez-Chacon et al., 2001; Mackler et al., 2002; Wang et al., 2003), motifs that constitute the regulatory calcium binding domain for a wide variety of proteins. The C2-domains accounts for the majority of

Syt I sequence, and both C2A and C2B domains function as separate Ca^{2+} binding domains, in complex with phospholipids (Chapman and Jahn, 1994: Daveltov and Sudhof, 1993; Fernandez et al., 2001). C2-domains are widespread modules of 130-140 residues that were initially defined as the second constant sequence (hence "C2") in protein kinase C isoforms (Coussens et al., 1986). Atomic structures revealed that the synaptotagmin C2A- and C2B-domains are similarly composed of a -sandwich containing eight-strands, with flexible loops emerging from the top and the bottom (Sutton et al., 1994; Fernandez et al., 2001). C2A-domains generally bind three Ca^{2+} ions, whereas C2B-domains bind only two Ca^{2+} ions (Fernandez et al., 2001; Ubach et al., 1998). All C2B-domains contain a bottom-helix between the 7th and 8th -strands that is absent from C2A-domains (Sutton et al, 1994; Fernandez et al, 2001; Ubach et al, 1998; 1999). The fact that the differences between the C2A- and C2B-domains are conserved indicates a common ancestry and suggests that the C2-domains are functionally specialized in all Syts (Südhof and Rizo, 1996).

Ca^{2+} binding to the C2-domains causes Syt I to bind to phospholipid membranes and to individual t-SNAREs (syntaxin and SNAP-25) in a Ca^{2+} dependent manner (Li et al., 1995; Schiavo et al., 1997; Gerona et al., 2000; Zhang et al., 2002). In addition, Syts undergo homo-oligomerization via Ca^{2+}dependent activation of its C2B domain (Chapman et al., 1996; Sugita et al., 1996, Littleton et al., 1999). Syt I has also been proposed to interact with N -and P/Q-type calcium channels (Charvin et al., 1997; Sheng et al., 1997), either of them contributing 30% of calcium influx in mouse chromaffin cells (Aldea et al., 2002). This interaction might serve as a molecular link between synaptic vesicles and the calcium channels and in addition might modulate channel function (Seagar et al., 1999).

Perhaps the most compelling evidence supporting the proposed role of Syt I as the major Ca^{2+} sensor that triggers synchronous neurotransmitter release was provided by the observation that in *Drosophila* synapses that express Syt I lacking the C2B domain exhibit partially recovered regulated release compared to Syt I null mutants (Yoshihara and Littleton, 2002). Moreover, mutating the calcium-binding site in the C2B domain severely reduced the fast synaptic transmission (Mackler, 2002). A mutation in a Ca^{2+}-binding loop

of the C2A domain (R233Q) causes a parallel decrease in the apparent Ca^{2+} affinity of Syt I in the presence of phospholipids in vitro and in the Ca^{2+} sensitivity of release in vivo (Fernandez-Chacon et al., 2001). However, rescue experiments in *Drosophila* have led to the proposal that Syt I promotes release independently of the C2A domain (Robinson et al., 2002). Calcium influx has long been shown to be the key trigger of release. However, calcium alone cannot regulate the degree of vesicle content release. For example, only a limited number of docked vesicles releases neurotransmitters when calcium entry occurs; this suggests that exocytosis is regulated by other factors besides calcium influx. Regulation of the degree of release is best explained by looking at the many enzymatic proteins that interact with the SNARE complex. These proteins have been hypothesized to regulate the formation, stability, or disassembly of the SNARE complex and therefore may regulate neurotransmitter release. One group of enzymatic regulators is the protein kinases. These proteins phosphorylate sites on both SNARE proteins and proteins that interact with SNARE proteins (Hilfiker and Augustine, 1999; Nagy et al, 2004). Recent research has identified some of the specific effects that phosphorylation (or dephosphorylation) at these sites can produce (Snyder et al., 2006).

1.7 Protein phosphorylation

Protein phosphorylation is a common and important mechanism for regulating a variety of cellular processes, including synaptic transmission. The balance of phosphorylation and dephosphorylation of certain proteins carried out by protein kinase and phosphatase activities plays a pivotal role in the modulation of signal transduction and synaptic transmission. Many studies over the past 20 years have shown that exocytosis is modulated by protein kinases in almost all regulated secretory cell types, including neurons and neuroendocrine cells (Turner et al., 1999). If Ca^{2+} is the trigger for exocytosis, protein phosphorylation can be thought as a ubiquitous regulator of exocytosis. Although a wide range of serine/threonine and tyrosine kinases has been implicated, only PKA and PKC

have been shown to modulate exocytosis in almost all regulated secretory cell types examined (Morgan et al., 2005).

1.7.1 Protein Kinases

Protein Kinases (PKs) are ubiquitous types of enzymes that modify proteins and regulate their function by catalyzing the addition of a phosphate group. PKs transfer the terminal phosphoryl group of the compound adenosine triphosphate (ATP) to an amino acid in the target protein. After proteins are produced on the ribosomal machinery in the cell by translation from their corresponding messenger RNA (mRNA), they are still subject to a variety of post-translational modifications, which regulate their function. These post-translational modifications can switch cellular activity from one state to another. The superfamily of PKs is classified into a number of families (Hanks and Hunter, 1995). A major criterion in this classification is the target amino acid: serine/threonine, or tyrosine. PKs phosphorylate other proteins, but can in many cases also undergo autophosphorylation and regulate their own activity. The multiple families of serine/threonine kinases include PKs regulated by cyclic nucleotides e.g. cyclic adenosine monophosphate (cAMP)-dependent PK (PKA); diacylglycerole-activated/phospholipids-dependent PKs (PKC); calcium/ calmodulin-dependent PKs (CaMK); and mitogen activated PKs (MAPK).

The biochemistry and molecular biology of PKs is complex. In the context of memory mechanisms some generalizations can however be made. PKs respond, either directly or indirectly to extracellular stimuli. This means that they fit to serve as components of the molecular acquisition or retrieval machinery in neurons. Some types of PKs can be converted into a persistently active form that is autonomous of the activating signal. This implies that these PKs can serve as molecular information storage devices in neurons, and retain activity dependent information over time.

PKs have been shown to phosphorylate a variety of presynaptic proteins. The most thoroughly characterized example is the phosphorylation of synapsin I by CaMKII (Greengard et al., 1993). This reaction results in the release of synapsin I from both actin

filaments and synaptic vesicles and may regulate their availability for the docking and the fusion event that result in neurotransmitter release. Docking and fusion reactions are also potential targets for phosphorylation-mediated regulation of synaptic transmission.

Several presynaptic proteins have been shown to be substrates for PKs, including Syt I (Hilfiker et al., 1999), SNAP-25 (Shimazaki et al., 1996; Hirling and Scheller, 1996; Risinger and Bennett, 1999), VAMP (Nielander et al., 1995; Hirling and Scheller, 1996), and syntaxin (Hirling and Scheller, 1996; Risinger and Bennett, 1999). Although these studies were performed with purified components *in vitro*, they do indicate that protein phosphorylation may be involved in multiple steps in synaptic transmission. Activation of PKC has been suggested to enhance release at step downstream of calcium entry in several preparations (Nichols et al., 1987; Hilfiker and Augustine, 1999).

Despite recent advances in the understanding of the molecular mechanisms underlying transmitter release, the mode of action of PKC in nerve terminals remains unclear. PKC activation might enhance the presynaptic Ca^{2+} signal that triggers release, for example by modulating ion channels to increase Ca^{2+} influx or by decreasing Ca^{2+} buffering or removal. Alternatively, PKC could act independently of Ca^{2+} entry, by increasing the number of release sites or releasable vesicles, or by making individual vesicles more sensitive to entering Ca^{2+}. Moreover PKA, Ca^{2+}, PKC and CaMKII have all been implicated in different aspects of long-term changes in synaptic efficacy (Morgan et al., 2005).

At the biochemical level serotonin activates second messenger cAMP in the sensory neurons causing PKA dependent protein phosphorylation. Serotonin also causes the translocation and thereby activation of PKC from cytosol to the membrane in the sensory cells (Braha et al., 1990; Sactor and Schwartz, 1990). Activation of either PKA or PKC leads to increased transmitter release from the sensory neurons, but under different conditions (Ghirardi et al., 1992).

In *Aplysia* sensory neurons, 5-HT can enhance synaptic transmission by both PKC and PKA- mediated processes (Byrne and Kandel, 1996). PKA-mediated processes dominate

facilitation in non-depressed synapses, whereas PKC-mediated processes are more important in depressed synapses (fig. 5).

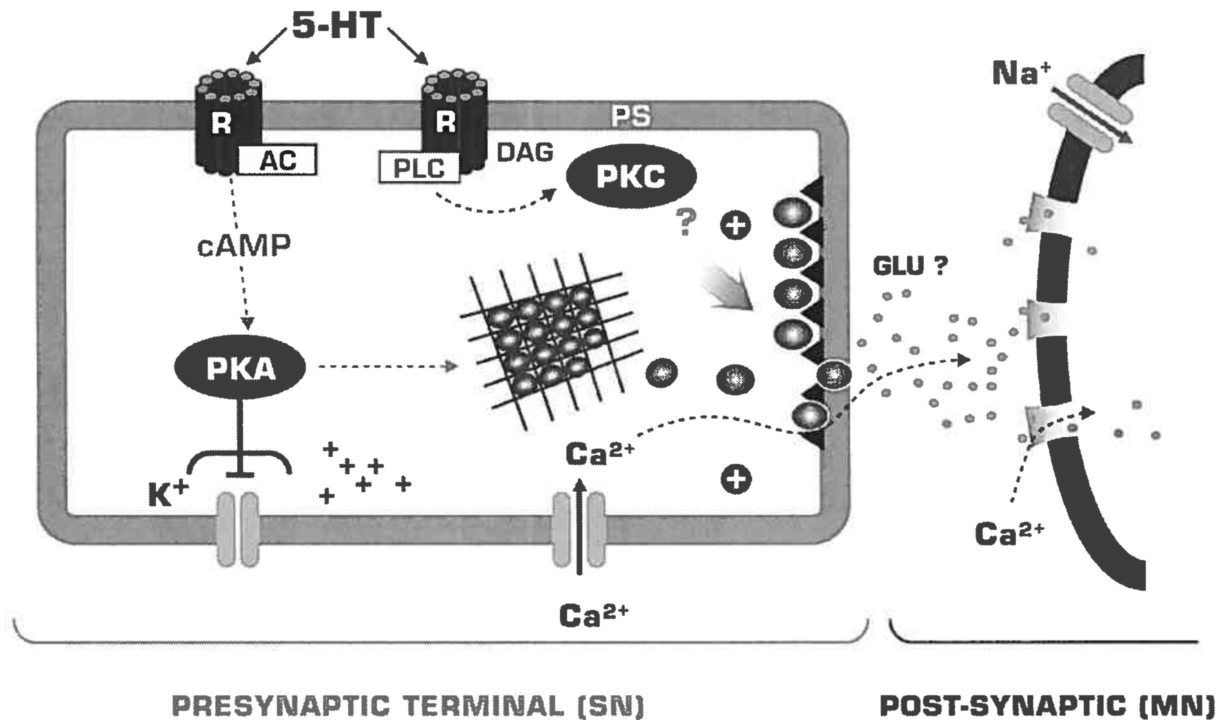


Fig. 5. In *Aplysia*, the effect of the facilitating neurotransmitter serotonin (5-HT) on sensorimotor (SM) synapses is mediated through multiple kinase pathways that become involved at different times according to the state of the neurons. Serotonin binds to two receptors. One activates a G protein (G_s), which activates the enzyme adenylyl cyclase (AC). AC converts ATP to the second messenger cyclic AMP (cAMP). cAMP binds to the PKA regulatory subunits, causing them to undergo a conformational change so that they can dissociate and free the catalytic subunit, allowing the free catalytic subunits to phosphorylate substrates, including a serotonin sensitive K^+ channel (K_s), thereby decreasing the K^+ current, thus prolonging the action potential (AP) and increasing the Ca^{2+} influx, which contributes to spike broadening.

At depressed *Aplysia* synapses, pools of releasable neurotransmitter vesicles are depleted, and spike broadening is not effective at enhancing secretion. Homosynaptic depression is reversed by the neurotransmitter 5-HT through activation of a Ca^{2+}-independent form of PKC, AplII. Here, serotonin acts through a second receptor, engaging the G protein G_o that activates phospholipase C (PLC). PLC cleaves phosphatidylinisitol 1,4,5-bisphosphate (PIP_2) into two second messengers: inisitol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). DAG remains in the membrane where it activates PKC. In turn PKC acts on various unidentified molecular targets to increase transmitter release.

1.7.2 PKC

PKC was originally isolated as a Ca^{2+} and phospholipid-dependent protein kinase (Takai et al., 1977) and exerts a wide range of physiological functions (Nishizuka, 1992), from the regulation of gene transcription to the modulation of stimulus secretion coupling in hormone and neurotransmitter release (Majewski and Iannazzo, 1998). The signaling pathway that activates PKC is evolutionary well conserved (Mellor and Parker, 1998). Briefly, neurotransmitters or hormones transiently activate phospholipases such as PLC, which leads to the metabolism of phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the transient production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Newton 2001; Nishizuka, 2001). The formation of IP_3 leads to the release of calcium from internal stores. DAG binds PKC and increases its affinity for phospholipids to provoke its activation (Newton 2001; Nishizuka, 2001). PKC is widely expressed in the brain and distributes differently in neurons in an isotype –specific manner. Eleven mammalian isoforms have been identified to date. The PKC family is divided into three types: conventional PKCs (α, β and γ isoforms), novel PKCs (δ, ε, η and θ isoforms), and atypical PKCs (ζ and λ isoforms) (Nishizuka, 1992). All PKC isoforms contain one or two cysteine-rich domains (CDRs) near the amino-terminal. DAG and phorbol ester (PE) binding to CDR activate PKC with the exception of the atypical PKC. PEs induce a remarkable enhancement of neurotransmitter release (Nichols et al., 1987; Stevens and Sullivan, 1998)

and it has been widely believed that the release is stimulated through the activation of PKC (Takahashi et al., 2003). However, all Unc-13/Munc13 isoforms also bind PEs and DAG with high affinity and in common with PKCs translocate to the plasma membrane in response to PE binding *(Betz et al., 1998; Ashery et al., 2000)*. Munc13 proteins are functional presynaptic phorbol-ester receptors and targets of the DAG second messenger pathway that act in parallel with PKCs to regulate transmitter release (Betz et al., 1998). Munc13 are specifically localised to presynaptic active zones (Brose et al., 1995; Augustin et al., 1999; Betz et al., 1998). At the molecular level, Munc13s act by unfolding and activating the SNARE protein syntaxin and thereby promoting SNARE complex formation (Betz et al., 1997; Brose et al., 2000; Richmond et al., 2001). In the absence of Unc-13/Munc13-mediated vesicle priming, synapses are completely unable to secrete neurotransmitters (Richmond et al., 1999; Aravamudan et al., 1999; Varoqueaux et al., 2002). Pharmacological studies on PKC must therefore be complemented with alternative experimental approaches to allow the separation of PKC-mediated effects from those caused by alternative targets of the DAG second messenger pathway. Although PKCs have been suggested to play a key role in the plasticity of vertebrates (Stevens and Sullivan, 1998; Waters and Smith, 2001) and invertebrate nervous system (Byrne and Kandel, 1996; Houeland et al., 2006), the multiplicity of isoforms and the absence of specific inhibitors have limited the understanding of their function. By contrast there are only two major PKC isoforms present in the nervous system of *Aplysia* (Sossin et al., 1993; Figure 6). This allows for more detailed assessment of the role of PKC isoforms in synaptic plasticity (Manseau et al., 2001; Zhao et al., 2006). Moreover, physiological effects mediated by PKC in *Aplysia* are similar to actions of PKC in vertebrates (Ghirardi et al., 1992). Manseau et al. (2001) found that short-term synaptic facilitation of depressed synapses is mediated by Ca^{2+}-independendent PKC (AplII). Recently, Zhao et al. (2006) discovered that dominant-negative *Apl* I, but not *Apl* II, blocks intermediate-term facilitation. Thus, different isoforms of PKC translocate under different conditions to mediate distinct types of synaptic plasticity: Ca^{2+}-independent Apl II is involved in short-term facilitation, and Ca^{2+}-

dependent Apl I contributes to intermediate-term facilitation. Recently a third atypical PKC (Apl III) has been added to the list (Bougie et al., 2006).

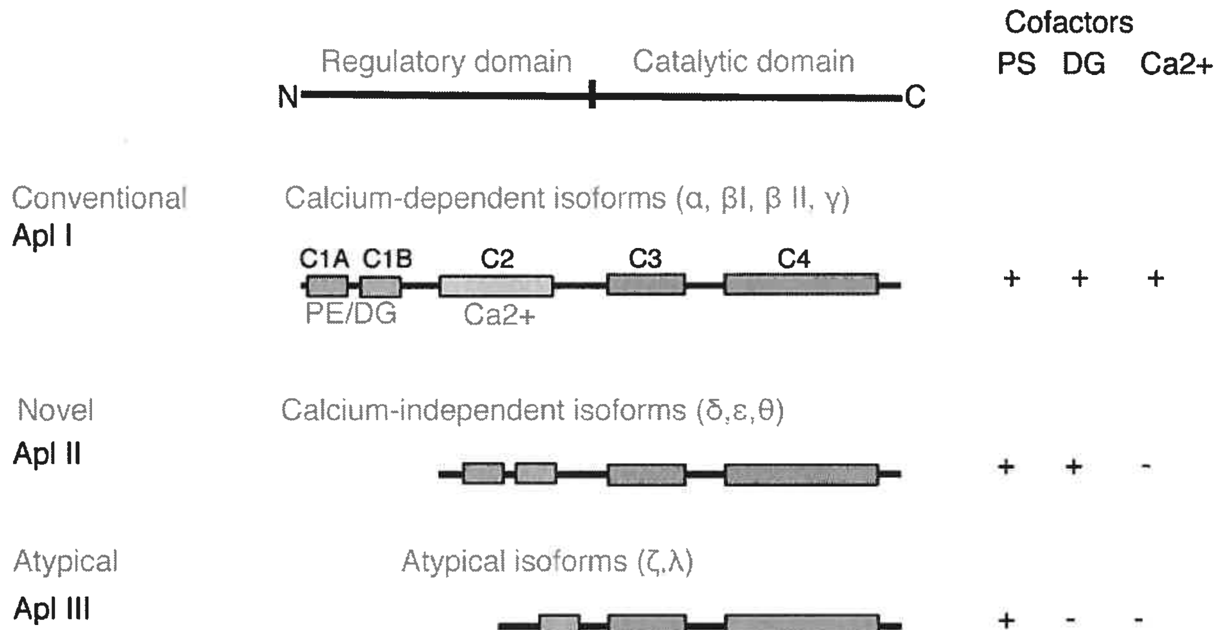


Fig. 6. PKC isoforms.

1.7.3 PKA

PKA phosphorylates many substrate proteins in the sensory neurons, including the S-type K^+ channel. The original model for action of serotonin in synaptic facilitation at sensory-motor-neuron synapses in *Aplysia* leads to phosphorylation of potassium channels. Potassium current is thereby reduced, resulting in a slowing of the repolarization of action potentials and consequent prolongation of transmitter release (Klein et al., 1980). The reduction in potassium current leads to a reduction in potassium conductance and thus an increase in neuron excitability (Klein et al., 1986). PKA-mediated phosphorylation of SNAP-25 may also increase synaptic strength (Hepp et al., 2002; Nagy et al., 2004).

1.8. Principal aims of my thesis

The principal aim of my research was to study the synaptic plasticity at the sensori-motor synapse of *Aplysia*, and how it contributes to behavior, learning and memory. In my studies I focused on the role of protein kinase C (PKC) in synaptic transmission. I sought to define the step or steps in the transmitter release process that involve PKC phosphorylation and that contribute to synaptic plasticity, with the ultimate goal of identifying the PKC posttranslational (phosphorylation) modifications of specific proteins in the exocytotic pathway that regulate the number of vesicles available for release. I studied these questions in cultured *Aplysia* sensory-motor neurons using molecular biological, cellular and electrophysiological techniques.

In the first paper (chapter 2), I have studied the differential regulation of transmitter release by alternatively spliced forms of synaptotagmin I.

In the second paper (chapter 3) I have identified and characterized a novel C2B splice variant of synaptotagmin I.

Finally in the third paper (chapter 4) I have studied the PKC modulation of transmitter release by SNAP-25 at sensory to motor synapse.

II ARTICLE 1

Differential regulation of transmitter release by alternatively spliced forms of synaptotagmin I.

Nakhost A, Houeland G, Castellucci VF, Sossin WS (2003).
J Neurosci 23:6238-44.

6238 • The Journal of Neuroscience, July 16, 2003 • 23(15):6238–6244

Cellular/Molecular

Differential Regulation of Transmitter Release by Alternatively Spliced Forms of Synaptotagmin I

Arash Nakhost,[1]* Gry Houeland,[2]* Vincent F. Castellucci,[2] and Wayne S. Sossin[1]

[1]Department of Neurology and Neurosurgery, McGill University, Montreal Neurological Institute, Montreal, Quebec, Canada H3A 2B4, and [2]Department of Physiology, University of Montreal, Montreal, Quebec, Canada H3C 3J7

We discovered a novel alternatively spliced form of synaptotagmin I (Syt I). This splicing event is conserved over evolution and, in *Aplysia*, results in a two amino acid insert in the juxtamembrane domain of Syt I (Syt I_{VQ}). Both Syt I and Syt I_{VQ} are localized to synaptic vesicles; however, we also observed punctae that contained one or the other spliced products. Both Syt I and Syt I_{VQ} are phosphorylated at the adjacent PKC site. Overexpression of Syt I_{VQ}, but not of Syt I, in *Aplysia* neurons blocked the ability of serotonin to reverse synaptic depression. This effect is upstream of PKC activation, because neither Syt I_{VQ} nor Syt I blocked the effects of phorbol esters on reversing synaptic depression or the effects of serotonin on facilitating nondepressed synapses. Our results demonstrate a physiological role for splicing in the juxtamembrane domain of Syt I.

Key words: **synaptotagmin; transmitter release; protein kinase C; PKC; *Aplysia*; depressed synapses; alternative splicing**

Introduction

Synaptotagmins (Syts) are membrane proteins thought to act as calcium sensors during membrane fusion. In particular, Syt I is important for the release of neurotransmitters from synaptic vesicles because loss of Syt I function removes the fast Ca^{2+}-dependent phase of neurotransmitter release (Nonet et al., 1993; DiAntonio and Schwarz, 1994; Geppert et al., 1994). Moreover, as would be expected from a Ca^{2+} sensor, mutations that affect the Ca^{2+} sensitivity of Syt I affect the Ca^{2+} sensitivity of transmitter release (Fernandez-Chacon et al., 2001; Mackler et al., 2002). So far, 13 isoforms of Syts have been identified in mammals (Syt I–Syt XIII), as well as six to eight homologues in *Drosophila* and *Caenorhabditis elegans* (for review, see Schiavo et al., 1998; Adolfsen and Littleton, 2001; Sudhof, 2002). Syts comprise a lumenal N terminal, a transmembrane domain, and a short juxtamembrane linker, followed by two C2 domains (C2A and C2B). The function of synaptotagmin in membrane fusion is mediated by protein–protein and protein–lipid interactions of these C2 domains (for review, see Schiavo et al., 1998; Adolfsen and Littleton, 2001).

The juxtamembrane domain of Syt I is well conserved within Syt I-like isoforms (Syt II, Syt IX, and invertebrate Syt Is) but is not conserved in other Syts. This suggests that the juxtamembrane region may play a role in specific functions of Syt I-like isoforms. Indeed, this segment has been proposed to play a role in the specific cellular localization of Syts (Fukuda et al., 2001). The juxtamembrane domain is also the location of all of the well characterized sites for Syt I phosphorylation (Bennett et al., 1993; Davletov et al., 1993; Hilfiker et al., 1999).

Aplysia sensorimotor (SM) neuron synapses show a remarkable synaptic depression to repeated stimulation that is thought to underlie behavioral depression (Byrne and Kandel, 1996). Both depression and the reversal of this depression by serotonin (5-HT) involve regulation of the release machinery (Byrne and Kandel, 1996). While recloning *Aplysia* Syt I to examine a possible role for Syt I phosphorylation in regulating the reversal of depression, we discovered a novel alternatively spliced form of this protein with two amino acids, V and Q, added in the juxtamembrane domain. This splicing event is conserved in *Drosophila* and mammalian Syt I. Overexpression of this synaptotagmin spliced product (Syt I_{VQ}), but not of Syt I, blocked 5-HT-mediated reversal of depression. These results reveal the first evidence for the importance of splicing in the juxtamembrane domain of Syt I and suggest additional roles for Syt I in regulating membrane trafficking.

Materials and Methods

Aplysia californica (50–200 gm) were purchased from Marine Specimens Unlimited (Pacific Palisades, CA) or the *Aplysia* resource facility at the University of Miami (Miami, FL) and kept in an aquarium for at least 3 d before experimentation. Dissections and isolation of tissues and cultured neurons was as described previously (Manseau et al., 2001).

Plasmid construction. We designed exact primers to the cytoplasmic domain of *Aplysia* Syt I (5′, CGCGAATTCAAGAAGGAGGGCAAGAAAGG; 3′, GCGCCCGGGTTAGTTCTTCTCTGGCA) based on the published sequence including restriction sites, allowing us to insert the PCR product into pGEX-5X-1 vector (Amersham Biosciences, Oakville, Ontario, Canada). The full-length Syt I was amplified by PCR using

Received Oct. 17, 2002; revised May 13, 2003; accepted May 21, 2003.

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Grant 187018 (W.S.S.) and Canadian Institutes of Health Research (CIHR) Grants MOP-12046 (W.S.S.) and MOP-14142 (V.F.C.). A.N. is supported by an NSERC graduate studentship, and W.S.S. is a Killiam Scholar and supported by a CIHR Scientist award. We thank Peter McPherson, Ted Fon, Luc Desgroseillers, and Louis-Eric Trudeau for helpful comments on this manuscript.

*A.N. and G.H. contributed equally to this work.

Correspondence should be addressed to Dr. Wayne S. Sossin, Department of Neurology and Neurosurgery, McGill University, Montreal Neurological Institute, BT 110, 3801 rue University, Montreal, Quebec, Canada H3A 2B4. E-mail: [redacted]

Copyright © 2003 Society for Neuroscience 0270-6474/03/236238-07$15.00/0

distinct 5′ primers, again based on the published sequence (CGCGAATTCACCATGGACTCCCTTCTGGCG). These constructs were subsequently excised from pGEX-5X-1 and inserted into enhanced green fluorescent protein (EGFP)-C2 vectors (Clontech, Palo Alto, CA) using *Eco*RI and *Sma*I. The EGFP-C2 Syt I clones were then excised by *Nhe*I and *Sma*I and inserted into the *Aplysia* expression vector pNEX-3 (Manseau et al., 2001) cut with *Xba*I and *Sma*I. All pNEX-3 EGFP-Syt I clones were checked by sequencing over their entire length. EGFP was replaced by enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) (Clontech) using *Sph*I and BsrGI. The S→A mutant was generated using the Syt I cytoplasmic domain of Syt I_{VQ} cloned into pGEX-5X-1 or the pNEX-3 EGFP-Syt I_{VQ} in a two-step mutagenic procedure as described previously (Manseau et al., 2001). A new *Mlu*I site was formed in Syt $I_{VQ;\,S\text{-}A}$ mutant. All constructs were sequenced over the entire amplified region to confirm that no changes were made. The DsRed VAMP (vesicle-associated membrane protein) was made using primers based on the published *Aplysia* VAMP sequence (Yamasaki et al., 1994) for insertion into EGFP-C2 and then insertion into pNEX-3. The EGFP was then replaced with DsRed (Clontech) with appropriate enzymes.

Quantitative reverse transcription-PCR. The relative amounts of Syt I_{VQ} and Syt I mRNA was determined by quantitative reverse transcription (RT)-PCR. RNA isolation was performed using the RNAqueous-4PCR kit (Ambion, Austin, TX) according to the protocol provided by the manufacturer. Common forward and reverse primers were used, followed by an *Rsa*I digest (addition of VQ introduces a new *Rsa*I site into Syt I DNA sequence). Mixes of plasmids encoding Syt I_{VQ} and Syt I were used in control reactions as part of each PCR set to generate a standard curve. The PCR products were subjected to *Rsa*I digest and separated on agarose gels, illuminated under UV light, digitally scanned, and quantified using NIH Image.

In vitro *phosphorylation assay.* Phosphorylation was initiated by the addition of purified protein kinase C (PKC) Apl II (Sossin et al., 1996) to the phosphorylation mix [50 nM 12-*O*-tetradecanoylphorbol-13-acetate, 5 μg/ml phosphatidylserine, 500 μM $CaCl_2$, 10 μM ATP, 1–3 μCi [γ-^{32}P]ATP, 45 mM $MgCl_2$, 180 mM Tris, pH 7.5, and various amounts of glutathione *S*-transferase (GST) fusion proteins]. Nonphosphorylated controls were incubated in a control mix (phosphorylation mix without ATP). These reactions were allowed to proceed at 25°C for 30 min and were stopped by the addition of 20 μl of Laemmli buffer and then loaded onto 9% SDS-polyacrylamide gels. After transfer to nitrocellulose, the blots were exposed to film to visualize the incorporation of radioactive phosphate.

Cell culture preparation. Injections of plasmid DNA and physiological paradigms were as described previously (Manseau et al., 2001). An arbitrary scale of fluorescence (from 0 to 5) was established to evaluate the labeling of each sensory neuron (Manseau et al., 2001). Sensory neurons that were positive for plasmid expression (3–5 on the scale) were individually paired with motor neurons (kept aside until then in the refrigerator at 4°C to prevent them from retracting their axons), in a 10% hemolymph-enriched medium. The day after the pairing, the Petri dish solution was exchanged with fresh 10% hemolymph–L-15. Electrophysiological recordings started on the fifth day, 2 d after pairing, to allow the formation of new synaptic contacts and a full maturation of the PKC transduction pathway involved in short-term plasticity (Sun and Schacher, 1996). All recordings were done in L-15 at room temperature (21–24°C) using Axoclamp-2A and Axoprobe-1A amplifiers (Axon Instruments, Foster City, CA) in the current-clamp mode. Membrane resistance of both sensory and motor neurons and the resting potential of motor neurons were measured at the start and at the end of each recording. The resting potential of sensory neurons was not measured until the end of experiment to prevent the generation of unwanted spikes. The major criterion for selection of healthy neurons was a stable resting potential. Throughout the experiments, the sensory neuron was held at −50 mV, and the siphon motor neuron of the LF cluster, which was impaled first, was hyperpolarized to −80 mV. In experiments on short-term facilitation of depressed synapses, a hyperpolarizing current was passed (glass pipette, 10–15 MΩ, filled with 2 M KAc) to prevent spike generation during the sensory neuron impalement. Short intracellular pulses were delivered, and, once the threshold for action potential was reached, the stimulation intensity and interval was kept constant through the experiment. We continued the experiment when the initial EPSP amplitude exceeded 2 mV. The series of EPSPs were evoked every 20 sec in the motor neuron. 5-HT (10 μM final concentration) was added directly to the bath near the cells and mixed gently after 40 EPSPs. Ten additional EPSPs were recorded. In another set of cocultures, 12,13-dibutyrate (PDBu) (100 nM final concentration) was added instead of 5-HT to determine whether the inhibition of facilitation was before or after PKC activation.

Changes in synaptic transmission. EPSPs were always normalized to the size of the initial EPSP. The amount of facilitation was calculated as the difference between EPSPs after treatment (averages of EPSPs 41–43) and EPSPs before treatment (averages of EPSPs 38–40). In experiments on short-term facilitation of rested synapses, a single depolarizing stimulus was applied to the sensory neuron, and the initial EPSP amplitude was recorded. At 2 min, 5-HT was applied to the bath (final concentration of 10 μM), and a second EPSP was recorded 3 min later in the presence of 5-HT. The amount of facilitation was calculated as the difference between EPSP 2 and EPSP 1 (EPSP 1 normalized to 100%). Data were acquired and analyzed digitally using CLAMPEX 7 and a modified version of pCLAMP (Axon Instruments) (Manseau et al., 2001). Experiments comparing the effects of the various constructs were always done in parallel.

Confocal laser microscopy on living cells. The cells were coinjected with constructs tagged with either ECFP or EYFP and were visualized with a Zeiss (Jena, Germany) LSM 510 confocal laser microscope. EYFP and ECFP were chosen because their emission spectra overlap minimally, so they can be distinguished when used simultaneously. For dual imaging of EYFP- and ECFP-injected cells, the cells were excited successively with multi-line argon lasers at 514 and 458 nm, respectively. Images were analyzed using Zeiss LSM 510 software. For EYFP, the cells were light collected through a 530 nm long-pass emission filter, passing by an infrared 480–520 nm bandpass dichroic mirror. For ECFP, the cells were light collected with a 480–520 nm bandpass emission filter. A DsRed-VAMP construct was used as an indicator for synapse localization. In these experiments, the cells were coinjected with DsRed and ECFP Syt I or ECFP Syt I_{VQ}. For Ds-Red, the cells were excited with a helium–neon laser unit at 543 nm and light collected through a 558–583 bandpass filter.

Antibody production and immunoblotting. Antibodies were raised against a GST fusion protein consisting of the cytoplasmic domain of Syt I_{VQ} (nucleotides 279–1284). The antibodies were affinity purified using MBP-Syt I_{VQ} fusion proteins encoding the cytoplasmic domain of Syt I. The MBP Syt I fusion proteins were immobilized on polyvinylidene difluoride membrane, and Syt I antibody was purified in a two-step purification procedure as described previously (Ramjaun et al., 1997).

We also generated a phospho-specific antibody against a peptide sequence [CQLLGNS(p)YKEK] from *Aplysia* Syt I, with serine 123 converted to a phosphoserine as described previously (Nakhost et al., 1999). Immunoblotting was performed as described previously (Nakhost et al., 1999).

Results

Identification of a novel alternatively spliced form of *Aplysia* synaptotagmin I

Aplysia Syt I was cloned previously and shares the putative domain structure of all other Syt isoforms (Martin et al., 1995). In the process of recloning the cytoplasmic domain of *Aplysia* Syt I, we identified an alternatively spliced form. Figure 1*A* shows the nucleotide alignment of the published Syt I sequence and our initial Syt I clone Syt I_{VQ} in the region of the alternative splice. Our sequence has six additional nucleotides resulting in a VQ insert in the amino acid sequence. Interestingly, the VQ is also seen in the published sequences of squid and *Drosophila* Syt I (Fig. 1*B*). Searching the *Drosophila* Expressed Sequence Tag (EST) database, we found a number of cDNAs that are identical to the published Syt I but specifically lack the VQ residue (Fig.

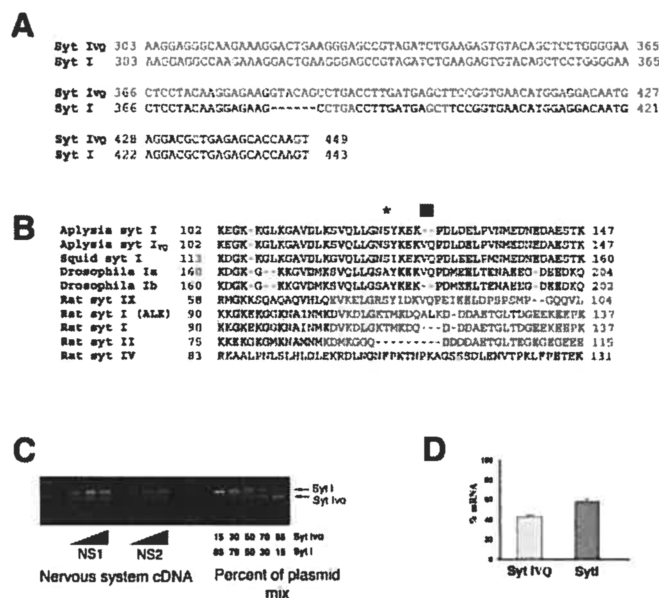


Figure 1. Cloning of a novel spliced isoform of Syt I. *A*, Nucleotide sequence of two clones amplified from a nervous system library showing the insertion of six amino acids. Nucleotide numbering is from residues 303–449. *B*, Alignment of juxtamembrane domain from a number of species highlighting the conservation of this region in Syt Is and the conservation of the spliced forms. *Drosophila* Ib (Dros Ib) (from EST clones; accession numbers 15484159, 15504610, and 15505802) and Syt I_{ALK} (Perin et al., 1990). Syt IX has also been called Syt V in other publications. The black bar represents the site of alternative splicing, and the star represents the site of PKC phosphorylation. *C*, RT-PCR of Syt I and Syt I_{VQ} demonstrates approximately equal amounts of both splice forms. The insertion of the VQ generates an *RsaI* site. We used PCR primers flanking the insert for RT-PCR from the *Aplysia* nervous system. The amplified product was then cut with *RsaI* to determine the proportion of RNAs with the insert. Different amounts of nervous system template were used to ensure that PCR amplification was in the linear range. Results are shown for two different animals (NS1 and NS2). To generate a standard curve, mixes of plasmids containing different proportions of Syt I_{VQ} and Syt I were used as the template for PCR. *D*, The proportion of the two RNAs was calculated based on the standard curves. Values are mean ± SEM for four independent RT-PCRs from four individual animals.

1 *B*). Although we have not cloned the genomic sequence of *Aplysia* synaptotagmin, examination of the *Drosophila* genome sequence reveals that the VQ sequence begins an exon of Syt I and thus suggests that alternative exon entry can explain the alternatively spliced forms. Interestingly, the VQ sequence is also located at the exon start in the Syt I-related Syt IX (also called Syt V) (Craxton et al., 1997). Alternative exon entry has also been described in this exact region of the linker region of rat Syt I in which the amino acids ALK are inserted instead of VQ (Perin et al., 1990) [Fig. 1*B*, Syt I(ALK)]. Syt I, II, and IX are more closely related to each other than to invertebrate Syt I isoforms, suggesting that they have diverged after the vertebrate–invertebrate separation (Marqueze et al., 2000). The juxtamembrane domain is well conserved in all Syt-I like isoforms (vertebrate and invertebrate) but not in other Syts, such as Syt IV (Fig. 1*B*). Although Syt I and Syt II are highly conserved in the juxtamembrane domain, it is also striking that, in the region of the VQ insertion, Syt II has a sizable deletion (Fig. 1*B*). Thus, there is an alternative exon entry site in the linker domain of Syt I that is conserved over evolution. However, the role for this splicing is unknown.

Quantitative reverse transcription (RT-PCR) studies from the *Aplysia* nervous system indicate that mRNAs encoding Syt I and Syt I_{VQ} are present in the nervous system of *Aplysia* at approximately a 1:1 ratio (Fig. 1*C*; quantitated in *D*). Similar results were obtained when RT-PCR was done with RNA isolated from sensory neuron clusters (data not shown). Treatment of sensory clusters with a paradigm that induces long-term facilitation [5 min pulses for five times each of 20 μM 5-HT (Montarolo et al., 1986)] did not alter the ratio of Syt I and Syt I_{VQ} (percentage change in Syt I/Syt I_{VQ} ratio, $-4 \pm 6\%$; $n = 4$).

Syt I and Syt I_{VQ} are both localized to synaptic vesicles

Splicing may effect localization of Syt Is as the juxtamembrane domain has been implicated in the localization of Syts (Fukuda et al., 2001). To test this, we examined colocalization of the expressed Syt Is with the synaptic vesicle protein VAMP/synaptobrevin. We coexpressed DsRed-tagged VAMP and either ECFP-Syt I_{VQ} or ECFP-Syt I in sensory neurons and examined their localization after 5 d in coculture with motor neurons. FP-tagged VAMP has been used to mark synaptic vesicle pools in many systems (Nonet, 1999; Ahmari et al., 2000), and, in *Aplysia* sensory neurons, tagged VAMP colocalizes with antibodies to clustered glutamate at sensory-to-motor neuron synapses (data not shown). Both Syt I isoforms are colocalized with VAMP at concentrations of VAMP likely to mark pools of synaptic vesicles (Fig. 2*A*,*B*). This is not surprising because both Syt I and Syt I_{VQ} contain the putative AP2 (adaptor protein 2) binding site required for endocytosis into synaptic vesicles (Zhang et al., 1994). However, because the juxtamembrane domain has been postulated to determine sorting from the trans-Golgi network (Fukuda et al., 2001), we examined whether Syt I and Syt I_{VQ} were always localized together. In these experiments, we used colocalization of EYFP-Syt I and ECFP-Syt I_{VQ} or ECFP-Syt I and EYFP-Syt I_{VQ} to control for variations in the intensities of the different fluorescent proteins. Approximately 5% of punctae contained one or the other isoform (Fig. 2*C*), regardless of the combination of tags, whereas most punctae colocalized (Fig. 2*D*). Although the number of non-colocalized punctae is small, we did not observe any non-colocalized punctae in control experiments when we examined colocalization of ECFP-Syt I and EYFP-Syt I or ECFP-Syt I_{VQ} and EYFP-Syt I_{VQ} (data not shown). The punctae containing only Syt I or only Syt I_{VQ} may be transport vesicles. The number of these vesicles in processes is probably small compared with the number of synaptic vesicles. The localization of ECFP- and EYFP-tagged proteins in the cell bodies was not possible because of the pigment granules in the cell body that could be seen even in the absence of injections.

Syt I and Syt I_{VQ} are both phosphorylated *in vitro* by PKC at serine 123

Serine 123 in *Aplysia* Syt I corresponds to the site phosphorylated by PKC in vertebrate Syt I (Hilfiker et al., 1999) and is located very close to the insertion of VQ (Fig. 1*B*). To examine whether the insertion of VQ affected PKC phosphorylation, we incubated PKC and PKC activators with GST fusion proteins with the cytoplasmic domain of Syt I, Syt I_{VQ}, or Syt I_{VQ} with serine 123 converted to alanine (Syt $I_{VQ;\,S\text{-}A}$). Although these results confirm that serine 123 is a conserved PKC site because PKC phosphorylates both Syt I and Syt I_{VQ}, but not Syt $I_{VQ;\,S\text{-}A}$ (Fig. 3*A*), there were no differences in the *in vitro* phosphorylation of Syt I and Syt I_{VQ} (Fig. 3*A*). Further confirming serine 123 as an *in vitro* PKC site, a phospho-specific antibody raised to the serine 123 site in *Aplysia* Syt I recognized bacterially expressed Syt I only after phosphorylation by PKC (Fig. 3*B*).

Overexpression of Syt I_{VQ} specifically blocks the facilitation of depressed synapses upstream of PKC activation

We investigated the physiological role(s) of different Syt I isoforms *in vivo* by overexpressing them in *Aplysia* sensory neurons.

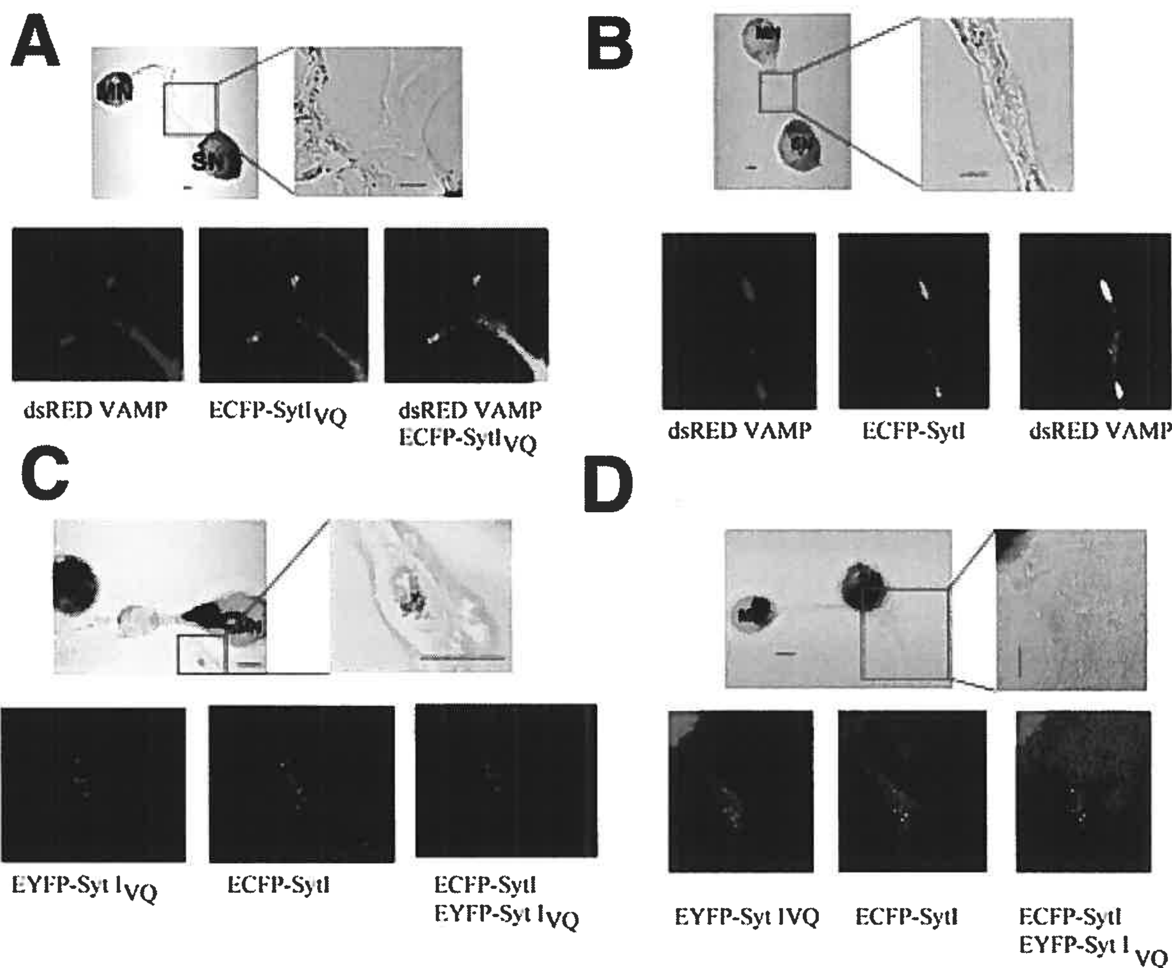


Figure 2. Localization of Syt I and Syt I_{VQ} in sensory neurons. Plasmids encoding DsRed-labeled *Aplysia* VAMP and ECFP-labeled Syt I (*A*) or DsRed-labeld *Aplysia* VAMP and ECFP-labeled Syt I_{VQ} (*B*) were injected into sensory neurons. Expressing neurons were then paired with motor neurons and visualized 3–5 d later. For five cells expressing Syt I and four cells expressing Syt I_{VQ}, all DsRed VAMP clusters were completely overlapped with ECFP-Syt I (43 clusters) or ECFP-Syt I_{VQ} (14 clusters). *C*, Plasmids encoding ECFP-labeled Syt I and EYFP-labeled Syt I_{VQ} or ECFP-labeled Syt I_{VQ} and EYFP-labeled Syt I were injected into sensory neurons. Expressing neurons were then paired with motor neurons and visualized 3–5 d later. *D*, In the majority of images, EYFP Syt I_{VQ} and ECFP Syt I did completely overlap. Scale bars, 20 μm.

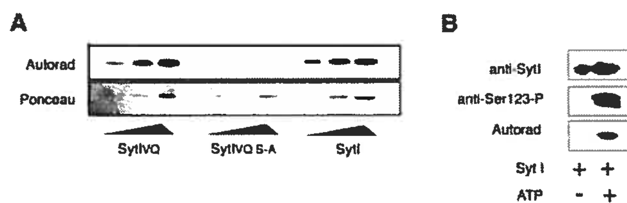


Figure 3. Syt I and Syt I_{VQ} are phosphorylated at serine 123 by PKC *in vitro*. *A*, GST fusion proteins (0.15, 0.3, and 0.6 μg) encoding the intracellular region of Syt I_{VQ}, Syt $I_{VQ;S\text{-}A}$, or Syt I were incubated with purified PKC Apl II, PKC activators, and radioactive ATP for 30 min at room temperature. The proteins were separated on 9% SDS-PAGE, blotted to nitrocellulose, and then stained with Ponceau to visualize the fusion proteins. The blots were then exposed to radiography to visualize incorporation of ATP. This experiment was repeated three times with similar results. *B*, GST-Syt I_{VQ} (0.6 μg) was incubated with purified PKC Apl II and PKC activators in the presence or absence of radioactive ATP. The proteins were separated on 9% SDS-PAGE, blotted to nitrocellulose, and then probed first with a phospho-peptide-specific antibody raised to the serine 123 site in Syt I. The blot was then stripped and probed with an antibody raised to a peptide from Syt I. The blot was then exposed to radiography to visualize incorporation of ATP (Autorad).

Various plasmids encoding EGFP or EGFP-tagged Syt I constructs (EGFP-Syt I, EGFP-Syt I_{VQ}, EGFP-Syt $I_{S\text{-}A}$, or EGFP-Syt $I_{VQ;\,S\text{-}A}$) were injected into *Aplysia* sensory neurons, which were subsequently used to make SM cell cultures. First, we examined the ability of 5-HT to reverse synaptic depression. Synaptic depression was produced by 40 repeated stimulations of the sensory cell. 5-HT (10 μM final concentration) was added to induce PKC-dependent facilitation (Ghirardi et al., 1992). Our electrophysiological results show that, in cells expressing EGFP-Syt I, EGFP-Syt $I_{S\text{-}A}$, or EGFP alone, 5-HT-induced facilitation of depressed SM synapses was normal (Fig. 4*A*,*B*). Injection of plasmids encoding EGFP alone had no effect on transmitter release or the reversal of synaptic depression in *Aplysia* (Manseau et al., 2001). Conversely, overexpression of EGFP-Syt I_{VQ} blocked the facilitating effect of 5-HT (Fig. 4*A*,*B*). Overexpression of EGFP-Syt $I_{VQ;\,S\text{-}A}$ also blocked the facilitating effect of 5-HT but appeared somewhat less effective than EGFP-Syt I_{VQ} (Fig. 4*A*,*B*). The difference between EGFP-Syt I_{VQ} and EGFP-Syt $I_{VQ;\,S\text{-}A}$ became significant when examined a few stimuli after the 5-HT pulse [amount of facilitation measured using EPSPs (42–44 or 43–45) EGFP-Syt I_{VQ} vs EGFP-Syt $I_{VQ;\,S\text{-}A}$; $p < 0.05$; Tukey's *post hoc* test]. This result is not consistent with Syt I_{VQ} being the PKC substrate that is important for the reversal of synaptic depression, because, in this case, converting the serine to alanine should reduce the ability to reverse synaptic depression.

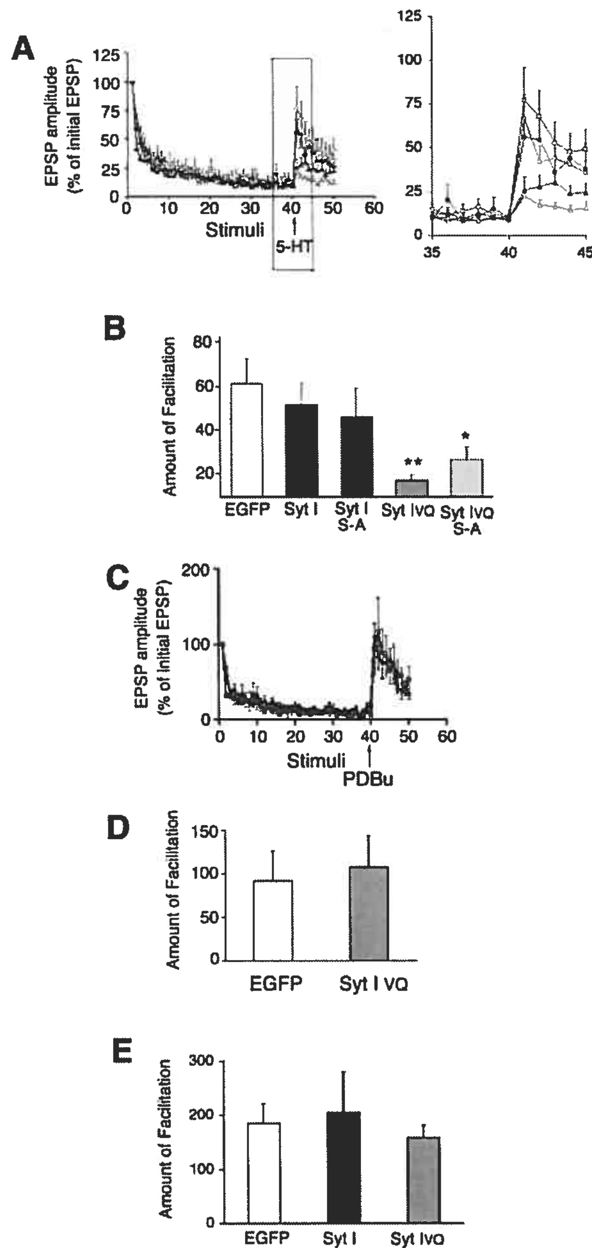


Figure 4. Syt I_{VQ} blocks the reversal of synaptic depression at a step upstream of PKC activation. *A*, Short-term facilitation of depressed synapses is blocked by overexpression of Syt I_{VQ} but not Syt I. Sensory to motor transmission was depressed by a series of 40 repeated intracellular stimuli (interstimulus interval, 20 sec), and 5-HT (10 μM) was applied to induce synaptic facilitation, followed by an additional 10 stimuli. Averaged normalized EPSPs (mean ± SEM) for cells expressing EGFP (open squares; $n = 23$), Syt I (open circles; $n = 8$), Syt I_{S-A} (filled circles; $n = 5$), Syt I_{VQ} (open triangles; $n = 19$), or Syt $I_{VQ;\,S-A}$ (filled triangles; $n = 12$) are shown. The region around 5-HT addition has been expanded for clarity below. *B*, The amount of facilitation was calculated as the difference between the average of the three normalized EPSPs after 5-HT and the three normalized EPSPs before 5-HT [ANOVA; $p < 0.005$; ***post hoc* Tukey's test showed that Syt I_{VQ} ($p < 0.01$) and Syt $I_{VQ;\,S-A}$ ($p < 0.05$) were significantly different from EGFP]. *C*, Same as in *A*, but PDBu (100 nM) was added after the 40th stimuli in cells expressing EGFP (open squares; $n = 4$) or Syt I_{VQ} (filled triangles; $n = 6$). *D*, The amount of facilitation was calculated as the difference between the average of the three normalized EPSPs after PDBu and the three normalized EPSPs before PDBu. There was no significant difference between EGFP- and Syt I_{VQ}-expressing cells (ANOVA; $p > 0.5$). *E*, Facilitation of rested SM synapses is unaffected by overexpression of either Syt I or Syt I_{VQ}. An initial EPSP was induced by single extracellular stimulation to the sensory neuron. After 5-HT (10 μM), a second EPSP was recorded. The interstimulus interval between the two EPSPs was 5 min. The EPSP amplitude was normalized to the initial control value. Facilitation was determined by comparing the difference between the two normalized EPSPs [EPSP 2 (after 5-HT) − EPSP 1 (before 5-HT); EGFP, $n = 5$; Syt I, $n = 8$; Syt I_{VQ}, $n = 6$]. No significant differences were observed (ANOVA; $p > 0.5$).

Moreover, mutating the serine to alanine in Syt I had no effect on the ability of 5-HT to reverse depression.

Overexpression of EGFP-Syt I, EGFP-Syt I_{VQ}, EGFP-Syt I_{S-A}, or EGFP-Syt $I_{VQ;\,S-A}$ did not affect the resting membrane potential or the rate of synaptic depression (Table 1). Overexpression of EGFP-Syt I showed a trend to lower initial EPSPs, although, because of the large variability in initial EPSPs, this was not significant in an ANOVA (Table 1). The reduction in EPSP size is similar to that seen in a previous study in which Syt I was overexpressed in this system (Martin et al., 1995).

Whereas overexpression of EGFP-Syt I_{VQ} significantly decreased the magnitude of the response to 5-HT in depressed synapses, the kinetics of the remaining effect of 5-HT was normal (Fig. 4*A*). This is in contrast to what is observed with overexpression of a dominant-negative form of PKC Apl II, in which the onset of facilitation was considerably delayed as might be expected for a true dominant-negative effect (Manseau et al., 2001). This suggested that the blockade was not attributable to a competition between wild-type and overexpressed EGFP-Syt I_{VQ} for PKC phosphorylation, but instead, that 5-HT does not activate PKC as well in the cells expressing EGFP-Syt I_{VQ}. To test whether facilitation downstream of PKC was intact, we examined whether EGFP-Syt I_{VQ} could block the effect of PDBu, which activates PKC independently of 5-HT. Indeed, EGFP-Syt I_{VQ} did not block phorbol ester-mediated reversal of synaptic depression (Fig. 4*C*,*D*), suggesting that the deficit lay in the ability of 5-HT to activate PKC. It should be noted that, although PDBu can increase transmitter release independently of PKC in some systems, the effect of PDBu on transmitter release at these synapses is blocked by inhibitors of PKC (Braha et al., 1990).

To determine whether there was a general deficit in 5-HT-mediated signal transduction, we looked at the facilitation of nondepressed synapses. This effect is meditated by 5-HT activation of PKA and not PKC (Ghirardi et al., 1992). In this case, facilitation was not affected by the overexpression of either EGFP-Syt I or EGFP-Syt I_{VQ} (Fig. 4*E*). This demonstrates that overexpression of EGFP-Syt I_{VQ} specifically interferes with the ability of 5-HT to reverse synaptic depression without generally affecting synaptic transmission or the ability of 5-HT to activate PKA.

Discussion

We found a novel alternative splice form of Syt I with a two amino acid VQ insert in the juxtamembrane region that joins the transmembrane region to C2A. Both Syt I and Syt I_{VQ} are expressed at equal levels. This splicing is evolutionary well conserved and physiologically significant because expression of Syt I_{VQ}, but not Syt I, blocked the reversal of synaptic depression.

A novel but conserved splice form in the juxtamembrane domain of Syt I

The juxtamembrane region between the transmembrane domain and C2A has not been studied extensively. Using antibodies for Syt I and Syt IV, Fukuda et al. (2001) found that these two isoforms were localized to distinct subcellular fractions in PC12 cells. Using chimeras, they showed that the localization signal was in the juxtamembrane region (Fukuda et al., 2001). Whereas invertebrates appear to have only one Syt I-like form, vertebrates have three isoforms, Syt I, II, and IX. Remarkably, although the juxtamembrane region of all three vertebrate Syt I-like isoforms is generally conserved, they are remarkably distinct in the segment in which the VQ splice exists, suggesting that this may be an important distinction between the different Syt I-like vertebrate

J. Neurosci., July 16, 2003 • 23(15):6238–6244 • 6243

Table 1. Comparison of intrinsic and synaptic properties of sensory neurons expressing EGFP or various EGFP-Syt I constructs

	Syt I_{VQ}	Syt I	Syt I_{S-A}	Syt $I_{VQ:S-A}$	EGFP	ANOVA
I_{hold} (pA)	−0.19 ± 0.16	−0.16 ± 0.07	−0.19 ± 0.10	−0.19 ± 0.13	−0.16 ± 0.15	NS
R_{in} (MΩ)	79 ± 35	71 ± 30	71 ± 29	96 ± 45	79 ± 31	NS
V_r (mV)	−32 ± 9.0	−30 ± 7.5	−40 ± 10	−32 ± 5.3	−32 ± 4.8	NS
EGFP levels	3.2 ± 0.4	3.1 ± 0.4	3.2 ± 0.3	3.3 ± 0.5	4.2 ± 0.8	NS
Initial EPSP (mV)	11.4 ± 9.8	6.2 ± 4.0	17.1 ± 10.5	14.3 ± 10.5	10.3 ± 6.3	NS
EPSPs 6–10 (% of initial)	25.1 ± 10.6	30.8 ± 14.9	22.2 ± 7.3	28.0 ± 12.4	26.8 ± 17.0	NS
EPSPs 36–40 (% of initial)	14.5 ± 7.9	11.6 ± 10.9	13.1 ± 9.0	10.9 ± 6.8	11.1 ± 11	NS

The holding current (I_{hold}) is the current needed to hold the sensory neuron (SN) at −50 mV before the first stimulus. Resting potential (V_r) and input resistance (R_{in}) were taken at the end of each experiment. The values for V_r are therefore underestimated because, at this point, 5-HT or PDBu was present in the bathing solution and the SNs were often spontaneously active when hyperpolarization was removed. EGFP levels were scored from 1 to 5; only cells expressing levels over 3 were used. EPSPs 6–10, Early depression; EPSPs 36–40, late depression; ANOVA, One-way ANOVA; NS, no significance; $p > 0.05$. All values are mean ± SD. Rows 1–5, Syt I_{VQ}, $n = 27$; Syt I, $n = 21$; Syt I_{S-A}, $n = 5$; Syt $I_{VQ:S-A}$, $n = 12$; EGFP, $n = 30$. Rows 6 and 7, Syt I_{VQ}, $n = 23$; Syt I, $n = 13$; Syt I_{S-A}, $n = 5$; Syt $I_{VQ:S-A}$, $n = 12$; EGFP, $n = 24$.

isoforms. Moreover, the conservation of this splicing event throughout evolution points to an important role for this domain.

Overexpression of Syt I_{VQ} blocks the reversal of depression mediated by 5-HT, but Syt I is not the PKC substrate important for the reversal of depression

The evidence that 5-HT mediates the reversal of synaptic depression through activation of PKC is strongly supported by both pharmacological inhibitors and activators of PKC and dominant-negative experiments (Braha et al., 1990; Ghirardi et al., 1992; Manseau et al., 2001). Syt I is a conserved PKC substrate involved in transmitter release, and the insertion is located quite near the PKC phosphorylation site. Thus, regulation of PKC phosphorylation represented an attractive step at which insertion of VQ could regulate Syt I. However, PKC phosphorylation *in vitro* does not differentiate between Syt I and Syt I_{VQ}, and Syt I_{VQ} does not block the reversal of synaptic depression mediated by phorbol esters. Moreover, the reversal of synaptic depression is not blocked by Syt I_{S-A}. Thus, the block by Syt I_{VQ} likely acts before PKC activation. For example, this could be attributable to less available 5-HT receptors that could activate PKC. Indeed, it has been suggested that these receptors were variably active early in synapse formation, suggesting that the receptors may be rate limiting in the ability of 5-HT to reverse synaptic depression (Sun and Schacher, 1996).

Interestingly, whereas PKC activity is required for the reversal of synaptic depression, overexpression of active PKC actually inhibited the ability of 5-HT to reverse synaptic depression (Manseau et al., 2001). This inhibition appeared kinetically similar to the block by Syt I_{VQ} and may be attributable to an effect of PKC on regulating the availability of 5-HT receptors (Manseau et al., 2001). Our results would be consistent with Syt I_{VQ} being the PKC substrate involved in this phenomenon because Syt $I_{VQ;\,S-A}$ showed reduced inhibition compared with Syt I_{VQ}.

In summary, we discovered a well conserved splice in the juxtamembrane region of Syt I. We demonstrated different physiological effects attributable to overexpression of the two distinct products of this splicing. Our results demonstrate an important undiscovered role for the juxtamembrane domain of Syt I.

References

Adolfsen B, Littleton JT (2001) Genetic and molecular analysis of the synaptotagmin family. Cell Mol Life Sci 58:393–402.

Ahmari SE, Buchanan J, Smith SJ (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. Nat Neurosci 3:445–451.

Bennett MK, Miller KG, Scheller RH (1993) Casein kinase II phosphorylates the synaptic vesicle protein p65. J Neurosci 13:1701–1707.

Braha O, Dale N, Hochner B, Klein M, Abrams TW, Kandel ER (1990) Second messengers involved in the two processes of presynaptic facilitation that contribute to sensitization and dishabituation in *Aplysia* sensory neurons. Proc Natl Acad Sci USA 87:2040–2044.

Byrne JH, Kandel ER (1996) Presynaptic facilitation revisited: state and time dependence. J Neurosci 16:425–435.

Craxton M, Olsen A, Goedert M (1997) Human synaptotagmin V (SYT5): sequence, genomic structure, and chromosomal location. Genomics 42:165–169.

Davletov B, Sontag JM, Hata Y, Petrenko AG, Fykse EM, Jahn R, Sudhof TC (1993) Phosphorylation of synaptotagmin I by casein kinase II. J Biol Chem 268:6816–6822.

DiAntonio A, Schwarz TL (1994) The effect on synaptic physiology of synaptotagmin mutations in *Drosophila*. Neuron 12:909–920.

Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Sudhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability. Nature 410:41–49.

Fukuda M, Ibata K, Mikoshiba K (2001) A unique spacer domain of synaptotagmin IV is essential for Golgi localization. J Neurochem 77:730–740.

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Sudhof TC (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell 79:717–727.

Ghirardi M, Braha O, Hochner B, Montarolo PG, Kandel ER, Dale N (1992) Roles of PKA and PKC in facilitation of evoked and spontaneous transmitter release at depressed and nondepressed synapses in *Aplysia* sensory neurons. Neuron 9:479–489.

Hilfiker S, Pieribone VA, Nordstedt C, Greengard P, Czernik AJ (1999) Regulation of synaptotagmin I phosphorylation by multiple protein kinases. J Neurochem 73:921–932.

Mackler JM, Drummond JA, Loewen CA, Robinson IM, Reist NE (2002) The C2BCa^{2+}-binding motif of synaptotagmin is required for synaptic transmission in vivo. Nature 418:340–344.

Manseau F, Fan X, Hueftlein T, Sossin WS, Castellucci VF (2001) Ca^{2+}-independent PKC Apl II mediates the serotonin induced facilitation at depressed synapses in *Aplysia*. J Neurosci 21:1247–1256.

Marqueze B, Berton F, Seagar M (2000) Synaptotagmins in membrane traffic: which vesicles do the tagmins tag? Biochimie 82:409–420.

Martin KC, Hu Y, Armitage BA, Siegelbaum SA, Kandel ER, Kaang BK (1995) Evidence for synaptotagmin as an inhibitory clamp on synaptic vesicle release in *Aplysia* neurons. Proc Natl Acad Sci USA 92:11307–11311.

Montarolo PG, Goelet P, Castellucci VF, Morgan J, Kandel ER, Schacher S (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. Science 234:1249–1254.

Nakhost A, Dyer JR, Pepio AM, Fan X, Sossin WS (1999) Protein kinase C phosphorylated at a conserved threonine is retained in the cytoplasm. J Biol Chem 274:28944–28949.

Nonet ML (1999) Visualization of synaptic specializations in live *C. elegans* with synaptic vesicle protein-GFP fusions. J Neurosci Methods 89:33–40.

Nonet ML, Grundahl K, Meyer BJ, Rand JB (1993) Synaptic function is impaired but not eliminated in *C. elegans* mutants lacking synaptotagmin. Cell 73:1291–1305.

Perin MS, Fried VA, Mignery GA, Jahn R, Sudhof TC (1990) Phospholipid

binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature 345:260–263.
Ramjaun AR, Micheva KD, Bouchelet I, McPherson PS (1997) Identification and characterization of a nerve terminal-enriched amphiphysin isoform. J Biol Chem 272:16700–16706.
Schiavo G, Osborne SL, Sgouros JG (1998) Synaptotagmins: more isoforms than functions? Biochem Biophys Res Commum 248:1–8.
Sossin WS, Fan XT, Saberi F (1996) Expression and characterization of *Aplysia* protein kinase C: a negative regulatory role for the E region. J Neurosci 16:10–18.
Sudhof TC (2002) Synaptotagmins: why so many? J Biol Chem 277:7629–7632.
Sun ZY, Schacher S (1996) Development of short-term heterosynaptic facilitation at *Aplysia* sensorimotor synapses in vitro is accompanied by changes in the functional expression of presynaptic serotonin receptors. J Neurophysiol 76:2250–2261.
Yamasaki S, Hu Y, Binz T, Kalkuhl A, Kurazono H, Tamura T, Jahn R, Kandel E, Niemann H (1994) Synaptobrevin/vesicle-associated membrane protein (VAMP) of *Aplysia* californica: structure and proteolysis by tetanus toxin and botulinal neurotoxins type D and F. Proc Natl Acad Sci USA 91:4688–4692.
Zhang JZ, Davletov BA, Sudhof TC, Anderson RG (1994) Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. Cell 78:751–760.

III ARTICLE 2

Identification and characterization of a novel C2B splice variant of synaptotagmin I.

Arash Nakhost, Gry Houeland, Vanessa E. Blandford
Vincent F. Castellucci and Wayne S. Sossin

The Journal of Neurochemistry (2004), 89(2):354-63.

Journal of Neurochemistry, 2004, **89**, 354–363 doi:10.1111/j.1471-4159.2004.02325.x

Identification and characterization of a novel C2B splice variant of synaptotagmin I

Arash Nakhost,* Gry Houeland,† Vanessa E. Blandford,* Vincent F. Castellucci† and Wayne S. Sossin*

**Department of Neurology and Neurosurgery, McGill University, Montreal Neurological Institute*
†Department of Physiology, University of Montreal, Montreal, QC, Canada

Abstract

We have identified an alternatively spliced form of synaptotagmin I in *Aplysia* neurons. This isoform, synaptotagmin I C2B-β, is generated by alternative exon usage in the C2B domain leading to nine amino acid changes in the C2B sequence from the previously characterized synaptotagmin I, now designated as synaptotagmin I C2B-α. Quantitative reverse transcriptase-polymerase chain reaction demonstrated that approximately 25% of mRNA encoding synaptotagmin I contained the C2B-β exon in the nervous system. Synaptotagmin I C2B-β showed greater resistance to digestion by chymotrypsin in the absence of calcium than did synaptotagmin I C2B-α, although both isoforms required the same amount of calcium to resist chymotrypsin digestion. The source of these changes in C2B properties was mapped to a single amino acid (threonine 358). We have also cloned SNAP 25 in *Aplysia* and show that it binds synaptotagmin I C2B-β with a higher affinity than synaptotagmin I C2B-α. These results suggest that this splicing alters biochemical properties of the C2B domain, affecting a number of its important known interactions.

Keywords: *Aplysia*, C2B domain, synaptotagmin, transmitter release.

J. Neurochem. (2004) **89**, 354–363.

Synaptotagmins (Syts) are a family of proteins that function in cellular trafficking and membrane fusion (Sudhof 2002). At least 13 Syt isoforms have been identified in mammals (Craxton 2001) as well as eight isoforms in *Drosophila* (Littleton 2000) and *enorhabditis. elegans* (Craxton 2001). Syt I is thought to act as the calcium sensor during exocytosis (Sudhof 2002). Syts are characterized by a short lumenal amino terminal, a single transmembrane region and a large cytoplasmic domain containing two C2 domains, C2A and C2B. C2 domains are protein motifs of about 130 amino acids that are found in a large number of proteins (Nalefski and Falke 1996; Rizo and Sudhof 1998). The three dimensional structure of the C2 domain consists of a β-sandwich formed by eight β-sheets with loops emerging from the top and bottom. In many cases, including Syt I, C2 domains bind calcium and phospholipids via their top loops (Davletov and Sudhof 1993; Sutton *et al.* 1995; Chapman and Davis 1998; Fernandez *et al.* 2001; Fernandez-Chacon *et al.* 2001). C2 domains also interact with a variety of other protein partners. In the case of Syt I, C2A binds to syntaxin in a calcium-dependant manner (Li *et al.* 1995), whereas C2B interacts with SNAP 25 (Schiavo *et al.* 1997) and AP2 (Zhang *et al.* 1994). The C2B domain also mediates the calcium dependent homo- and hetero-dimerization of synaptotagmins (Desai *et al.* 2000). The oligomerization of Syt I is thought to play an important role during membrane fusion (Littleton *et al.* 2001; Wu *et al.* 2003).

We study synaptic release and plasticity in *Aplysia californica*, which is a well-established model for investigating these events (Byrne and Kandel 1996). Based on its published sequence, *Aplysia* Syt I is a polypeptide of 426 residues that shares the putative domain structure of all other

Received August 6, 2003; revised manuscript received November 5, 2003; accepted December 9, 2003.

Address correspondence and reprint requests to Dr Wayne S. Sossin, Department of Neurology and Neurosurgery, McGill University, Montreal Neurological Institute, BT 110, 3801 rue University, Montreal, Quebec, Canada H3A 2B4. E-mail:

Abbreviations used: ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; GST, glutathione S-transferase; PCRs, polymerase chain reactions; RT, reverse transcriptase; Syt I, Synaptotagmin I; TBS, Tris-buffered saline; TX-100, Triton-X-100; VAMP, vesicle associated membrane protein.

 © 2004 International Society for Neurochemistry, *J. Neurochem.* (2004) **89**, 354–363

synaptotagmin isoforms (Martin *et al.* 1995). We have previously identified a spliced form of Syt I with two amino acids, V and Q, added to its juxtamembrane domain (Syt I_{VQ}) (Nakhost *et al.* 2003). Overexpression of Syt I_{VQ}, but not Syt I, blocks serotonin (5-HT)-mediated reversal of depression, demonstrating a functional difference for this evolutionarily conserved splicing event (Nakhost *et al.* 2003). Here, we report the identification and characterization of a second alternatively spliced form of Syt I (Syt I C2B-β) in the nervous system of *Aplysia.* This splicing results in nine amino acid changes, mainly in the non-conserved parts of the C2B domain. This alternative exon usage is also found in *C. elegans*, but does not appear to be present in *Drosophila* or mammals. The splice at the C2B domain does not appear to change the calcium affinity of Syt I. However, it enhances the ability of Syt I C2B-β to oligomerize and resist chymotryptic degradation in the absence of calcium. It also increases the ability of Syt I C2B-β to bind SNAP 25. Taken together, these results suggests that the amino acid changes that are introduced by splicing can cause a significant change in the C2B domain of Syt I, which affects a number of its important interactions.

Materials and methods

Constructs

The cytoplasmic domain of *Aplysia* Syt I and the full-length Syt I were cloned and put into pGEX-5X-1 vector (Amersham/Pharmacia, Oakville, Ont., Canada) as described (Nakhost *et al.* 2003). Enhanced green fluorescent protein (EGFP), enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) (Clontech Palo Alto, CA, USA) constructs of Syt I C2B-α and C2B-β in pNEX-3 were also generated as described previously Nakhost *et al.* 2003).

Constructs of both C2B domains, including the linker region between C2A and C2B, were made in pGEX-5X-1 (residues 828–1284), by using specific forward (F1: GGGGATCCCAGACACA GAGTCTGAGAAG) and reverse primers (R1: GGGAATTCT-TAGTTCTTCTCTTGGCACCCT) in polymerase chain reactions (PCRs) where the cytoplasmic domains of Syt I C2B-α or Syt I C2B-β in pGEX-5X-1 were used as template.

Two chimeras of these CB2 domains were also made, C2B-αβ and C2B-βα, using a two-step mutagenic procedure (Manseau *et al.* 2001). In the case of C2B-αβ, in the first step, R1 and the external primer F2 (ACTATCAAGAAATGTACCCTG) were used in a PCR reaction and Syt I C2B-β in pGEX-5X-1 was used as the template. In another PCR reaction using Syt I C2B-α in pGEX-5X-1 as template F1 and R2 (TTTCTTGATAGTTGTTTTCTTCTTCTTCA-GACG) primers were used. In the second step, these PCR products were mixed together and used as a template in a PCR reaction where F1 and R1 were used. The product of this set of PCR was subsequently cloned into pGEX-5X-1 using *Eco*RI and *Bam*H1 digests. In order to make C2B-βα: in the first step, the external R1 and internal F3: (ACCATTAAGAAAAACACCCTC) primers were used in a PCR, where Syt I C2B-α in pGEX-5X-1 was used as the template. In another set of PCR, the external F1, internal R3 (TTTCTTAATGGTGGTCTTCTTCTTTTTCACTCG) and Syt I C2B-β in pGEX-5X-1 were used. The second step was performed as described previously (Manseau *et al.* 2001).

C2B-α N_{347}-C and C2B-α G_{358}-T constructs were generated in a two-step mutagenic procedure as described (Manseau *et al.* 2001). In both cases, F1 and R1 were used as the external primers and C2B-α in pGEX-5X-1 was used as the PCR template. The internal primers for C2B-α N-C mutants were (F4: ACCATTAAGAAGTGCAC CCTCAATCCC, R4: ATTGAGGGTGCACTTCTTAATGGTTGT). The internal primers for C2B-α G-T mutants were (F5: GAG-TCATTTACCTTTGAGGTTCCCTTTGAG, R5: AACCTCAAAG-GTAAATGACTCGTTGAA). All constructs were sequenced over their entire amplified regions to confirm their correctness.

Quantitative reverse transcription (RT)-PCR

The relative amounts of Syt I C2B-α and Syt I C2B-β mRNAs were determined by quantitative reverse transcriptase (RT)-PCR. RNA isolation was performed using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA) according to the protocol provided by the manufacturer. Specific forward primers and a common reverse primer were used [(C2B-α-F: 1056-TTCAACGAGTCATTTGGC) (C2B-β-F: 984-TCCCTGATGCTCAATGGA) and (R: GCGCC CGGGTTAGTTCTTCTCTGGCA-1267)]. Mixes of plasmids encoding the cytoplasmic domains of Syt I C2B-α and Syt I C2B-β were used in control reactions as part of each PCR set in order to generate a standard curve. The PCR products were separated on agarose gels, illuminated under ultraviolet light, digitally scanned and quantified using NIH Image (http://rsb.info.nih.gov/nih-image/).

Recombinant soluble proteins and binding assays

Glutathione S-transferase (GST) fusion proteins were expressed in DH5α cells and subsequently purified using GST beads (Amersham). Soluble Syt I fragments were generated by factor Xa (Novagen, Madison, WI, USA) digest of GST bead-bound fusion proteins according to the protocol provided by the manufacturer. In brief, every 88 μL of GST-bound fusion protein was incubated with 10 μL of 10 × cleavage and capture buffer (500 mM Tris-HCl pH 8.0, 1 M NaCl, 50 mM $CaCl_2$) and 4 units of factor Xa for 4 h at room temperature (25°C) and put on a shaker. After the cleavage, factor Xa was removed with addition of Novagen Xarrest agarose (100 μL of slurry per 4 units of enzyme). The soluble target protein was recovered by using the spin filter provided with the kit.

To investigate the interaction between GST-SNAP 25 and the soluble cytoplasmic domains of Syt I C2B-α and Syt I C2B-β, the soluble forms of the cytoplasmic domain of Syt I C2B-α and β were generated by factor Xa cleavage of GST fusion proteins. Ten micrograms of these proteins were incubated with various concentrations of GST-SNAP 25 immobilized on glutathione sepharose beads in TBS plus 1% Triton X-100 in presence of either 20% factor Xa cleavage and capture buffer (1 mM calcium) in the presence or absence of 10 mM EGTA. These reactions were incubated at room temperature on a shaker for 1 h. The beads were subsequently washed 3 × 5 min with either 1 mL of TBS + 20% factor Xa in the presence or absence of 10 mM EGTA. After the last wash, 100 μL of the final buffer wash was left in each reaction and 20 μL of Laemmli (10% β-mercaptoethanol) buffer was added to each tube and boiled for 10 min. Twenty per cent of each reaction was subjected to

sodium dodecyl sulfate – gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane. Western blots were performed and analyzed as previously described (Nakhost *et al.* 1999) using a Syt I-specific antibody (Nakhost *et al.* 2003).

Immunoblots were quantitated using NIH Image using the uncalibrated OD function. We have shown earlier that this results in linear results over a wide range of protein concentrations (Nakhost *et al.* 1999). Images of Coomassie or Ponseau stained gels were quantitated using NIH Image without the uncalibrated OD function. The amounts bound were standardized to the level of fusion protein as determined by Ponseau or Coomassie staining.

Limited chymotrypsin proteolysis

Soluble forms of the cytoplasmic domains and various constructs of C2B domains of Syt I were generated by factor Xa cleavage of GST-fusion proteins (10–20 μL) and subjected to limited proteolysis in the presence or absence of 10 mm EGTA at various concentrations of chymotrypsin ranging from 0 to 100 ng/μL for 1 h at room temperature. All samples were put on a nutator during the proteolysis. Samples were subsequently boiled in Laemmli buffer (10% β-mercaptoethanol) and subjected to SDS page analysis on 12% gels and stained with Coomassie blue. These gels were digitally scanned and quantified using NIH Image.

Calcium dose–response experiments were carried out using a similar protocol, under a fixed concentration of chymotrypsin (50 ng/μL). As factor Xa cleavage-capture buffer contains calcium, all preparations of various soluble Syt I fusion proteins also contained calcium. In these experiments, calcium concentration was reduced by addition of EGTA. Each reaction contained 20 μg of protein (final volume of 100 μL with the starting calcium concentration of 1 mm). These reactions were stopped by the addition of Laemmli buffer (10% β-mercaptoethanol). They were subsequently boiled and subjected to SDS page analysis on 9% gels and stained with Coomassie blue.

Confocal laser microscopy on living cells

The cells that were co-injected with constructs tagged with either enhanced cyan fluorescent protein (ECFP) or the enhanced yellow fluorescent protein (EYFP), or Ds-Red and were visualized with a Zeiss LSM 510 confocal laser microscope (GmBh, Jena) as described (Nakhost *et al.* 2003).

Cloning of SNAP 25

Aplysia nervous system cDNA was generated using pleural, pedal, and abdominal ganglia from the animal. These ganglia were dissected and immediately frozen in liquid nitrogen, and then processed using the Qiagen RNEasy Minikit (Qiagen, Santa Clara, CA, USA) to obtain total RNA. cDNA template was made using Superscript II RT (Life Technologies, Gaithersburg, MD, USA). The cDNA product was subsequently used as a template in PCR to amplify fragments of SNAP 25 using degenerate primers. The following primers were used: SNAP 25 degenerate 5′ primers F1 [GA(T/C)GANCA(A/G)GGNGA(A/G)CA], F2 [GA(T/C)ATGA(A/G)GA(A/G)GCNGA], degenerate 3′ primers R1 [CAT(A/G)TCNA(T/A/G)NGCCAT(A/G)T] and R2 [TCNTCCAT(T/C)TC(G/A)T(T/C)(T/C)TC]. The nested degenerate PCR provided a fragment of SNAP 25 that was then extended using rapid amplification of cDNA ends (RACE) to generate the full-length sequence. GST-SNAP 25 was then generated using PCR to insert the full length *Aplysia* SNAP 25 into pGEX-5X-1. All sequencing was done on both strands using the services Bio S & T Inc. (Montreal, Québec, Canada).

Results

Identification of a novel alternatively spliced form of *Aplysia* synaptotagmin I

While cloning Syt I from the nervous system of *Aplysia*, we identified two alternatively spliced forms. The first, termed Syt I_{VQ} involved an alternative exon entry in the juxtamembrane domain of Syt I and is well conserved over evolution (Nakhost *et al.* 2003). Here, we report on a novel alternative splice that is found in the C2B domain. Figure 1 shows the nucleotide alignment of the C2B domain of the published Syt I (Syt I C2B-α) (Martin *et al.* 1995) and the new C2B spliced form (Syt I C2B-β). The C2B domains are of identical size, but there are 39 nucleotide changes in the C2B domain of Syt I C2B-β. However, because of many third-base-pair substitutions, this alters only nine amino acid changes and these are mainly in non-conserved residues (Fig. 1b). Interestingly, the segment of C2B where changes occur maps to a single exon that is conserved within all of the known synaptotagmins (Craxton 2001), suggesting that duplication of this exon is the source of the alternative splicing. There is alternative splicing of a similar duplicated exon in *C. elegans*, but this duplication is not present in the human or *Drosophila* genomes (Craxton 2001). All of the known C2B residues that are important for Syt I interactions with calcium, other Syts, AP2 and synprint are conserved between Syt I C2B-α and Syt I C2B-β (Chapman *et al.* 1998).

Quantitative reverse transcription (RT)-PCR studies from the *Aplysia* nervous system indicate that both forms of Syt I are present in the nervous system where 75% of Syt I mRNAs encode C2B-α, while the remaining 25% encodes C2B-β (Fig. 2). Similar results were obtained when RT-PCR was done with RNA isolated from sensory neuron clusters (data not shown). Treatment of sensory clusters with a paradigm that induces long-term facilitation (five pulses of 20 μm 5HT, each 5-min pulse separated by 15 min) did not alter the ratio of C2B-α and C2B-β measured 3 hours later (percentage change in ratio 10 ± 9%, $n = 4$, data not shown).

Chymotrypsin proteolysis of the cytoplasmic domains of Syt I C2B-α and Syt I C2B-β

One mechanism to look for differences between the two isoforms is to examine the protease sensitivity of these proteins in the presence or absence of calcium. It has been previously shown that binding of calcium to the C2B domain stabilizes it, resulting in the formation of a protease-resistant fragment (Littleton *et al.* 2001). We performed these studies

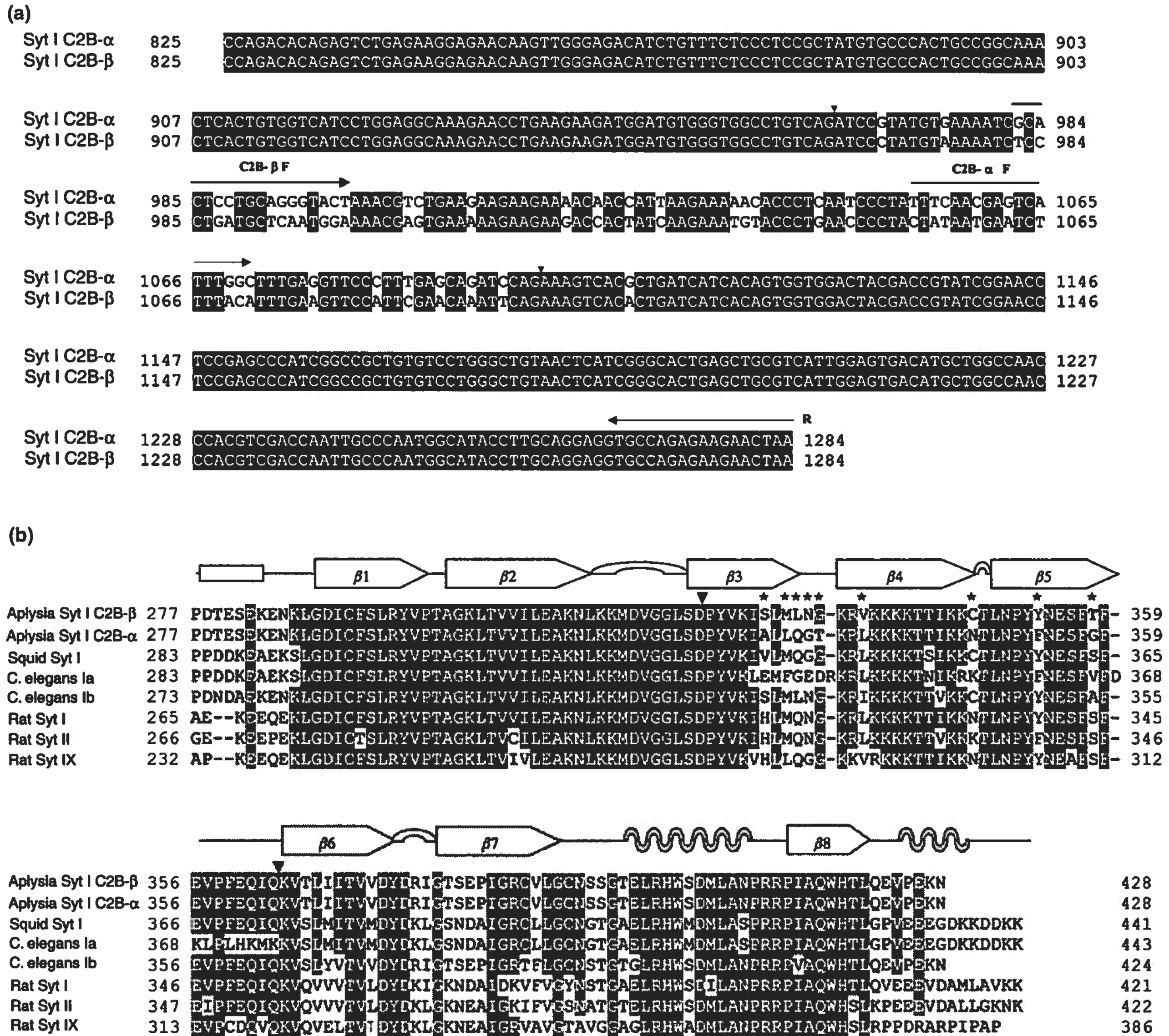


Fig. 1 Cloning of a novel splice isoform of Syt I. (a) Nucleotide sequence of two clones amplified from a nervous system library showing the entire C2B domain. Nucleotide numbering is from (825–1284). Primers used for quantitative RT-PCR are shown. Arrowheads indicate the position of the duplicated exon based on its conserved position throughout evolution (Craxton 2001). (b) Alignment of the Syt I-like C2B domain from vertebrate and invertebrate species. Conserved residues (5/8) are shaded. The amino acid differences between wild-type and spliced form Syt I are marked by stars. The beginning and the end of the exon where the splicing occurs is marked by arrow heads.

using a similar method. Soluble versions of the cytoplasmic domains of Syt I C2B-α and Syt I C2B-β were generated. These proteins were subsequently subjected to limited proteolysis in the presence of 10 mM EGTA or 1 mM calcium at various concentrations of chymotrypsin. Both isoforms are equally resistant to proteolysis in the presence of calcium (Fig. 3). However, Syt I C2B-β shows a significantly higher degree of resistance to proteolysis in the absence of calcium (Fig. 3).

Calcium dose–response of chymotrypsin proteolysis

The interaction of C2B with calcium regulates Syt I function. Syt I binds five calcium ions with low intrinsic affinity via the top loops of its C2 domains. C2A has three distinct calcium binding sites [~ 60 μM, 500 μM and > 20 mM, respectively (Ubach *et al.* 1998)]. C2B has two calcium binding sites with calcium affinities of 300–600 μM (Fernandez *et al.* 2001). Many of the C2B interactions with other proteins are regulated by calcium. Therefore, it is

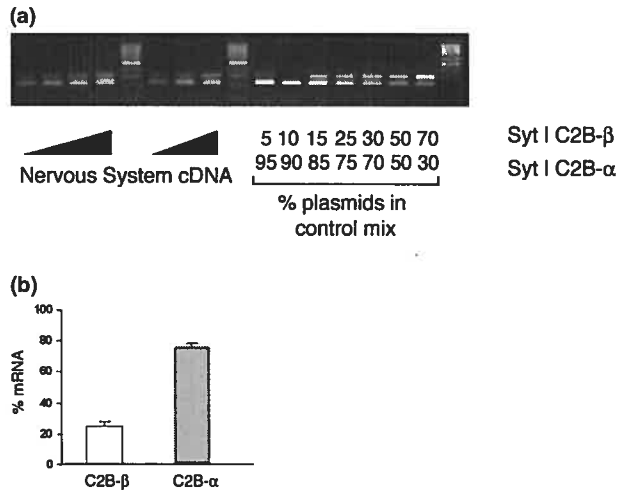


Fig. 2 RT-PCR of Syt I C2B-α and Syt I C2B-β. We used PCR primers flanking the splice region for RT-PCR from the *Aplysia* nervous system, two unique forward primers (C2B-αF and Syt I C2B-βF) and a common reverse primer. The positions of these primers are indicated on Fig. 1(a). (a) To generate a standard curve, mixes of plasmids containing different proportions of Syt I C2B-α and Syt I C2B-β were used as the template for PCR. At the same time, different amounts of nervous system template were used to ensure PCR amplification was in the linear range. (b) The relative amount of the two splice form mRNAs in the nervous system were determined according to a standard curve run in the same experiment. Values are mean ± SEM for four independent RT-PCRs from four individual animals.

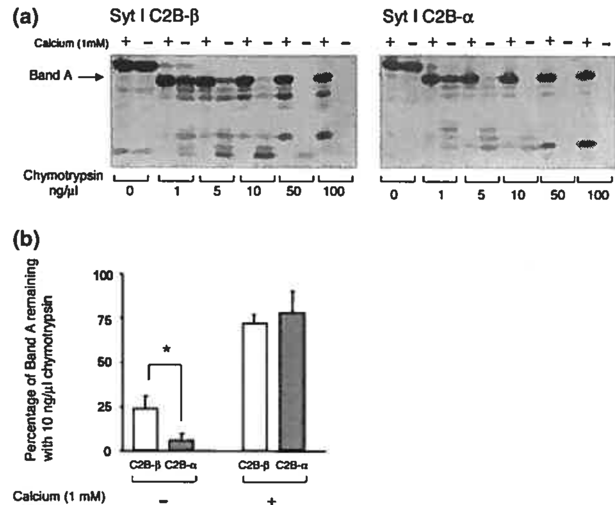


Fig. 3 Chymotrypsin proteolysis of the cytoplasmic domain of Syt I C2B-α and Syt I C2B-β. (a) Soluble forms of the cytoplasmic domains of Syt I C2B-α and Syt I C2B-β were generated by factor Xa cleavage of GST-fusion proteins and subjected to limited proteolysis in the presence of 10 mM EGTA (–) or 1 mM calcium (+). Samples were subsequently boiled in Laemmli buffer and subjected to SDS page and stained with Coomassie blue. Band A represents the stable proteolytic product. (b) The percentage of Band A remaining at 10 ng/μL chymotrypsin in the absence of calcium was significantly different for Syt I C2B-β and Syt I C2B-α (*two-tailed Students tailed *t*-test, $p < 0.01$, $n = 6$). There was no significant difference in cleavage in the presence of calcium.

possible that the differences that are observed between these two isoforms, occur because splicing has increased Syt I C2B-β's affinity for calcium such that it can be stabilized at relatively low calcium concentrations.

In order to address this question, we examined the concentration of calcium required for protection of soluble Syt I C2B-α and Syt I C2B-β using a high concentration of chymotrypsin (50 ng/μL). Both isoforms have a similar dose–response for calcium and their calcium-bound forms are protected to the same extent (Fig. 4). Thus, amino acid changes in C2B do not regulate the ability of calcium to stabilize the C2 domain.

Investigating the effect of splicing in C2B on binding of Syt I to SNAP 25

Previous studies have shown that SNAP 25 binds Syt I (Gerona *et al.* 2000; Zhang *et al.* 2002). In order to further investigate the structural differences between the two splice variants, the ability of each to bind SNAP 25 was investigated. We have cloned SNAP 25 from the nervous system of *Aplysia* (Houeland *et al.*, in preparation). The interaction between the entire cytoplasmic domain of Syt I with SNAP 25 was examined using GST-SNAP 25 fusion protein as the binding partner for soluble Syt I. These studies demonstrated that both forms of Syt I's binding to SNAP 25 increased about 2–4-fold in the presence of calcium (Figs 5a and c). This result is similar to previous findings, where calcium stimulated this interaction by 2.5–5-fold (Gerona *et al.* 2000; Zhang *et al.* 2002). However, Syt I C2B-β showed significantly more binding to GST-SNAP 25 irrespective of calcium (Figs 5a and b).

Mutational analysis of wild-type and spliced C2B domains

In order to determine the residues in the C2B domain conferring differences in structure, we generated C2B-α and C2B-β C2B domain constructs, as opposed to the entire cytoplasmic domain constructs used to this point. The chymotrypsin sensitivity was enhanced in the isolated C2B domains and again C2B-β was more resistant to proteolysis in the absence of calcium (Figs 6a and c). Chimeras of these two C2B domains were then generated, in which the most C-terminal three amino acids differing between the two domains were swapped (referred to as αβ and βα) (Fig. 7). Switching the last three differing amino acids of C2B-β to α (βα) resulted in the loss of C2B-β resistance to proteolysis (Figs 6a–c). Moreover, introduction of these residues in the wild-type sequence (C2B-αβ) enhanced the ability of C2B-α to resist chymotrypsin proteolysis in the absence of calcium (Figs 6a–c).

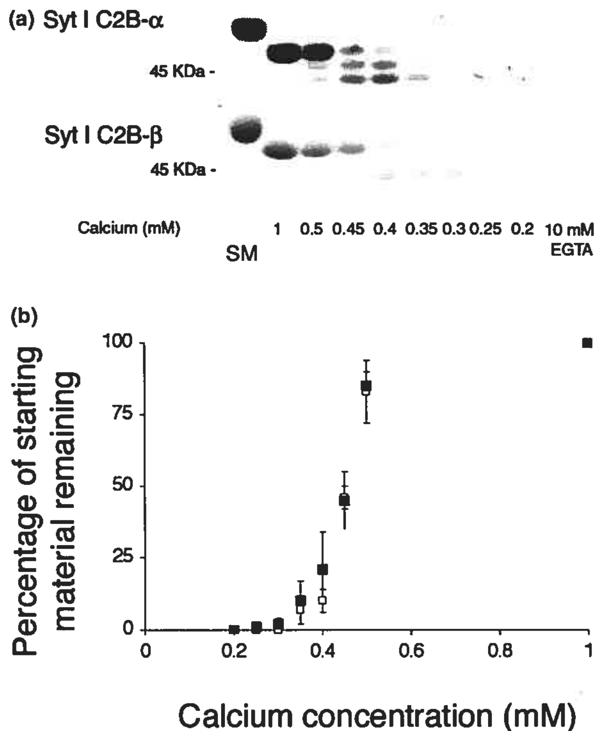


Fig. 4 Calcium dose–response experiments. (a) Soluble forms of the cytoplasmic domains of Syt I C2B-α and Syt I C2B-β were generated by factor Xa cleavage of GST-fusion proteins and subjected to limited proteolysis in the presence of different levels of calcium with a fixed concentration of chymotrypsin (50 ng/μL) for 1 hour at room temperature. Starting material (SM) was incubated without chymotrypsin but in the presence of 1 mM Ca^{2+}. As factor Xa cleavage-capture buffer contains calcium, in these experiments Ca^{+2} concentrations were changed by addition of EGTA. The effect of Ca^{+2} concentration on proteolysis was studied by monitoring a fragment of Syt I that is protected against proteolysis in the presence of Ca^{+2}. (b) Quantitation of experiments similar to the one shown above. Syt I C2B-α (open squares), Syt I C2B-β (closed squares). Values are mean ± SEM, $n = 4$.

Both F353 and G358 are part of the β5 sheet and N347 is part of loop two (Fig. 1). Based on the structural similarity of F and Y, the switching of these amino acids should not result in a major structural change. For that reason we focused our attention on other two C2B-α residues, N347 and G358. In general, these two amino acids are not well conserved among Syt I homologues or different synaptotagmin isoforms. In order to identify which one (or combination) of these residues is important for the structural difference between C2B domains, C2B-α G-T and C2B-αN-C constructs were generated and tested. The C2B-α G-T mutation has enhanced ability to resist proteolysis in the absence of calcium, similar to C2B-β (Figs 6a and c). Therefore, introduction of the threonine in the C2B-α was sufficient to switch its behavior to that of Syt I C2B-β (Fig. 6d). In contrast, the N-C mutation did not affect the stability of C2B-α in the absence of calcium but, surprisingly, made the C2B domain more sensitive to chymotrypsin digestion even in the presence of calcium (Figs 6a and b). Comparing the C2B-αβ construct to the C2B-α N-C construct again showed a large difference in stability based on the residue at 358 (Fig. 6e). The presence of the cysteine residue was not destabilizing in the presence of G358, but was unstable in the presence of T358. This interaction between a residue in β sheet 5 and in loop 2 suggests long-distance effects due to changes in the residue at position 358.

Consistent with changes in the proteins due to threonine 358, SDS–PAGE analysis of various soluble Syt I fusion proteins shows that both cytoplasmic and C2B domains of the wild-type and splice forms migrate differently (Fig. 7). Interestingly, the C2B-α G-T and C2B-β have a similar migration pattern on an SDS–PAGE, consistent with an important role for T358 in determining this property of Syt I (Fig. 7). Together, these results point to the T358 as the main amino acid residue that gives the spliced C2B its unique structural features.

Localization of Syt I C2B-β

The different properties of Syt I C2B-α and Syt I C2B-β could define differences in synaptic vesicle properties. We attempted to determine if they were present in the same synaptic vesicles, but were unsuccessful in generating antibodies specific for Syt I C2B-α and Syt I C2B-β. As an alternative strategy, we expressed fluorescently tagged versions of the full-length proteins into sensory neurons. Both versions contained the VQ insert. Co-localization of full-length ECFP-Syt I C2B-β and dsRED-tagged VAMP demonstrated that both ECFP-Syt I C2B-β (Fig. 8a) and ECFP-Syt I C2B-α (Nakhost *et al.* 2003) were present on clusters of synaptic vesicles (Fig. 8a). While the majority of expressed ECFP-Syt I C2B-α and EYFP-Syt I C2B-β were co-localized to the same punctae (Fig. 8b), a subset of punctae showed differences in the relative proportion of the two expressed fusion proteins (Fig. 8c). We observed this subset of punctae in four out of eight experiments and in these four cells 22% (19/83) of the punctae showed a substantial preference for one of the fusion proteins.

Discussion

The major difference between the splice forms appears to be position 358. The conversion of a glycine in Syt I C2B-α to a threonine in Syt I C2B-β may cause a structural change as this residue is located in a beta-sheet and glycine is intrinsically de-stabilizing in beta sheets (Minor and Kim 1994). Glycine may also affect flexibility or other aspects of Syt I's biochemical properties. The ability of calcium to stabilize the Syt I C2B domain in the presence of a cysteine in loop 2 also depended on the residue at position 358 (Fig. 6e), suggesting that changes in this residue does have effects on the loops of Syt I.

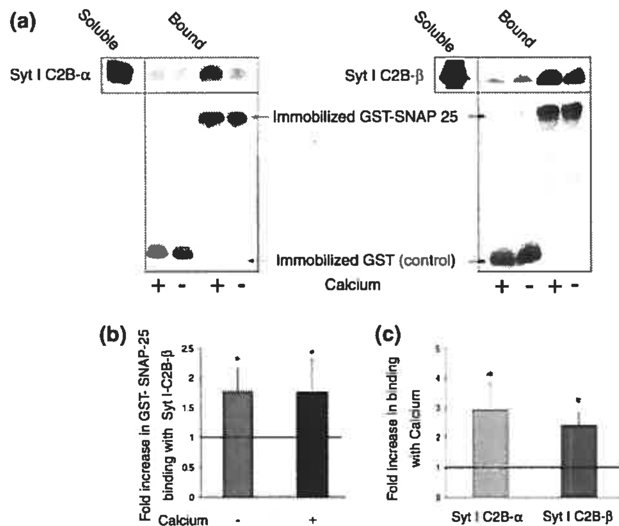


Fig. 5 Effect of splicing in C2B on binding to SNAP 25. (a). The amount of the cytoplasmic domain of Syt I C2B-α or β bound to either 5 μg of GST-SNAP 25 (experimental) or GST immobilized on glutathione sepharose beads in the presence of either 1 mM calcium or 10 mM EGTA was determined by immunoblotting. Ten per cent of the starting material was also separated on the gel. Ponseau-S staining of the same membrane prior to immuno-blotting to visualize amounts of GST and GST-SNAP 25 are shown below. (b) In each experiment, the amount of binding of equal amounts of Syt I C2B-β and Syt I C2B-α was compared with aliquots of GST-SNAP 25. The amount bound to GST was subtracted. The amount bound to Syt I C2B-α was set to 1 and the fold increase in binding to Syt I C2B-β was calculated. Values are mean + SEM, $n = 9$., There was significantly more Syt I C2B-β bound than Syt I C2B-α in the presence or absence of calcium (*two-tailed paired students *t*-test, $p < 0.05$ for both comparisons). (c) GST-SNAP 25 bound more soluble Syt I in the presence of calcium. The fold increase was the same for Syt I C2B-α and Syt I C2B-β and was significantly greater than 1 (*two-tailed one sample *t*-test, $p < 0.05$). Values are mean ± SEM, $n = 9$.

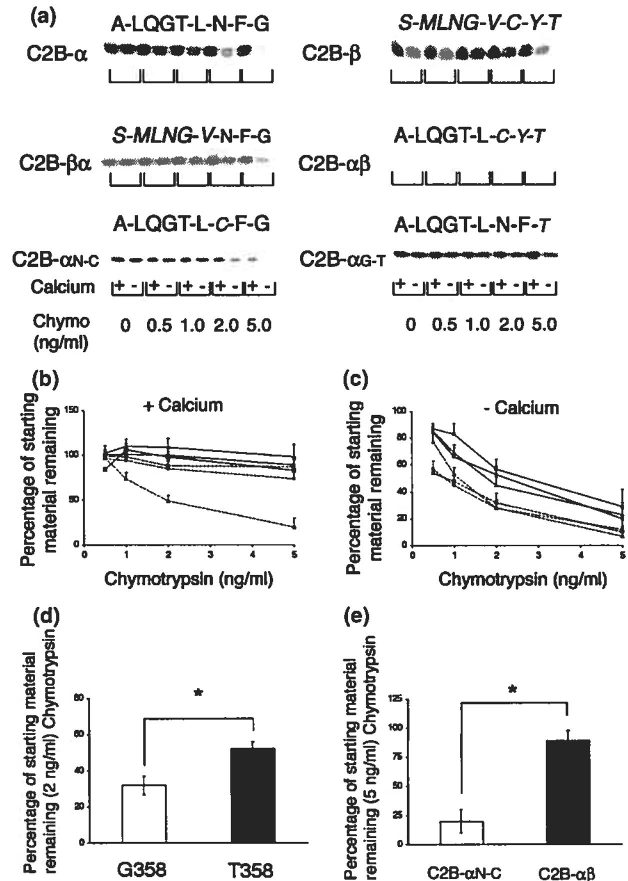


Fig. 6 Chymotrypsin proteolysis of the C2B domains of Syt I C2B-α and Syt I C2B-β. (a) Soluble forms of the regular and mutated C2B domains were generated by factor Xa cleavage of GST-fusion proteins and subjected to limited proteolysis in the presence of 10 mM EGTA (–) or 1 mM calcium (+) at various concentrations of chymotrypsin for 1 h at room temperature. Samples were subsequently boiled in Laemmli buffer, subjected to SDS page and stained with Coomassie blue. There are nine amino acid differences between C2B-α and C2B-β. These amino acid residues are shown in single letter amino acid code above each of the representative Coomassie stained gels. (b–c) Percentage of soluble protein remaining after digestion is plotted for the different fusion proteins (filled square, C2B-β, filled circle, C2B-αβ, filled triangle, C2B-α G-T, open circle C2B-α, open square C2B-βα, open triangles, C2B-α N-C. Results are shown either in the presence (b) or absence (c) of calcium. Values are SEM, $n = 6$. (d) The percentage of protein remaining at 2 ng/μL of chymotrypsin was compared between constructs containing G358 (C2B-α, C2B-βα, and C2B-α N-C) and those containing T358 (C2B-β, C2B-αβ, and C2Bα G-T). There was a significant difference in their sensitivity to chymotrypsin (*two tailed students *t*-test, $p < 0.01$, $n = 18$ for both groups). Values are mean ± SEM. (e) The percentage of protein remaining at 5 ng/μL of chymotrypsin was compared between C2B-αβ and C2B-α N-C. These constructs differ only in Y353 and G358. (*two tailed students *t*-test, $p < 0.01$, $n = 6$ for both groups).

The duplicated C2B exon present in *C. elegans* and *Aplysia* is not present in *Drosophila* or human genomes. This may be an ancient duplication that has been lost, or may have occurred more than once in evolution. Examination of other molluscan Syts in the database (squid and lymnae) show a sequence more similar to C2B-β than to C2B-α. In contrast, a partial sequence of sea urchin Syt I shows the presence of a glycine in the equivalent position to glycine 358, and thus may resemble C2B-α. Interestingly, this exon has recently been shown to be a target of RNA editing in *Drosophila*, suggesting that different C2B forms may also exist in this organism (Hoopengardner *et al.* 2003). It will be interesting to determine whether other organisms without splicing use RNA editing to alter this domain.

Based on the 3-D structure of Syt I (Fernandez *et al.* 2001), residue 358 is in close proximity to the polybasic region (part of the b4 sheet) that is implicated in many

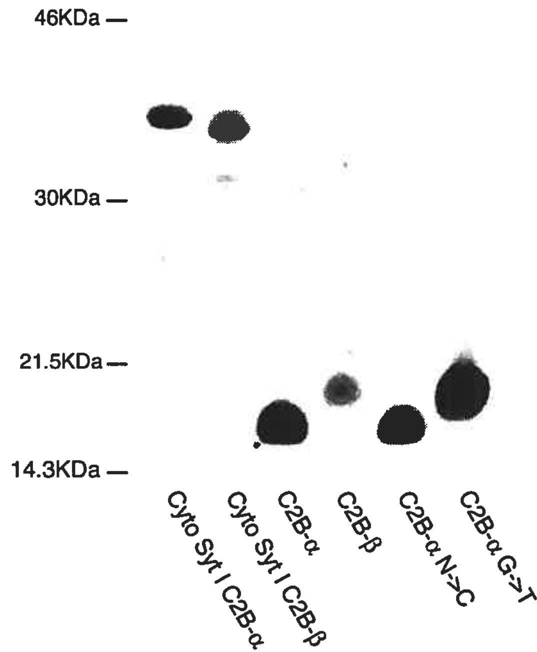


Fig. 7 SDS–PAGE analysis of various soluble Syt I proteins. Soluble forms of the cytoplasmic and C2B domains of Syt I C2B-α and Syt I C2B-β were generated by factor Xa cleavage of GST-fusion proteins. These soluble proteins were subjected to SDS–PAGE analysis and Coomassie staining. This experiment has been repeated three times with similar results.

protein–protein interactions (Chapman *et al.* 1998). However, studies now have shown that the calcium-dependent oligomerization of recombinant Syt I, and perhaps other interactions requiring the basic domain, may be due to bacterial contaminants that tightly bind to the C2B domain. This may suggest that differences between the splice isoforms are due to differences in binding to the bacterial contaminant. However, while the GST constructs used in our experiments do have nucleic acid contaminants based on high absorbance at 260 nM, the amount of contamination is the same in all the forms tested (Fig. 9). Thus, differing contamination is not an explanation for differences between the isoforms. Splicing in C2B also affected binding of *Aplysia* Syt I to SNAP 25. Although previous studies have shown that SNAP 25 binds Syt I in a calcium-dependent manner (Gerona *et al.* 2000; Zhang *et al.* 2002), the exact binding site on Syt I is not identified. While one study claims that this binding site is exclusively located on the C2B domain (Schiavo *et al.* 1997), another one shows that C2B does not bind SNAP 25 (Gerona *et al.* 2000). Gerona and co-workers show that, although C2A can bind SNAP 25 to some extent on its own, high-affinity interaction requires both C2A and C2B (Gerona *et al.* 2000). Our data is consistent with an important role for the C2B domain of Syt I in its binding to SNAP 25, as splicing of C2B affects this interaction.

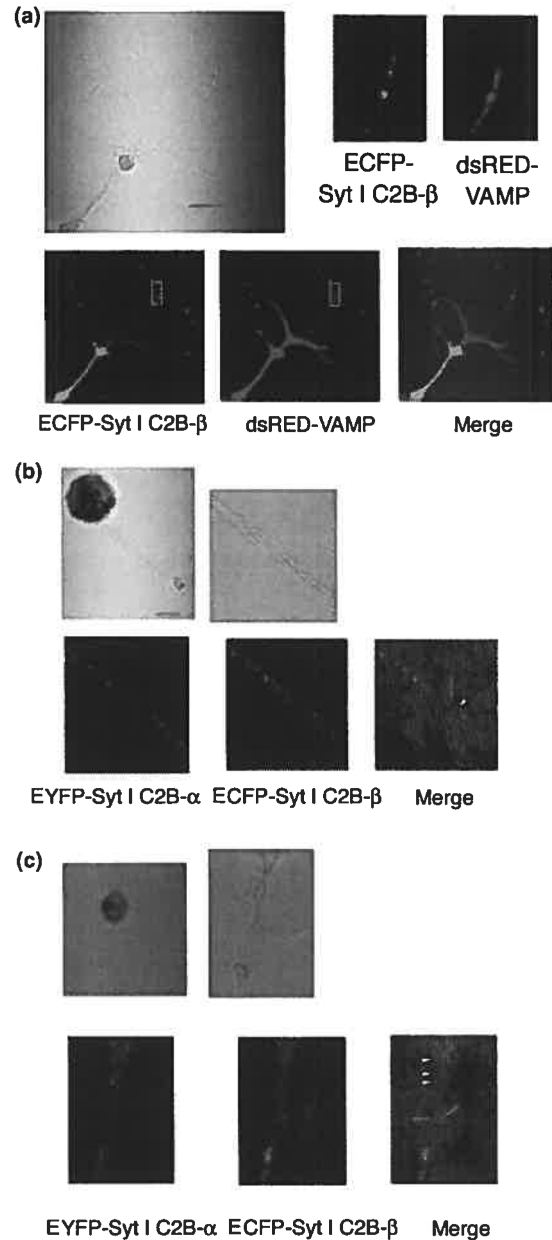


Fig. 8 Localization of CFP-tagged Syt I C2B-β in sensory neurons. (a) Nomarski images of the entire sensory cell and at higher magnification the region shown in the fluorescent images. Localization of ECFP-tagged Syt I C2B-β and ds-RED tagged *Aplysia* VAMP. Merge figures show good co-localization. (b) Nomarski images of the entire sensory cell and at higher magnification the region shown in the fluorescent images. Localization of EYFP-tagged Syt I C2B-α and ECFP-tagged Syt I C2B-β. Merge figures show good co-localization although there is one vesicle with low levels of ECFP-tagged Syt I C2B-β (arrowhead). (c) Nomarski images of the entire sensory cell and, at higher magnification, the region shown in the fluorescent images. Localization of EYFP-tagged Syt I C2B-α and ECFP-tagged Syt I C2B-β. Merge figures show some co-localization, although the levels of expressed fusion protein appear to differ between the punctae (see arrowheads for Syt I C2B-α enriched and arrows for Syt I C2B-β enriched).

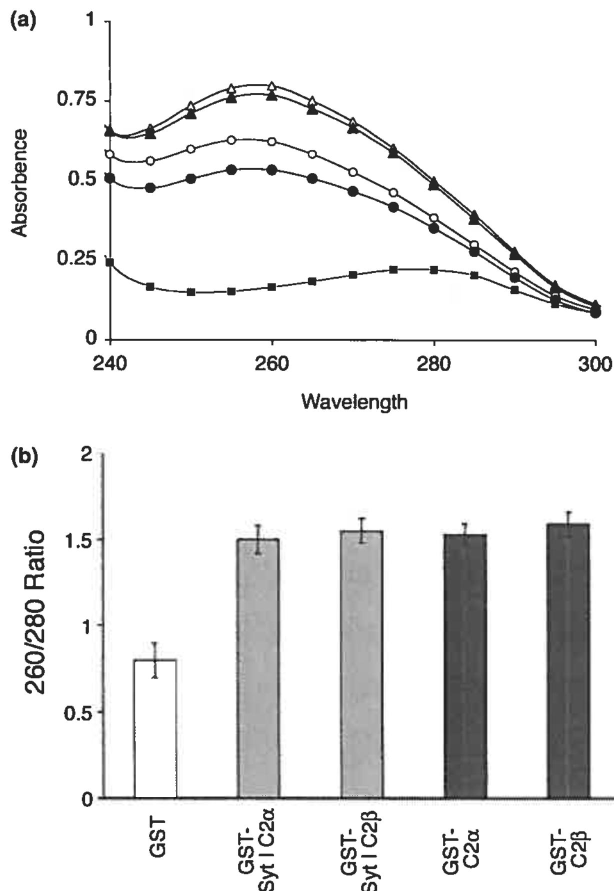


Fig. 9 Similar levels of bacterial contamination in the splice isoforms. (a) Absorbance spectrum for GST (circle) and GST fusion proteins containing either the entire cytoplasmic domain (GST-Syt I C2Bα, filled squares, or GST-Syt I C2Bβ, open squares) or just the C2B domain (GST-C2Bα, filled triangles, or GST-C2Bβ, open triangles). (b) Quantitation of the 260/280 ratio from independent preparations of the fusion proteins demonstrates no significant differences in the amount of bacterial contamination in the splice forms. Errors are SD, $n = 3$.

When overexpressed, the two splice isoforms appear to be present in different amounts in a subset of vesicles (Fig. 8). This may be due to differences in oligomerization between the isoforms. Indeed, the ability of the isoforms to oligomerize *in vitro* was different with C2B-β binding to itself more strongly than to C2B-α in the absence of calcium (data not shown). However, since this *in vitro* assay depends on the bacterial contamination (Ubach *et al.* 2001), further studies will be needed to determine if this difference is important in cells, where oligomerization depends on lipid and calcium interactions. But, as there are probably only a small number of synaptotagmin molecules in each synaptic vesicle (Fernandez-Chacon and Sudhof 1999), this observation may also reflect a stochastic distribution of the expressed proteins. Arguing against this possibility, we did not see this type of differential distribution when we overexpressed two forms that were identical except for the color of the fusion protein (Nakhost *et al.* 2003). Moreover, although there are a subset of punctae in which Syt I_{VQ} C2B-α and Syt I C2B-α did not co-localize, the number of these vesicles was smaller than that seen with the difference in the C2B splice. It is important to note that we cannot differentiate vesicles originated from the cell body (transport vesicles) and synaptic vesicles that are formed by endocytosis. One possible role proposed for the splice in the juxtamembrane domain was differential sorting in transport vesicles (Nakhost *et al.* 2003). This would affect the distribution in transport vesicles, but not in synaptic vesicles. The more numerous vesicles exhibiting differences in levels of the C2B spliced isoform would suggest that these are synaptic vesicles. If the properties of the C2B domain lead to differences in the efficiency of transmitter release, it would suggest that there could be subtle differences in the fusion abilities of individual synaptic vesicles based on their relative content of splice forms.

In summary, we have identified a novel alternative splice form of Syt I that results in a number of amino acid changes in the C2B domain. This spliced form is expressed in *Aplysia* neurons. The splice forms differ in a number of biochemical properties, such as sensitivity to chymotrypsin and binding to SNAP 25. Further studies will determine whether this splicing affects the physiological function of Syt I.

Acknowledgements

We thank Xiaotang Fan for technical assistance and Dr John Dyer, Dr Peter McPherson and Dr Phil Barker for comments on the manuscript. This work was supported by NSERC grant 187018 to WSS and CIHR grant MOP-14142 to VC. AN. is supported by a NSERC graduate studentship and WSS is a Killiam Scholar and supported by a CIHR Investigator award.

References

Byrne J. H. and Kandel E. R. (1996) Presynaptic facilitation revisited: state and time dependence. *J. Neurosci.* **16**, 425–435.

Chapman E. R. and Davis A. F. (1998) Direct interaction of a Ca2+-binding loop of synaptotagmin with lipid bilayers. *J. Biol. Chem.* **273**, 13995–14001.

Chapman E. R., Desai R. C., Davis A. F. and Tornehl C. K. (1998) Delineation of the oligomerization, AP-2 binding, and synprint binding region of the C2B domain of synaptotagmin. *J. Biol. Chem.* **273**, 32966–32972.

Craxton M. (2001) Genomic analysis of synaptotagmin genes. *Genomics* **77**, 43–49.

Davletov B. A. and Sudhof T. C. (1993) A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding. *J. Biol. Chem.* **268**, 26386–26390.

Desai R. C., Vyas B., Earles C. A., Littleton J. T., Kowalchyck J. A., Martin T. F. and Chapman E. R. (2000) The C2B domain of synaptotagmin is a Ca(2+)-sensing module essential for exocytosis. *J. Cell Biol.* **150**, 1125–1136.

Fernandez I., Arac D., Ubach J., Gerber S. H., Shin O., Gao Y., Anderson R. G., Sudhof T. C. and Rizo J. (2001) Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine. *Neuron* **32**, 1057–1069.

Fernandez-Chacon R. and Sudhof T. C. (1999) Genetics of synaptic vesicle function: toward the complete functional anatomy of an organelle. *Annu. Rev. Physiol.* **61**, 753–776.

Fernandez-Chacon R., Konigstorfer A., Gerber S. H., Garcia J., Matos M. F., Stevens C. F., Brose N., Rizo J., Rosenmund C. and Sudhof T. C. (2001) Synaptotagmin I functions as a calcium regulator of release probability. *Nature* **410**, 41–49.

Gerona R. R., Larsen E. C., Kowalchyk J. A. and Martin T. F. (2000) The C terminus of SNAP25 is essential for Ca(2+)-dependent binding of synaptotagmin to SNARE complexes. *J. Biol. Chem.* **275**, 6328–6336.

Hoopengardner B., Bhalla T., Staber C. and Reenan R. (2003) Nervous system targets of RNA editing identified by comparative genomics. *Science* **301**, 832–836.

Li C., Ullrich B., Zhang J. Z., Anderson R. G., Brose N. and Sudhof T. C. (1995) Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* **375**, 594–599.

Littleton J. T. (2000) A genomic analysis of membrane trafficking and neurotransmitter release in *Drosophila. J. Cell Biol.* **150**, F77–F82.

Littleton J. T., Bai J., Vyas B., Desai R., Baltus A. E., Garment M. B., Carlson S. D., Ganetzky B. and Chapman E. R. (2001) Synaptotagmin mutants reveal essential functions for the C2B domain in Ca2+-triggered fusion and recycling of synaptic vesicles *in vivo*. *J. Neurosci.* **21**, 1421–1433.

Manseau F., Fan X., Hueftlein T., Sossin W. S. and Castellucci V. F. (2001) Ca2+-independent PKC Apl II mediates the serotonin induced facilitation at depressed synapses in *Aplysia*. *J. Neurosci.* **21**, 1247–1256.

Martin K. C., Hu Y., Armitage B. A., Siegelbaum S. A., Kandel E. R. and Kaang B. K. (1995) Evidence for synaptotagmin as an inhibitory clamp on synaptic vesicle release in *Aplysia* neurons. *Proc. Natl Acad. Sci. USA* **92**, 11307–11311.

Minor D. L. Jr and Kim P. S. (1994) Measurement of the β-sheet-forming propensities of amino acids. *Nature* **367**, 660–663.

Nakhost A., Dyer J. R., Pepio A. M., Fan X. and Sossin W. S. (1999) Protein kinase C phosphorylated at a conserved threonine is retained in the cytoplasm. *J. Biol. Chem.* **274**, 28944–28949.

Nakhost A., Houeland G., Castellucci V. F. and Sossin W. S. (2003) Differential regulation of transmitter release by alternatively spliced forms of synaptotagmin I. *J. Neurosci.* **23**, 6238–6244.

Nalefski E. A. and Falke J. J. (1996) The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci.* **5**, 2375–2390.

Rizo J. and Sudhof T. C. (1998) C2-domains, structure and function of a universal Ca2+-binding domain. *J. Biol. Chem.* **273**, 15879–15882.

Schiavo G., Stenbeck G., Rothman J. E. and Sollner T. H. (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated. *Proc. Natl Acad. Sci. USA* **94**, 997–1001.

Sudhof T. C. (2002) Synaptotagmins: why so many? *J. Biol. Chem.* **277**, 7629–7632.

Sutton R. B., Davletov B. A., Berghuis A. M., Sudhof T. C. and Sprang S. R. (1995) Structure of the first C2 domain of synaptotagmin I: a novel Ca2+/phospholipid-binding fold. *Cell* **80**, 929–938.

Ubach J., Zhang X., Shao X., Sudhof T. C. and Rizo J. (1998) Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain? *EMBO J.* **17**, 3921–3930.

Ubach J., Lao Y., Fernandez I., Arac D., Sudhof T. C. and Rizo J. (2001) The C2B domain of synaptotagmin I is a Ca2+-binding module. *Biochemistry* **40**, 5854–5860.

Wu Y., He Y., Bai J., Ji S. R., Tucker W. C., Chapman E. R. and Sui S. F. (2003) Visualization of synaptotagmin I oligomers assembled onto lipid monolayers. *Proc. Natl Acad. Sci. USA* **100**, 2082–2087.

Zhang J. Z., Davletov B. A., Sudhof T. C. and Anderson R. G. (1994) Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. *Cell* **78**, 751–760.

Zhang X., Kim-Miller M. J., Fukuda M., Kowalchyk J. A. and Martin T. F. (2002) Ca2+-dependent synaptotagmin binding to SNAP-25 is essential for Ca2+-triggered exocytosis. *Neuron* **34**, 599–611.

IV ARTICLE 3

PKC modulation of transmitter release by SNAP-25 at sensory to motor synapses in *Aplysia*.

Gry Houeland, Arash Nakhost, Wayne S. Sossin, Vincent F. Castellucci

The Journal of Neurophysiology (2007), 97:134-143.

V. Discussion

The analysis of sensitization in *Aplysia* is a classical example of a reductive approach. It has permitted the identification of molecular devices that could perform functions of acquisition of cellular information (e.g. serotonin receptors), storage (e.g. protein kinases),

J Neurophysiol 97: 134–143, 2007.
First published September 13, 2006; doi:10.1152/jn.00122.2006.

PKC Modulation of Transmitter Release by SNAP-25 at Sensory-to-Motor Synapses in *Aplysia*

Gry Houeland,[1] Arash Nakhost,[2] Wayne S. Sossin,[2] and Vincent F. Castellucci[1]
[1]*Department of Physiology, University of Montreal; and* [2]*Department of Neurology and Neurosurgery, McGill University, Montreal Neurological Institute, Montreal, Quebec, Canada*

Submitted 2 February 2006; accepted in final form 11 September 2006

Houeland G, Nakhost A, Sossin WS, Castellucci VF. PKC modulation of transmitter release by SNAP-25 at sensory-to-motor synapses in *Aplysia. J Neurophysiol* 97: 134–143, 2007. First published September 13, 2006; doi:10.1152/jn.00122.2006. Activation of phosphokinase C (PKC) can increase transmitter release at sensory–motor neuron synapses in *Aplysia*, but the target of PKC phosphorylation has not been determined. One putative target of PKC at synapses is the synaptosomal-associated protein of 25 kDa (SNAP-25), a member of the SNARE protein complex implicated in synaptic vesicle docking and fusion. To determine whether PKC regulated transmitter release through phosphorylation of SNAP-25, we cloned *Aplysia* SNAP-25 and expressed enhanced green fluorescent protein (EGFP)–coupled SNAP-25 constructs mutated at the PKC phosphorylation site Ser198 in *Aplysia* sensory neurons. We found several distinct effects of expression of EGFP–SNAP-25 constructs. First, the rates of synaptic depression were slowed when cells contained SNAP-25 with phosphomimetic residues Glu or Asp. Second, PDBu-mediated increases in transmitter release at naïve synapses were blocked in cells expressing nonphosphorylated-state SNAP-25. Finally, expression of EGFP-coupled SNAP-25 but not uncoupled SNAP-25 inhibited 5-HT–mediated reversal of depression and the ability of EGFP-coupled SNAP-25 to inhibit the reversal of depression was affected by changes at Ser198. These results suggest SNAP-25 and phosphorylation of SNAP-25 by PKC can regulate transmitter release at *Aplysia* sensory–motor neuron synapses by a number of distinct processes.

INTRODUCTION

PKC is a Ca^{2+} phospholipid-dependent protein kinase and plays a prominent role in many neuronal processes including the regulation of transmitter release (Hilfiker and Augustine 1999; Waters and Smith 2000). Activation of PKC increases both the size and the rate at which the readily releasable pool (RRP) refills in cultured hippocampal autapses (Stevens and Sullivan 1998) and in chromaffin cells (Smith et al. 1998). In chromaffin cells, PKC enhances exocytosis both by increasing the RRP and by shifting vesicles to a highly Ca^{2+} sensitive state, enabling exocytosis at sites relatively distant from Ca^{2+} channels (Yang et al. 2002).

Aplysia sensory-to-motor neuron synapses show a marked depression at low frequencies that contributes to behavioral habituation (Byrne et al. 1978). Homosynaptic depression is reversed by the neurotransmitter 5-hydroxytryptamine (5-HT, serotonin) through activation of a Ca^{2+}-independent form of PKC, PKC Apl II (Ghirardi et al. 1992; Manseau et al. 2001). Whereas activation of PKC by 5-HT increases transmitter release only at depressed synapses, activation of PKC by phorbol esters increases transmitter release at both naïve and depressed synapses (Braha et al. 1990; Ghirardi et al. 1992; Nakhost et al. 2003). However, the molecular targets of PKC at either depressed or naïve synapses have not been identified. We previously examined the role of the potential target synaptotagmin I (Syt I) in synaptic modulation of the sensory–motor synapse (Nakhost et al. 2003). However, although PKC phosphorylates Syt I in *Aplysia* and splicing of Syt I regulates transmitter release, synaptotagmin was not the phosphorylation target for PKC (Nakhost et al. 2003). Here, we focus on another possible substrate, synaptosomal-associated protein of 25 kDa (SNAP-25).

SNAP-25 is essential for evoked synaptic transmission. In SNAP-25–deficient mice, spontaneous release persists, whereas evoked release is abolished (Sørensen et al. 2003; Washbourne et al. 2002). SNAP-25 is involved in the molecular regulation of neurotransmitter release through its association with vehicle-associated membrane protein (VAMP, synaptobrevin) and syntaxin (Südhof 1995). Together these three factors form a stable SNARE complex of proteins (Fernàndez-Chacon and Südhof 1999), which is essential for evoked neurotransmitter release. In vitro evidence indicates that the SNARE complex can promote fusion between lipid vesicles (Schuette et al. 2004; Weber et al. 1998). Moreover, this complex is likely to provide a framework for a variety of protein interactions involved in assembly and recycling of synaptic vesicles (Brunger 2000).

Although the role of the SNARE complex in synaptic vesicle release is clear, there is less evidence that the physiological modulation of transmitter release arises from posttranslational modification of SNARE proteins. Perhaps the best evidence for this phenomenon is the phosphorylation of SNAP-25 at Ser187 by PKC (Genoud et al. 1999; Shimazaki et al. 1996). The amount of SNAP-25 associated with syntaxin decreases after PKC-induced phoshorylation of SNAP-25 in PC12 cells (Shimazaki et al. 1996) and phosphorylation at this site increases recruitment of dense-core vesicles in chromaffin cells (Nagy et al. 2002). However, SNAP-25 phosphorylation was not important for phorbol 12,13-dibutyrate (PDBu)–mediated increase in transmitter release in CA1 neurons (Finley et al. 2003), calling into question whether SNAP-25 phosphorylation is also important in regulating release of synaptic vesicles.

In the present paper, we used the plastic synapses between mechanoreceptor sensory neurons and siphon motor neurons of *Aplysia* as a model to investigate whether PKC phosphorylation of SNAP-25 plays a modulatory role in evoked release of synaptic vesicles. Our results suggest an important role for SNAP-25 and phosphorylation of SNAP-25 in multiple aspects

Address for reprint requests and other correspondence: V. F. Castellucci, Pavillon Paul-G. Desmarais, 2960, Chemin de la tour, CP 6128, Succ. Centre-ville, Montreal, Quebec, Canada H3C 3J7 (E-mail: [redacted]

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

0022-3077/07 $8.00 Copyright © 2007 The American Physiological Society

Downloaded from jn.physiology.org on January 17, 2007

of the regulation of synaptic vesicle release at sensory–motor neuron synapses.

METHODS

Aplysia californica (75–200 g) were purchased from Marine Specimens Unlimited (Pacific Palisades, CA) or the *Aplysia* Resource Facility at the University of Miami (Miami, FL). They were kept in an aquarium for ≥3 days before experimentation. Dissection and isolation of tissues and cultured neurons were as previously described (Manseau et al. 2001).

Cloning of SNAP-25

Aplysia nervous system cDNA was generated using pleural, pedal, and abdominal ganglia from the animal. These ganglia were dissected and immediately frozen in liquid nitrogen, and then processed using the Qiagen RNEasy Minikit (Qiagen, Santa Clara, CA) to obtain total RNA. cDNA template was made using Superscript II RT (Life Technologies, Gaithersburg, MD). The cDNA product was subsequently used as a template in PCR to amplify fragments of SNAP-25 using degenerate primers. The following primers were used: SNAP-25 degenerate 5′ primers: F1 [GA(T/C)GAN CA(A/G)GG NGA(A/G)CA], F2 [GA(T/C) ATGA(A/G) GA(A/G)GC NGA], degenerate 3′ primers R1 [CAT(A/G)TC NA(T/A/G)NGC CAT(A/G)T], and R2 [TCN TCC AT(T/C)TC(G/A) T(T/C) TC(G/A) T(T/C)(T/C)TC].

The nested degenerate PCR provided a fragment of SNAP-25 that was then extended using rapid amplification of cDNA ends (RACE) to generate the full-length sequence. GST–SNAP-25 was then generated using RT-PCR from total nervous system RNA to insert the full length *Aplysia* SNAP-25 into pGEX-5X-1. This plasmid was then cut with *Bam* HI and *Kpn* I to insert into the plasmid pNEX-3 for expression in *Aplysia* neurons and pGEX5.2 for generation of a GST–SNAP 25 fusion protein. To confirm that this isoform of SNAP-25 was expressed in sensory neurons we recloned SNAP-25 using RT-PCR from total sensory neuron RNA and examined three independent PCR products. All PCR products were identical to the previously isolated *Aplysia* SNAP-25. EGFP fusion proteins were generated by amplifying SNAP-25 with PCR and inserting into the Kpn site of pNEX3–EGFP. The lack of introduced errors was again confirmed with sequencing. Finally, mutations were made with overlap PCR and confirmed by sequencing. All sequencing was done on both strands using the services of Bio S&T (Montreal, Quebec, Canada). *Aplysia* SNAP-25 GenBank Accession Number: DQ382281.

Expression in SF9 cells

The pNEX-3 plasmids were expressed in SF9 cells by transfection with lipofectamine. By 48 h after transfection cells were harvested in sample buffer and separated on SDS–PAGE gels, transferred to nitrocellulose, and immunoblotted with an antibody to GFP.

Generation of an antibody to SNAP-25

The GST–SNAP 25 fusion protein was used as an immunogen for injection into rabbits. The resultant serum was used without purification.

Fluorescence microscopy

Injected sensory neurons were visualized at ×4 with a Nikon fluorescence microscope (Nikon Eclipse E600). The success rate of expression ranged from 50 to 90%. Expression was stable 24 h after injection and the cells remained fluorescent for about 1 wk.

Cell culture preparation

Injections of plasmid DNA and physiological paradigms were as previously described (Manseau et al. 2001). Briefly, 4 days after injection of the EGFP-tagged construct into the sensory neuron (SN) and 2 days after pairing with a motor neuron (MN), excitatory postsynaptic potentials (EPSPs) were evoked every 20 s in the motor neuron that was kept at −50 mV. After 40 evoked EPSPs, 5-HT (10 μM final concentration) was added to the bath and 10 additional EPSPs were sampled. In another set of experiments, the phorbol ester phorbol 12,13-dibutyrate (PDBu, 100 nM final concentration) was added after the first EPSP, a second EPSP was evoked 10 min later, followed by 38 evoked EPSPs to observe the rate of depression afterward. In some of the PDBu experiments a PKC inhibitor, bisinoolylmaleimide I (Bis) (10 μm final concentration), was added to the bath 10 min before the start of an experiment and remained in the bath throughout. All experiments were performed at room temperature and the initial EPSP amplitude had to be ≥2 mV for an experiment to progress. In a couple of experiments the MN spiked in the first EPSP or after the addition of PDBu. In this case we measured the EPSP at the shoulder of the spike.

Data acquisition and analysis

Data were acquired and analyzed digitally using CLAMPEX 9 and a modified version of pCLAMP (Axon Instruments) (Manseau et al. 2001). The effects of the various constructs were always tested in parallel. EPSPs were always normalized to the size of the initial EPSP.

Changes in synaptic transmission

The difference in homosynaptic depression was quantified using a nonparametric bootstrap test procedure with 10,000 repetitions and a criterion of $P < 0.05$ (Efron and Tibshirani 1986). The amount of facilitation was calculated as the difference between EPSPs after treatment (averages of EPSPs 41–43) and EPSPs before treatment (averages of EPSPs 38–40), or in the case of PDBu, the difference between EPSP 1 and EPSP 2 measured after PDBu. Significance was calculated by Student's *t*-test.

Immunofluorescence

To identify the localization of SNAP-25 wild-type and SNAP-25 mutants at *Aplysia* sensory–motor synapses, dissociated *Aplysia* SNs were plated on Matek (Ashland, MA) polylysine-coated glass-bottom culture dishes and coinjected with EGFP–SNAP-25 and DsRed–VAMP (as an indicator for synapse localization) or DsRed–VAMP alone. The cultures were subsequently labeled with an *Aplysia*-specific SNAP-25 antibody (Anti SNAP-25). Four days after injection of the EGFP-tagged construct into the SN and 2 days after pairing with an MN, the cells were fixed in 4% paraformaldehyde in a 30% sucrose-buffered PBS solution for 60 min. The cells were then solubilized and permeabilized in a 30% sucrose-buffered PBS containing 0.1% Triton for 10 min. The cultures were rinsed in PBS and free aldehydes were quenched with 50 mM NH_4Cl for 30 min, then incubated in 10% normal goat serum containing 0.5% Triton in PBS for 30 min to block nonspecific binding. After 1-h incubation with primary antibodies (Anti SNAP-25) diluted (1:50) in blocking buffer, cultures were rinsed in PBS and incubated for 1 h in Alexa Fluor 644–conjugated goat anti-rabbit secondary antibodies (1:200) (Molecular Probes, Eugene, OR) diluted in blocking buffer. Cultures were rinsed in PBS and then mounted with Shandon Immu-Mount (Thermo, Pittsburg, PA) mounting media. All steps were performed at room temperature.

Downloaded from jn.physiology.org on January 17, 2007

Confocal microscopy

The triple-labeled sections were visualized simultaneously with a Zeiss LSM 510 confocal laser microscope (Carl Zeiss, Jena, Germany), equipped with one argon (488 nm) and two He/Ne lasers of 543 and 633 nm.

RESULTS

Cloning of Aplysia SNAP-25

Aplysia SNAP-25 was cloned using degenerate PCR and RACE and confirmed by sequencing three clones isolated using RT-PCR from sensory neurons. We found that the PKC phosphorylation site Ser198 in *Aplysia* (the equivalent of mammalian Ser187) is conserved over evolution. Figure 1*A* shows the amino acid alignment with other published SNAP-25 sequences. To study the effect of the phosphorylation of Ser198 in SNAP-25, various plasmid constructs encoding EGFP or EGFP-tagged SNAP-25 constructs were generated. We replaced *Aplysia* Ser198 with the negatively charged glutamate (S198E) or aspartate (S198D), both of which mimic the effect of PKC phosphorylation by introducing a negative charge, but may differ in their orientation based on possible rotamer possibilities, and thus may impose spatial constraints on the three-dimensional structure of SNAP-25. Conversely, we substituted serine with the nonpolar alanine (S198A) or cysteine (S198C), both of which cannot be phosphorylated. Cysteine is suggested to be a better substitute for the nonphosphorylated state because of its longer side chain, but there is a danger of forming inappropriate disulfide bonds. EGFP was coupled to the N-terminus of SNAP-25 as an indicator of expression. N-terminal–coupled EGFP–SNAP-25 was used in a number of functional studies in other systems and is reported to fully functionally replace SNAP-25 (Finley et al. 2002, 2003; Nagy et al. 2002, 2004; Sørensen et al. 2002). All EGFP–SNAP-25 constructs expressed well in SF9 cells and we did not observe any cleavage between the EGFP and SNAP-25 (Fig. 1*B*).

SNAP and SNAP-25 mutants are both localized to synaptic vesicles

We further examined the subcellular distribution of EGFP–SNAP-25 and EGFP–SNAP-25 mutants and tested whether these constructs localized at synaptic sites. Both wild-type and mutant EGFP–SNAP 25 co-localized with DsRed-tagged *Aplysia* VAMP at concentrations of VAMP likely to mark pools of synaptic vesicles (Fig. 2*A*). FP-tagged VAMP was previously used to mark synaptic vesicle pools in many systems (Ahmari et al. 2000; Nonet 1999) and in *Aplysia* sensory neurons, tagged VAMP co-localizes with antibodies to clustered glutamate at sensory-to-motor neuron synapses (data not shown). However, some of the puncta of tagged VAMP (see wt SNAP-25 in Fig. 2*A*) do not co-localize. These are most probably small clusters of synaptic vesicles traveling down the neurites (Ahmari et al. 2000). Using an antibody raised to endogenous SNAP-25 (Fig. 2*B*), we also show that, as expected, endogenous SNAP-25 also localizes to sites where VAMP is concentrated (Fig. 2*C*, asterisk), although this is less obvious because SNAP-25 immunoreactivity is also present in the motor neuron and the immunolocalization of endogenous protein has more background staining than the fluorescent proteins. These experiments also demonstrate that the levels of overexpressed EGFP–SNAP-25 are much greater than those of the endogenous protein, as when sensory neurons are expressing EGFP–SNAP-25 (or the mutants; data not shown), at laser levels five times lower than in Fig. 2*C* and more than sufficient

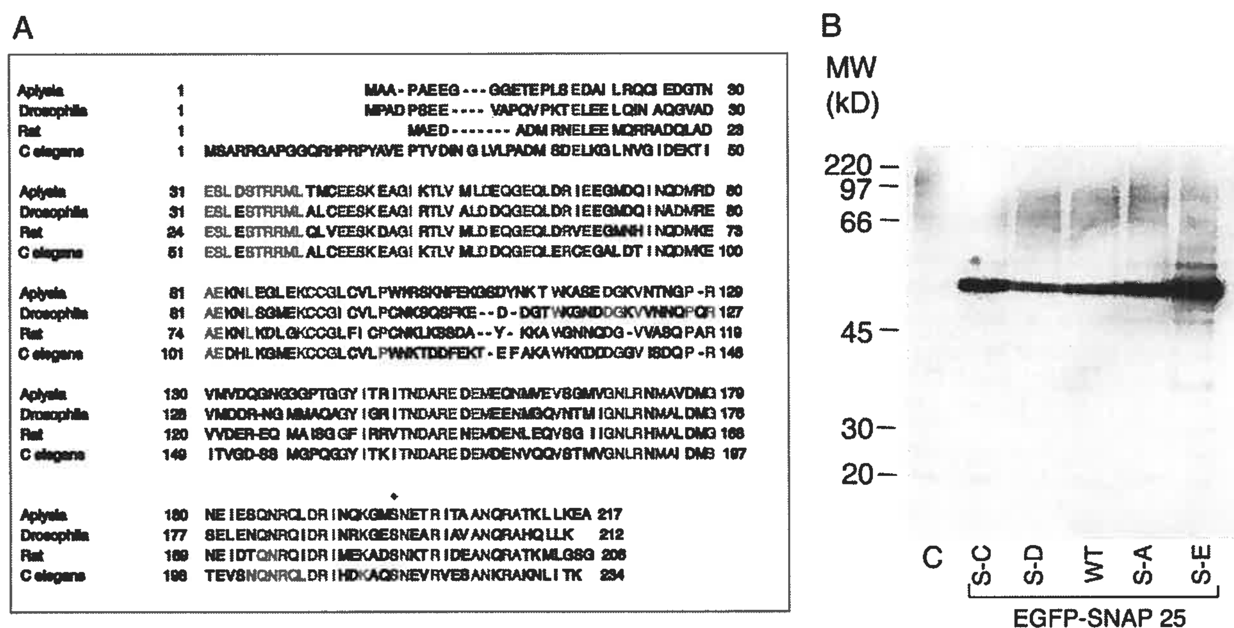


FIG. 1. Cloning of *Aplysia* synaptosomal-associated protein of 25 kDa (SNAP-25). *A*: protein sequence SNAP-25 amplified from a nervous system library. Alignment of SNAP-25s from a number of species highlighting the evolutionary similarity conserved nucleotides. A star identifies the PKC phosphorylation site at serine 187. *B*: plasmids encoding enhanced green fluorescent protein–coupled SNAP-25 constructs (EGFP–SNAP-25) and EGFP–SNAP-25 mutants were transfected into SF9 cells and lysates immunoblotted with an antibody to GFP. All constructs express at similar levels and that there is minimal or no cleavage between the EGFP and SNAP-25 under these conditions. Control cells were not transfected.

Downloaded from jn.physiology.org on January 17, 2007

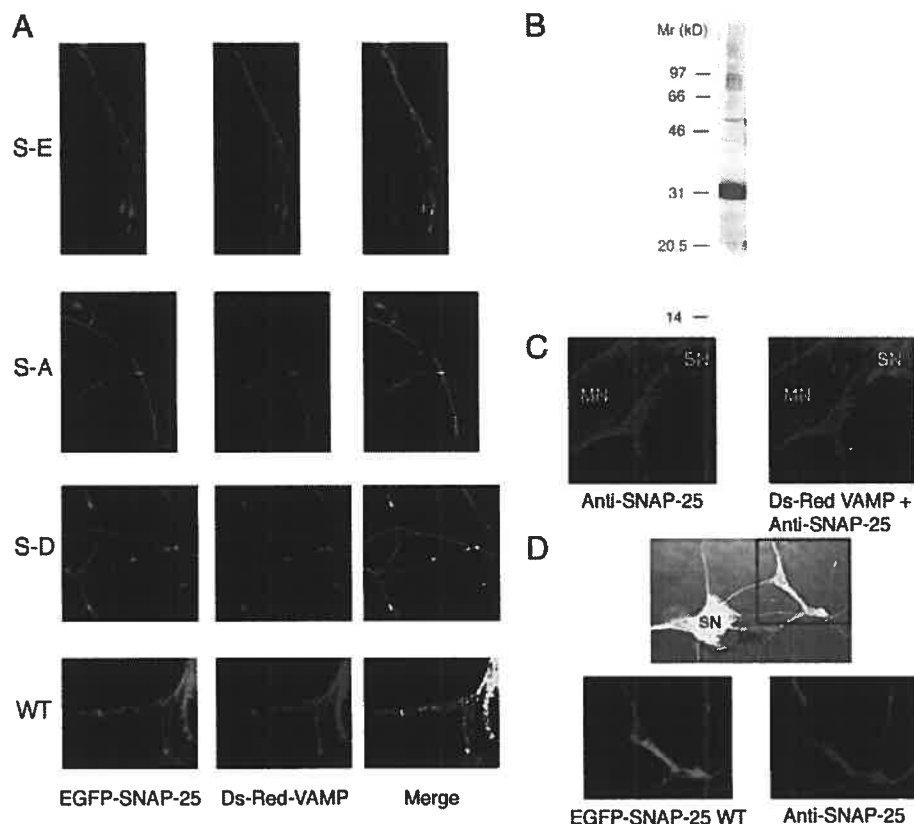


FIG. 2. Localization of SNAP-25 constructs in sensory neurons. Plasmids encoding DsRed-labeled *Aplysia* vehicle-associated membrane protein (VAMP, synaptobrevin) and EGFP-labeled *Aplysia* SNAP-25 were coinjected into sensory neurons. Expressing neurons were then paired with motor neurons and visualized 3 days later. High-power views of the synaptic regions are shown. Similar results were seen with staining of SNAP-25 as opposed to EGFP labeling. *B*: 10 μg of nervous system tissue were blotted with the anti-Apl-SNAP-25 antibody; one major band of about 30 kDa is observed. Although this is slightly higher than the molecular weight of SNAP-25 in vertebrates (25 kDa), it is within the range that one would expect the *Aplysia* protein to migrate given possible changes in posttranslational modification such as palimtylation between the species. *C*: DsRed–VAMP was injected into *Aplysia* sensory cells. After 2 days expressing neurons were paired with a motor neuron; 3 days later the cells were fixed and labeled with an *Aplysia* SNAP-25 antibody (Anti SNAP-25). Asterisk indicates potential synapse where endogenous SNAP-25 overlap with DsRed–VAMP. *D*: as in *C*, only here DsRed–VAMP and EGFP–SNAP-25 were injected into *Aplysia* sensory cells. First part shows a transmitted light image with superimposed fluorescent staining of DsRed–VAMP and EGFP–SNAP-25. High-power views of synaptic regions show the amount of overexpression. EGFP–SNAP-25 (green) and Anti-SNAP-25 (blue). Laser power was 5 times lower than that in *C*.

to detect the expressed protein, no staining is seen in the motor neuron (Fig. 2*D*).

SNAP phosphomimetic mutants decrease the rate of synaptic depression

Sensory neurons injected with various plasmids were paired with motor neurons to obtain functional synapses in isolated cell cultures (see Manseau et al. 2001). Synaptic depression was produced by 40 repeated intracellular stimulations of the sensory cell with an interstimulus interval of 20 s. Mutants mimicking the phosphorylated state (S-E, S-D) slowed down the rates of depression (Fig. 3*A*). Homosynaptic depression (HSD) in *Aplysia* is characterized by an initial rapid phase and a subsequent slow phase, during which the response remains fairly stationary, after the major part of HSD has already taken place (Armitage and Siegelbaum 1998; Royer et al. 2000). After seven to ten stimuli, the EPSP amplitude of the control EGFP–SNAP-25 was rapidly reduced to 27% of the initial EPSP amplitude and slowly decreased to 12% at the end of the stimulation period (EPSPs 38–40), whereas when sensory cells expressed the phosphomimetic mutant S-E or S-D, the plateau started at 48 and 40% of the initial EPSP, respectively, and decreased to about 22 and 18% after 40 stimuli. We observed a statistically significant difference ($P < 0.05$) for S-E throughout the stimulation period. For S-D we observed a statistical difference ($P < 0.05$) only through parts of the stimulation period (EPSPs 4–5, 7–8, 20–25, 28–31, and 36–37). In contrast, there was no significant difference on the rate of depression between mutants mimicking the nonphoshorylated state of SNAP-25 (S-A or S-C) and the control (Fig. 3*B*).

Whereas phosphomimetics of SNAP-25 decreased the rate of depression, SNAP-25 variants that could not be phosphorylated did not. This is not surprising because it is unlikely that PKC is activated by depression itself. To determine whether PKC-mediated phosphorylation of SNAP-25 could also decrease the rate of depression and to determine whether SNAP-25 could be a target for phorbol-ester–mediated increases in synaptic facilitation, the effect of wild-type SNAP-25 and one of the nonphosphorylated state mutants (SNAP-25 S-A) was examined in the presence of the PKC activator PDBu. Given the recent data from mammalian cells

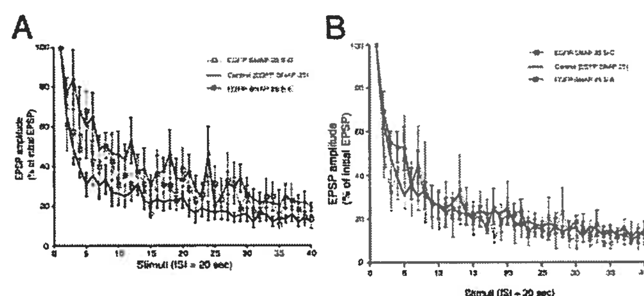


FIG. 3. Effects of SNAP-25 Ser198 mutations on the rates of depression. *A*: significant difference is observed among the phosphomimetic mutants compared with the wild-type (EGFP–SNAP-25) control. EGFP–SNAP-25 S-D ($n = 7$); EGFP–SNAP-25 S-E ($n = 7$); EGFP–SNAP-25 ($n = 14$). *B*: no significant difference is observed among the nonphoshorylated state mutants compared with the control ($n = 14$ as in *A*). EGFP–SNAP-25 S-C ($n = 6$); EGFP–SNAP-25 S-A ($n = 7$). Comparison of the rates of depression between the different constructs was quantified by subtracting the mean of the constructs from the reference control. Mean was calculated for every 2 normalized excitatory postsynaptic potential (EPSP) amplitudes, starting at the second stimulation, and was compared with the mean of the control. Difference was determined using a nonparametric bootstrap test procedure (Efron and Tibshirani 1986; Georgopoulos et al. 1988) with 10,000 repetitions and a criterion of $P < 0.05$. Briefly, each individual EPSP within the mean was shuffled randomly and compared with the equally shuffled control.

Downloaded from jn.physiology.org on January 17, 2007

that PDBu effects on transmitter release can also be mediated by Munc-13 (Rhee et al. 2002), we first confirmed that in *Aplysia* neurons the effects of PDBu on synaptic strength are mediated by PKC using a specific PKC inhibitor, Bisindoleamide I (Bis). Bis completely blocked the ability of PDBu to increase transmitter release at naïve synapses, confirming earlier results (Braha et al. 1990) that this effect is mediated by PKC (Fig. 4, *A* and *B*). Strikingly, the increase in synaptic strength seen after PDBu treatment was also significantly blocked in sensory neurons expressing the nonphosphorylated state mutant of SNAP-25 ($P < 0.01$), but not when wild-type EGFP–SNAP-25 was expressed (Fig. 4, *A* and *B*). The effect of expressing the nonphosphorylated state mutant of SNAP-25 was similar to that of Bis.

Because PDBu initially increased synaptic strength, it is difficult to determine the effect of PDBu on the rates of depression because one cannot directly compare the rates of depression after PDBu to control because they start at different points. Instead, we examined the effect of PDBu on the steady-state amount of depression (i.e., the percentage of the initial EPSP reached after 15–20 stimulations). In the presence of PDBu, there is less synaptic depression in the steady state (Fig. 4*A*), similar to the effect of overexpressing EGFP–SNAP-25 phosphomimetics. Moreover, although wild-type SNAP-25 does not affect the steady state of depression in the absence of PDBu (Fig. 3) or the increase in synaptic strength mediated by PDBu, wild-type EGFP–SNAP-25 did further decrease the amount of steady-state synaptic depression (Fig. 4*A*). The effect of PDBu on the steady state of depression is blocked by Bis ($P < 0.05$) and by EGFP–SNAP-25 S-A, (Fig. 4*A*). These results are consistent with a model where PKC phosphorylation of SNAP-25 leads to an increase in synaptic strength in naïve synapses and to a lower level of steady-state depression after repeated stimulations; both of these effects are mediated mainly through phosphorylation of SNAP-25.

Overexpression of SNAP-25 or phosphomutants does not affect initial synaptic strength

If phosphorylation of SNAP-25 was sufficient to increase synaptic strength, one might expect that neurons expressing phosphomimetics of SNAP-25 would have higher initial synaptic strength and the nonphosphorylated-state SNAP-25 would have lower initial synaptic strength. Although, similar to other studies (Nakhost et al. 2003), we observed large variabilities in initial synaptic strength, there was certainly not a trend for any of the phosphomimetic constructs to increase synaptic strength ($P > 0.05$) (Fig. 5). One hypothesis proposed by Waters and Smith (2002) argues that the proportion of recycling vesicles in the readily releasable pool differs from synapse to synapse (Waters and Smith 2002; Zhao and Klein 2003) and this may account for the heterogeneity of our data.

Effect of SNAP-25 expression on the 5-HT–induced reversal of depression

We next examined the ability of 5-HT to reverse synaptic depression in paired neurons where the sensory cells were preinjected with various constructs. 5-HT (10 μM, final concentration) was added to induce PKC-dependent facilitation (Ghirardi et al. 1992). Our electrophysiological results show that the reversal of depression was reduced by overexpression of EGFP–SNAP-25 (Fig. 6*A*). This was a surprising result; EGFP–SNAP-25 was previously reported to functionally replace SNAP-25 in a number of systems, yet here it appeared to act as a dominant negative construct. To determine whether the EGFP attachment at the N-terminal end was responsible for this effect, we separately injected EGFP and SNAP-25 (SNAP-25 + EGFP in Fig. 6). This did not interfere with the reversal of depression, suggesting that the addition of EGFP to the N-terminal converted SNAP-25 to a dominant negative construct. This effect is specific for the reversal of depression because EGFP–SNAP 25 did not affect initial synaptic strength (Fig. 5), PDBu-mediated increases in synaptic strength (Fig. 4*A*), or the normal rate of synaptic depression (Fig. 3). Interestingly, overexpression of EGFP–SNAP-25 with mutations at the PKC phosphorylation site, with the exception of the Ser-Asp (S198D) mutation, did not interfere with facilitation, suggesting that this amino acid was important for the ability of SNAP-25 to act as a dominant negative (Fig. 6*B*).

DISCUSSION

Phosphorylation of SNAP-25 slows down the rate of depression in SM synapses

Although the data corroborate that phosphorylation of Ser187 in dense-core vesicle release in chromaffin cells is strong, no previous evidence indicates that this site is important for release of synaptic vesicles. Synaptic vesicles like dense-core vesicles exhibit multiple kinetic components of release, but the rates are about tenfold faster for synaptic vesicles (Mennerick and Matthews 1996; Sakaba and Neher 2001).

Phosphomimetics of *Aplysia* SNAP-25 decreased the rate of depression (Fig. 3). Unlike in chromaffin cells where exocytosis can be measured directly using capacitance and amperometry measurement, we can measure exocytosis only indirectly using the EPSP in the motor cells. Nevertheless, this result is similar to a study in adrenal chromaffin cells where overexpression of phosphomimetic mutants at SNAP-25 Ser187 resulted in enhanced vesicle recruitment (Nagy et al. 2002). In contrast to our results, this study demonstrated that overexpression of nonphosphorylated-state mutants at SNAP-25 Ser187 resulted in inhibition of vesicle pool refilling (Nagy et al. 2002). However, in this study the stimulus used for release led to enhanced phosphorylation of SNAP-25. Because PKC is not activated during 0.05-Hz stimulation and there was no effect of the nonphosphorylated-state EGFP–SNAP-25 on depression, it is unlikely that PKC phosphorylation of SNAP 25 plays a role in depression in *Aplysia* sensory–motor neuron synapses.

Synaptic depression in *Aplysia* appears to consist of two separate mechanisms. Klein and colleagues compared the effect of depression on the readily releasable pool (as measured by transmitter released by sucrose) and the effect on overall release. They found that only a fraction of the decrease in synaptic transmission during depression was the result of a decrease in the readily releasable pool (RRP) and the remainder was the result of a decrease in calcium-secretion coupling (Fig. 6; Zhao and Klein 2002). The percentage of depression attributed to the decrease in the RRP is approximately the same as the amount of depression recovered by expressing the

Downloaded from jn.physiology.org on January 17, 2007

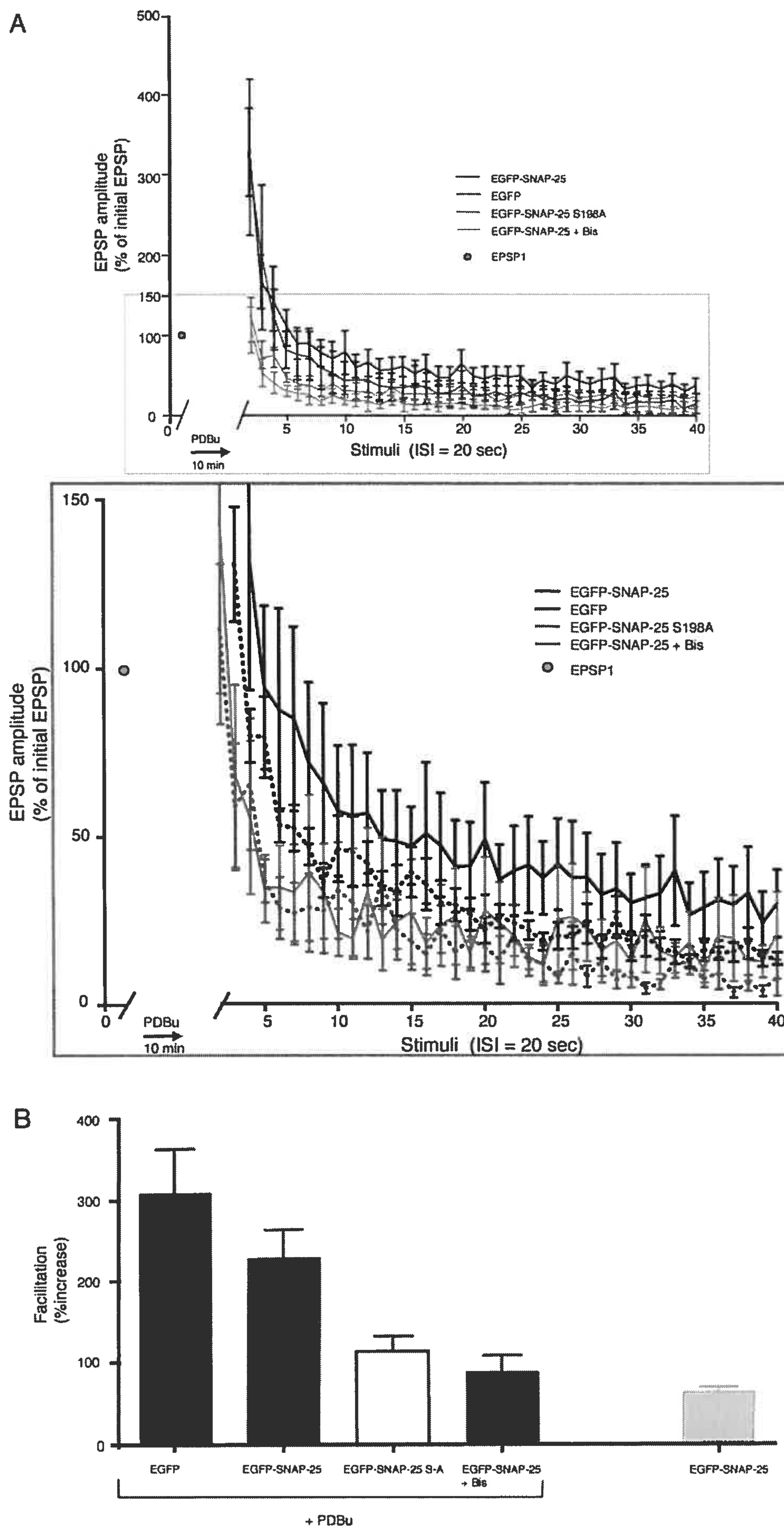


FIG. 4. Rates of depression. *A*: after the first EPSPs, phorbol 12,13-dibutyrate (PDBu, 100 nM) was applied to induce synaptic facilitation. Interstimulus interval (ISI) between the first and the second EPSP was 10 min. Sensory-to-motor transmission was then depressed by a series of 40 repeated intracellular stimuli (ISI = 20 s). EPSP amplitude was normalized to the initial control value. EGFP (black dotted line; $n = 11$); EGFP–SNAP-25 (black solid line; $n = 12$); EGFP–SNAP-25 S-A (gray dotted line; $n = 5$); EGFP–SNAP-25 + bisinolylmaleimide I (Bis; gray solid line; $n = 5$). Region between 0 and 150% on the *y*-axis was expanded for clarity below. We removed one anomalous experiment with an extremely high facilitation (13-fold) from the SNAP-25 group. Comparison of the steady-state level of depression (stimuli 15–20) between the EGFP–SNAP-25 and the EGFP control was quantified by using a nonparametric bootstrap test procedure (see Fig. 2). Bootstrap test showed that EGFP–SNAP-25 was significantly different from EGFP–SNAP-25 + Bis ($P < 0.005$) throughout the period of steady-state depression. With respect to EGFP–SNAP-25 S-A there was a significant difference throughout the entire stimulation period ($P < 0.05$), apart from stimuli 18 and 19 ($P = 0.0535$). Similarly, EGFP alone, compared with EGFP–SNAP-25 + Bis, showed a significant difference ($P < 0.005$) throughout the steady-state stimulation period and vs. EGFP–SNAP-25 S-A ($P < 0.05$). *B*: initial effect of PDBu. A measurement of the fold increase in EPSP amplitude by PDBu between EPSP1 and EPSP2. Unpaired one-tailed *t*-test with Welch correction showed that EGFP–SNAP-25 was significantly different from both EGFP–SNAP-25 S-A and Bis ($P < 0.01$) and that PDBu significantly increases the EPSP vs. the wild-type EGFP–SNAP-25, in the absence of PDBu ($P < 0.001$).

Downloaded from jn.physiology.org on January 17, 2007

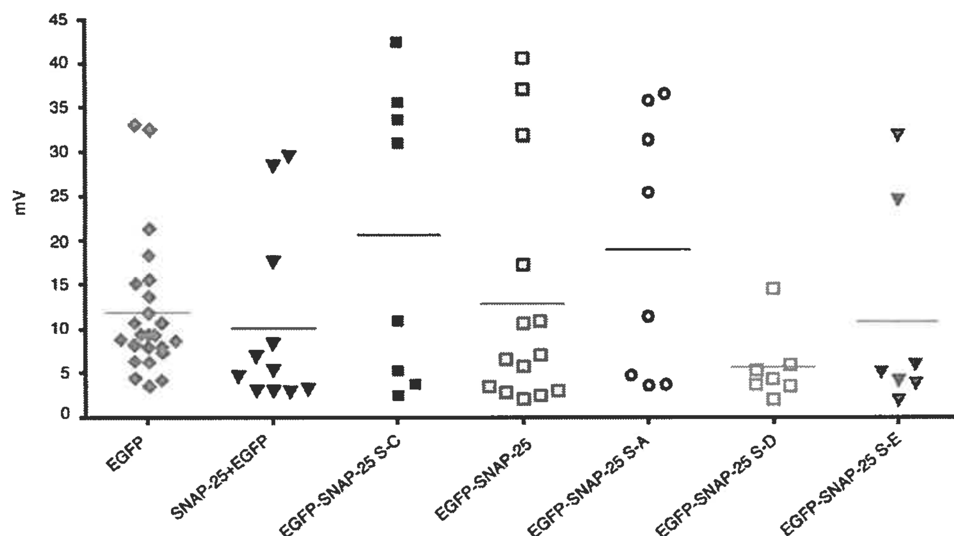


FIG. 5. Scatterplot of initial EPSP for all constructs. Despite large variability, none of the mutants significantly changed the initial EPSP. Horizontal lines indicate the average value in each group (ANOVA; $P > 0.5$). EGFP (n = 24); SNAP-25 + EGFP (n = 11); EGFP–SNAP-25 S-C (n = 8); EGFP–SNAP-25 (n = 14); EGFP–SNAP-25 S-A (n = 8); EGFP–SNAP-25 S-D (n = 7); EGFP–SNAP-25 S-E (n = 7).

phosphomimetic mutants, suggesting that these mutants act by blocking this decrease in the RRP seen with stimulation.

Phorbol esters increase transmission at naïve synapses through phosphorylation of SNAP-25

A striking result from this study is that the increase in transmitter release seen after PDBu treatment is almost completely blocked by expression of the SNAP-25 S-A mutant. This suggests that PDBu increases synaptic strength mainly through PKC phosphorylation of SNAP-25. Finley et al. (2003) examined the effect of replacement of SNAP-25 with phosphomimetic mutants in hippocampal neurons, but saw no effect of S187A mutants on PDBu increases in transmitter release and actually saw an increase in synaptic failures with an S187E

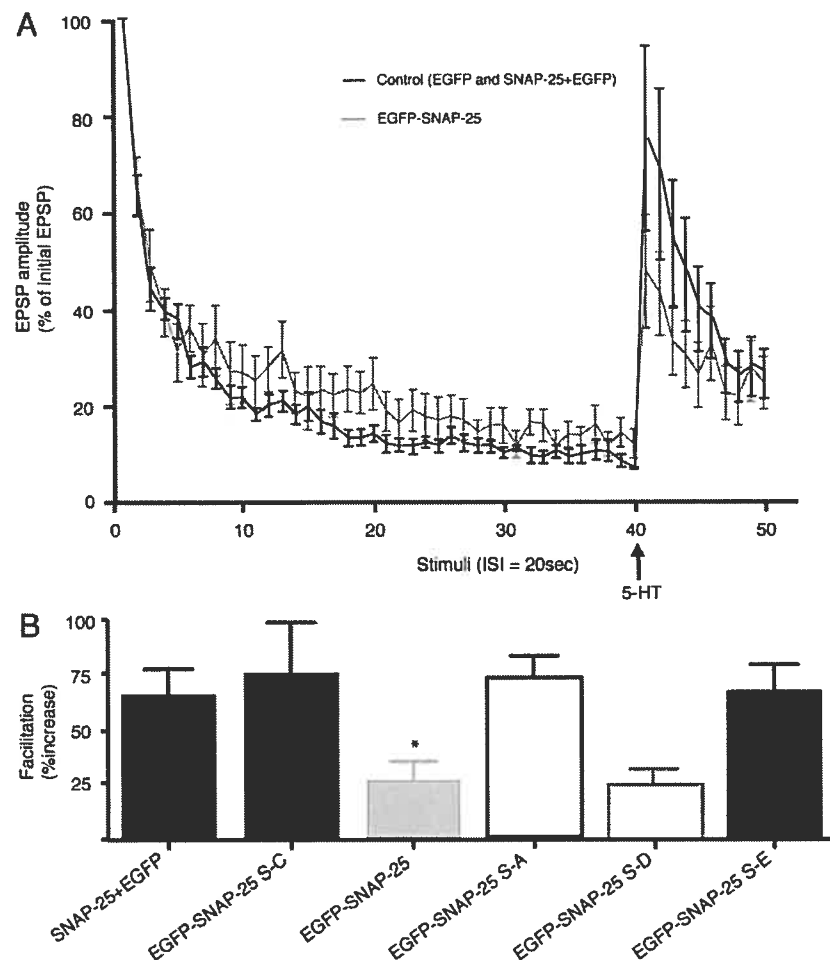


FIG. 6. Short-term facilitation of depressed synapses is partly blocked by overexpression of EGFP–SNAP-25 and EGFP–SNAP-25 S-D but not by any of the other mutants. *A*: sensory-to-motor transmission was depressed by a series of 40 repeated intracellular stimuli (ISI = 20 s) and 5-hydroxytryptamine (5-HT, 10 μM) was applied to induce synaptic facilitation, followed by an additional 10 stimuli. Control (n = 35), we pooled cells injected with EGFP alone with the cells where EGFP and SNAP-25 were injected separately: EGFP alone (n = 24); SNAP-25 + EGFP (n = 11). *B*: amount of facilitation was calculated as the difference between the average of the 3 normalized EPSPs after and before 5-HT application: EPSP 2 (after 5-HT) − EPSP 1 (before 5-HT). Unpaired one-tailed t-test with Welch's correction showed that EGFP–SNAP-25 was significantly different from control ($P < 0.05$) (SNAP-25 + EGFP, n = 35 as in *A*); EGFP–SNAP-25 S-C, n = 6; EGFP–SNAP-25, n = 14; EGFP–SNAP-25 S-A, n = 7; EGFP–SNAP-25 S-D, n = 7; EGFP–SNAP-25 S-E, n = 7.

Downloaded from jn.physiology.org on January 17, 2007

mutation. The lack of effect in this case may be explained by the fact that the majority of phorbol-ester–mediated enhancement of transmitter release in CA1 neurons is mediated through Munc13 and not PKC (Rhee et al. 2002). It should be noted that PDBu-mediated increases in transmitter release in CA1 neurons are rather modest, for example, compared with effects at other hippocampal synapses (Hussain and Carpenter 2003).

In *Aplysia* sensory–motor synapses, PDBu mediates increases in transmitter release through PKC because PKC inhibitors block the effect of PDBu on transmitter release at naïve and depressed synapses (Braha et al. 1990; Fig. 4). These inhibitors target the ATP-binding site of PKC and thus do not affect nonkinase diacylglycerol–binding proteins such as Munc13 (Morgan et al. 2005). However, if phosphorylation of SNAP-25 was sufficient to increase transmitter release, one would have expected the phosphomimetics to significantly increase initial synaptic strength, although there was no trend to suggest this effect. One possibility is that the phosphomimetic SNAP-25s do not effectively mimic phosphorylated SNAP-25 for this physiological effect. Another possibility is that prolonged expression of the phosphomimetic SNAP-25s causes homeostatic changes in transmitter release that mask changes that may be seen after acute changes in phosphorylation. Alternatively, it may be that phosphorylation of SNAP-25 is necessary, but not sufficient, for the phorbol-ester–mediated increases in synaptic strength. Thus additional substrates of PKC, or activation of other phorbol-ester targets such as Munc-13 or ras guanine exchange factors (Dyer et al. 2003) may also be required in addition to SNAP-25 phosphorylation for the increase in transmitter release. Because 5-HT increases synaptic strength at depressed synapses through PKC (Ghirardi et al. 1992; Manseau et al. 2001), why does 5-HT not increase the synaptic strength of naïve synapses through PKC activation, especially because phorbol esters can? Many explanations have been proposed for this conundrum. One possibility is that the isoforms of PKC activated by phorbol esters and 5-HT may be distinct (Zhao et al. 2006) and phosphorylation of SNAP-25 may be isoform specific. Another possibility is that the initial increase in synaptic strength caused by activation of PKA may make irrelevant the modifications induced by 5-HT. Indeed, some data suggest that prolonged applications of 5-HT do result in PKC-mediated increases in synaptic strength at naïve synapses (Jin et al. 2005). PKA-mediated phosphorylation of SNAP-25 at a separate site may also increase synaptic strength (Hepp et al. 2002; Nagy et al. 2004).

Does SNAP-25 modulation of calcium dynamics play a role in depression?

During HSD the depression of transmitter release occurs downstream of calcium influx (Armitage and Siegelbaum 1998) and probably affects calcium-secretion coupling. A recent study in hippocampal GABAergic synapses shows that SNAP-25 negatively regulates neuronal calcium responsiveness to depolarization (Verderio et al. 2004). These neurons naturally lack SNAP-25, but contain the related SNAP-23. Exogenously expressing SNAP-25 into GABAergic neurons was sufficient to decrease the sensitivity of release to depolarization. Most of the above effect was localized to the 180–197 residues in the C-terminal portion of SNAP-25 containing the PKC phosphorylation site. In chromaffin cells the role of PKC is to increase the proportion of vesicles that are highly sensitive to calcium (Yang et al. 2002). These results together suggest a model where SNAP-25 naturally inhibits a step in calcium secretion coupling and that PKC activation can reverse this step.

In our experiments, overexpression of EGFP-coupled SNAP-25 acted as a dominant negative for the 5-HT–mediated reversal of depression, but not for initial synaptic strength or PDBu-mediated increases in synaptic strength. The specificity of the dominant negative effect suggests a distinct role of SNAP-25 in regulating depression independent of its role in transmitter release and vesicle availability. Thus although somewhat speculative, one model that is consistent with our data posits that during depression SNAP-25 acts to inhibit release at a postpriming step and thus blocking calcium-secretion coupling. This inhibition by SNAP-25 is sensitive to the amino acid at the phosphorylation site and thus many of the mutants do not compete with endogenous SNAP-25 for this interaction. The fact that the EGFP–SNAP-25 S-D mutant can still act as a dominant negative does not address whether PKC phosphorylation of SNAP-25 plays a direct role because the wild-type protein acts as a dominant negative as well, despite the likelihood that it is still phosphorylated after serotonin addition. Indeed, in chromaffin cells both S187A and S187D mutants had decreases in the fast burst of release, presumably resulting from sensitivity for some SNAP-25 interaction dependent on the exact conformation of the amino acid at this position (Nagy et al. 2002). Presumably, because of some steric restraint from the EGFP-tag, inhibition mediated by SNAP-25 cannot be released through PKC phosphorylation. This may be attributable either to phosphorylation of SNAP-25 by PKC or to PKC phosphorylation of an interacting partner.

How could SNAP-25 mediate this inhibition? The carboxy-terminal region is also required for Ca^{2+}-dependent interaction of the calcium sensor Syt I and SNAP-25 during Ca^{2+}-triggered exocytosis (Gerona et al. 2000; Zhang et al. 2002). The interaction of Syt I with the SNARE complex is a leading candidate for the calcium-sensitive step regulating exocytosis (Bai et al. 2004; Tucker et al. 2004) and thus regulation of this interaction by phosphorylation could regulate calcium-secretion coupling. In previous work we showed that Syt I binding to SNAP-25 increases about two- to fourfold in the presence of calcium (Nakhost et al. 2004). However, we did not observe any differences in Syt I–SNAP-25 interactions with any of the mutations in the phosphorylation site (data not shown; see Nakhost et al. 2004). It should be noted though that these experiments investigated only the low-affinity interaction of Syt I with SNAP-25 compared with the high-affinity interaction of Syt I with the entire SNARE complex because SNAP-25 is largely unstructured as a monomer but acquires α-helicity in binary and ternary SNARE complexes (Fasshauer et al. 1997). The G-protein $\beta\gamma$ inhibits exocytosis downstream of priming through binding to the C- terminus of SNAP-25 (Gerachshenko et al. 2005), perhaps interfering with the Ca^{2+}-dependent binding to Syt I (Blackmer et al. 2005). Removal of this inhibition by 5-HT could be blocked by the EGFP–SNAP-25 dominant negative construct.

In conclusion, our data support that PKC phosphorylation of SNAP-25 is necessary, but probably not sufficient, for PDBu-mediated increases in transmitter release. Moreover, we provide suggestive evidence that SNAP-25 may play an important

Downloaded from jn.physiology.org on January 17, 2007

role in the block of calcium-secretion coupling that is important for HSD.

ACKNOWLEDGMENTS

We thank F. Fiumara for helpful comments on the manuscript.

GRANTS

This work was supported by Canadian Institutes of Health Research (CIHR) Grants MOP-12046 to W. S. Sossin, MOP-14142 to V. Castellucci, and group grant MGC-57079. W. S. Sossin is a Killiam and Dawson Scholar and supported by a CIHR Scientist award.

REFERENCES

Ahmari SE, Buchanan J, Smith SJ. Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat Neurosci* 3: 445–451, 2000.

Armitage BA, Siegelbaum SA. Presynaptic induction and expression of homosynaptic depression at *Aplysia* sensorimotor neuron synapses. *J Neurosci* 18: 8770–8779, 1998.

Bai J, Wang CT, Richards DA, Jackson MB, Chapman ER. Fusion pore dynamics are regulated by synaptotagmin t-SNARE interactions. *Neuron* 5: 929–942, 2004.

Blackmer T, Larsen EC, Bartleson C, Kowalchyk JA, Yoon E-J, Preininger AM, Alford S, Hamm HE, Martin TFJ. G protein $\beta\gamma$ directly regulates SNARE protein fusion machinery for secretory granule exocytosis. *Nat Neurosci* 8: 421–425, 2005.

Braha O, Dale N, Hochner B, Klein M, Abrams TW, Kandel ER. Second messengers involved in the two processes of presynaptic facilitation that contribute to sensitization and dishabituation in *Aplysia* sensory neurons. *Proc Natl Acad Sci USA* 87: 2040–2044, 1990.

Brunger AT. Structural insights into the molecular mechanism of Ca(2+)-dependent exocytosis. *Curr Opin Neurobiol* 3: 293–302, 2000.

Byrne JH, Castellucci VF, Kandel ER. Contribution of individual mechanoreceptor sensory neurons to defensive gill-withdrawal reflex in *Aplysia*. *J Neurophysiol* 41: 418–431, 1978.

Dyer JR, Manseau F, Castellucci VF, Sossin WS. Serotonin persistently activates the extracellular signal-related kinase in sensory neurons of *Aplysia* independently of cAMP or protein kinase C. *Neuroscience* 116: 13–17, 2003.

Efron B, Tibshirani R. Bootstrap methods for standard errors, confidence intervals, and other measures of statistical accuracy. *Stat Sci* 1: 54–77, 1986.

Fasshauer D, Bruns D, Shen B, Jahn R, Brunger AT. A structural change occurs upon binding of syntaxin to SNAP-25. *J Biol Chem* 272: 4582–4590, 1997.

Fernandez-Chacon R, Südhof TC. Genetics of synaptic vesicle function: toward the complete functional anatomy of an organelle. *Annu Rev Physiol* 61: 753–776, 1999.

Finley MF, Patel SM, Madison DV, Scheller RH. The core membrane fusion complex governs the probability of synaptic vesicle fusion but not transmitter release kinetics. *J Neurosci* 22: 1266–1272, 2002.

Finley MF, Scheller RH, Madison DV. SNAP-25 Ser187 does not mediate phorbol ester enhancement of hippocampal synaptic transmission. *Neuropharmacology* 45: 857–862, 2003.

Genoud S, Pralong W, Riederer BM, Eder L, Catsicas S, Muller D. Activity-dependent phosphorylation of SNAP-25 in hippocampal organotypic cultures. *J Neurochem* 72: 1699–1706, 1999.

Georgopoulos AP, Kettner RE, Schwartz AB. Primate motor cortex and free arm movements to visual targets in three-dimensional space. II. Coding of the direction of arm movement by a neural population. *J Neurosci* 8: 2928–2937, 1988.

Gerachshenko T, Blackmer T, Yoon E-J, Bartleson C, Hamm H, Alford S. $G\beta\gamma$ acts at the C-terminus of SNAP-25 to mediate presynaptic inhibition. *Nat Neurosci* 8: 597–605, 2005.

Gerona RRL, Larsen EC, Kowalchyk JA, Martin TFJ. The C terminus of SNAP-25 is essential for Ca^{2+}-dependent binding of synaptotagmin to SNARE complexes. *J Biol Chem* 275: 6328–6336, 2000.

Ghirardi M, Braha O, Hochner B, Montarolo PG, Kandel ER, Dale N. Roles of PKA and PKC in facilitation of evoked and spontaneous transmitter release at depressed and nondepressed synapses in *Aplysia* sensory neurons. *Neuron* 9: 479–489, 1992.

Hepp R, Cabaniols J-P, Roche PA. Differential phosphorylation of SNAP-25 in vivo by protein kinase C and protein kinase A. *FEBS Lett* 532: 52–56, 2002.

Hilfiker S, Augustine GJ. Regulation of synaptic vesicle fusion by protein kinase C. *J Physiol* 515: 169–180, 1999.

Hussain RJ, Carpenter DO. The effects of protein kinase C activity on synaptic transmission in two areas of rat hippocampus. *Brain Res* 990: 28–37, 2003.

Jin I, Kandel ER, Hawkins RD. The roles of PKA, PKC and CAMKII in facilitation at the *Aplysia* sensory motor neuron synapses depend on the duration of the exposure of 5-HT. *Soc Neurosci Abstr* 540.5, 2005.

Manseau F, Fan X, Hueftlein T, Sossin WS, Castellucci VF. Ca^{2+}-independent PKC Apl II mediates the serotonin induced facilitation at depressed synapses in *Aplysia*. *J Neurosci* 21: 1247–1256, 2001.

Mennerick S, Matthews G. Ultrafast exocytosis elicited by calcium current in synaptic terminals of retinal bipolar neurons. *Neuron* 17: 1241–1249, 1996.

Morgan A, Burgoyne RD, Barclay JW, Craig TJ, Prescott GR, Ciufo LF, Evans GJ, Graham ME. Regulation of exocytosis by protein kinase C. *Biochem Soc Trans* 33: 1341–1344, 2005.

Nagy G, Matti U, Nehring RB, Binz T, Retting J, Neher E, Sørensen JB. Protein kinase C-dependent phosphorylation of synaptosome associated protein of 25 kDa at Ser 187 potentiates vesicle recruitment. *J Neurosci* 22: 9278–9286, 2002.

Nagy G, Reim K, Matti U, Brose N, Binz T, Rettig J, Neher E, Sorensen JB. Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. *Neuron* 5: 417–429, 2004.

Nakhost A, Houeland G, Blandford VE Castellucci VF, Sossin WS. Identification and characterization of a novel splice variant of synaptotagmin I. *J Neurochem* 89: 354–363, 2004.

Nakhost A, Houeland G, Castellucci VF, Sossin WS. Differential regulation of transmitter release by alternatively spliced forms of synaptotagmin I. *J Neurosci* 23: 6238–6244, 2003.

Nonet ML. Visualization of synaptic specializations in live *C. elegans* with synaptic vesicle protein-GFP fusions. *J Neurosci Methods* 89: 33–40, 1999.

Rhee J-S, Betz A, Pyott S, Reim K, Varoqueaux F, Augustin I, Hesse D, Südhof TC, Takahashi M, Rosenmund R, Brose N. Phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKC. *Cell* 108: 121–133, 2002.

Royer S, Coulson RL, Klein M. Switching off and on of synaptic sites at *Aplysia* sensorimotor synapses. *J Neurosci* 20: 626–638, 2000.

Sakaba T, Neher E. Calmodulin mediates rapid recruitment of fast-releasing synaptic vesicles at a calyx-type synapse. *Neuron* 32: 1119–1131, 2001.

Schuette CG, Hatsuzawa K, Margittai M, Stein A, Riedel D, Kuster P, Konig M, Seidel C, Jahn R. Determinants of liposome fusion mediated by synaptic SNARE proteins. *Proc Natl Acad Sci USA* 101: 2858–2863, 2004.

Shimazaki Y, Nishiki T, Omori A, Sekiguchi M, Kamata Y, Kozaki S, Takahashi M. Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. *J Biol Chem* 271: 14548–14553, 1996.

Smith C, Moser T, Xu T, Neher E. Cytosolic Ca^{2+} acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells. *Neuron* 20: 1243–1253, 1998.

Sørensen JB, Matti U, Wei S, Nehring RB, Voets T, Ashery U, Binz T, Neher E, Rettig J. The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. *Proc Natl Acad Sci USA* 99: 1627–1632, 2002.

Sørensen JB, Nagy G, Varoqueaux F, Nehring RB, Brose N, Wilson MC, Neher E. Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. *Cell* 114: 75–86, 2003.

Stevens CF, Sullivan JM. Regulation of the readily releasable vesicle pool by protein kinase C. *Neuron* 21: 885–893, 1998.

Südhof TC. The synaptic vesicle cycle: a cascade of protein–protein interactions. *Nature* 375: 645–653, 1995.

Tucker WC, Edwardson JM, Bai J, Kim HJ, Martin TF, Chapman ER. Identification of synaptotagmin effectors via acute inhibition of secretion from cracked PC12 cells. *J Cell Biol* 162: 199–209, 2003.

Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C, Matteoli M. SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron* 41: 599–610, 2004.

Washbourne P, Thompson PM, Carta M, Costa ET, Mathews JR, Lopez-Bendito G, Molnar Z, Becher MW, Valenzuela CF, Partridge LD, Wilson MC. Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nat Neurosci* 5: 19–26, 2002.

Downloaded from jn.physiology.org on January 17, 2007

Waters J, Smith SJ. Phorbol esters potentiate evoked and spontaneous release by different presynaptic mechanisms. *J Neurosci* 20: 7863–7870, 2000.

Waters J, Smith SJ. Vesicle pool partitioning influences presynaptic diversity and weighing in rat hippocampal synapses. *J Physiol* 541: 811–823, 2002.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE. SNAREpins: minimal machinery for membrane fusion. *Cell* 20: 759–772, 1998.

Yang Y, Udayasankar S, Dunning J, Chen P, Gillis KD. A highly Ca^{2+}-sensitive pool of vesicles is regulated by protein kinase C in adrenal chromaffin cells. *Proc Natl Acad Sci USA* 99: 17060–17065, 2002.

Zhang X, Kim-Miller MJ, Fukuda M, Kowalchyk JA, Martin TFJ. Ca^{2+}-dependent synaptotagmin binding to SNAP-25 is essential for Ca^{2+}-triggered exocytosis. *Neuron* 34: 599–611, 2002.

Zhao Y, Klein M. Modulation of the readily releasable pool of transmitter and of excitation-secretion coupling by activity and by serotonin at *Aplysia* sensorimotor synapses in culture. *J Neurosci* 22: 10671–10679, 2002.

Zhao Y, Leal K, Abi-Farah C, Sossin WS, Martin KC, Klein M. Isoform specificity of PKC translocation in living Aplysia sensory neurons and a role for Ca^{2+}-dependent PKC APL I in the induction of intermediate-term facilitation. *J Neurosci* 26: 8847–8856, 2006.

Downloaded from jn.physiology.org on January 17, 2007

and readout, or retrieval of such information (e.g. potassium channels and components of the transmitter release machinery, like SNARE proteins). A sizeable part of what is so far known about the molecular and cellular mechanisms of short-and long-term memory is derived from the cellular analysis of short and long-term facilitation in *Aplysia*. The remarkable heterogeneity and plasticity of neurotransmitter release is made possible by the expression of various protein isoforms, products of pretranslational modifications (e.g., splicing), and the dynamic regulation of protein functions by posttranslational modifications, (e.g., phosphorylation). In the present study, we investigated these two aspects of neurotransmitter release as they apply to two different Syt I isoforms and the phosphorylation SNAP-25 at a PKC phosphorylation site.

5.1 Contribution of this thesis for the comprehension of transmitters release by PKC.

Although the time course between action potential arrival at the nerve terminal and synaptic vesicle fusion is too short for protein phosphorylation/dephosphorylation to carry out a direct and acute role in a single round of vesicle exocytosis, protein kinases and phosphatases may have significant effects on subsequent neurotransmitter release events. It is reasonable to speculate that the phosphorylation/dephosphorylation states of synaptic proteins that mediate vesicle exocytosis could regulate the biochemical pathways leading from synaptic vesicle docking to fusion.

5.1.1 Mutation of Syt I at phosphorylation sites does not alter transmitter release.

Syt I is a conserved PKC substrate involved in transmitter release. The evidence that 5-HT mediates the reversal of synaptic depression through activation of PKC is strongly supported by both pharmacological inhibitors and activators of PKC and dominant-negative experiments (Braha et al., 1990; Ghirardi et al., 1992; Manseau et al., 2001). To investigate if Syt could be a PKC substrate involved in transmitter release, we generated *Aplysia* Syt I with a mutation in the PKC phosphorylation site and overexpressed them in *Aplysia* sensory neurons. We discovered that the reversal of synaptic depression is not blocked by Syt $I_{S\text{-}A}$.

In addition, Syt $I_{S\text{-}A}$ did not affect the resting membrane potential, nor the rate of synaptic depression (Nakhost et al., 2003). Thus, we conclude that Syt I is not the PKC substrate important for the reversal of depression. In chromaffin cells, phosphorylation at the PKC site lead to an increase in releasable pool size, whereas the release rate constants from the pools was unaffected (Gillis et al., 1996; Smith et al., 1998; Yang et al., 2002). Similarly, using rescue of null cells with Syt I constructs mutated in phosphorylation sites (Nagy et al., 2006), did not show any modification of secretion in the presence or absence of PEs. Because in this study, even in Syt I null cells, PEs increased secretion. These results demonstrated that PEs act independently and upstream of Syt to regulate the releasable vesicle pool size in mouse chromaffin cells. They concluded that exocytosis from mouse chromaffin cells can be modified by the differential expression of Syt isoforms and by Syt abundance but not by phosphorylation of Syt I. However, in pituitary gonadotrophs and in the calyx of Held, PE treatment causes an increase in release rate constant in the absence of a change in RRP size (Wu and Wu 2001; Zhu et al., 2002; Lou et al., 2005). These results are consistent with a previous study at the cholinergic synapse in the chick ciliary ganglion (Yawo, 1999), but it is in conflict with another study at hippocampal cultured synapses in which PEs were suggested to increase the releasable pool size (Stevens and Sullivan, 1998). The reason for this apparent discrepancy is unclear. Because Hori et al. (1999) found that PEs may enhance transmitter release by the activation of PKC and by interaction with another synaptic protein Munc13-1 at the calyx of Held synapse (Hori et al., 1999), differential distribution of PKC and Munc13-1 in different tissues has been postulated to account for the discrepancy (Hilfiker and Augustine, 1999).

5.1.2 Phorbol esters increase transmission at naïve synapses through phosphorylation of SNAP-25.

While phosphomimetics of SNAP-25 slowed down the rate of depression, SNAP-25 variants that could not be phosphorylated did not. We artificially boosted the system by adding a phorbolester (PdBu). A striking result from this study is that the increase in

transmitter release seen after PDBu treatment is almost completely blocked by expression of the SNAP-25 S-A mutant. In addition, the PKC inhibitor Bis blocks the effect of PDBu on transmitter release at naïve and depressed synapses. Together these results suggest that PDBu increases synaptic strength mainly through PKC phosphorylation of SNAP-25. On the other hand, if phosphorylation of SNAP-25 was sufficient to increase transmitter release, one would have expected the phosphomimetics to significantly increase initial synaptic strength, and there was no trend to suggest this effect. One possibility is that the phosphomimetic SNAP-25s do not effectively mimic phosphorylated SNAP-25 for this physiological effect. Another possibility is that prolonged expression of the phosphomimetic SNAP-25s cause homeostatic changes in transmitter release that mask changes that may be seen after acute changes in phosphorylation. Alternatively, it may be that phosphorylation of SNAP-25 is necessary, but not sufficient for the phorbol-ester mediated increases in synaptic strength.

However, we did not observe PKC phosphorylation of SNAP-25 *in vitro* like we did with *Aplysia* Syt I (were we successfully showed that Syt I and Syt I_{VQ} are both phosphorylated in vitro by PKC at Serine 123). It should be pointed out that this was also not observed in other studies and SNAP-25 may need to be in the SNARE complex to be phosphorylated (Risinger and Bennett, 1999). Changes in amino acid around the phosphorylation site do not allow us to use the mammalian anti-phospho SNAP-25 antibody and our attempts to raise a phospho-specific antibody to this site in *Aplysia* were unsuccessful.

Finley et al. (2003) suggest that PKC phosphorylation of SNAP-25 is not responsible for the enhancement of neurotransmitter release. They examined the effect of replacement of SNAP-25 with phosphomimetic mutants in hippocampal neurons, but saw no effect of S187A mutants on PDBu increases in transmitter release and actually saw an increase in synaptic failures with a S187E mutation. The lack of effect in this case may be due to the fact that the majority of PE mediated enhancement of transmitter release in CA1 neurons is

mediated through Munc13 and not PKC (Rhee et al., 2002). Although, PDBu mediated increases in transmitter release in CA1 neurons are rather modest, for example, in comparison to effects at other hippocampal synapses (Hussain and Carpenter, 2003).

Synaptic depression in *Aplysia* appears to consist of two separate mechanisms. Klein and colleagues compared the effect of depression on the readily releasable pool (RRP) (as measured by transmitter released by sucrose) and the effect on overall release. They found that only a fraction of the decrease in synaptic transmission during depression was due to a decrease in the RRP, and the rest was due to a decrease in calcium-secretion coupling (Zhao and Klein, 2002). The percentage of depression due to the decrease in the readily releasable pool is approximately the same as the amount of depression recovered by expressing the phosphomimetic mutants suggesting that these mutants act by blocking this decrease in the RRP seen with stimulation.

In conclusion, our data support that PKC phosphorylation of SNAP-25 is necessary, but probably not sufficient for PDBu-mediated increases in transmitter release. Recently it has been reported that increased SNAP-25 phosphorylation at Ser187 occurs during periods rich in synaptogenesis and plays an important role in the formation of synapses in the early postnatal period (Kataoka et al., 2006), possibly by regulating neurite extensions and sprouting. This study also showed that SNAP-25 phosphorylation is regulated in the adult brain in a neuronal activity-dependent manner and decreases after the induction of seizure by kainic acid. Previous studies have reported that SNAP-25 phosphorylation increased after long-term potentiation induction and seizures (Genoud et al., 1999). Although it remains to be determined, the discrepancy is most probably due to differences in experimental conditions, including the age of the animals (neonatal versus adult) and experimental system (organotypic slice cultures versus anesthetized animals).

As PKC is abundant in the brain and SNAP-25 is essential for synaptic transmission, phosphorylation of SNAP-25 is likely to play an important role in the central nervous system. Kataoka et al. (2005) created a SNAP-25 SerS187A mutant mouse using a knock in

technology. These mutant mice manifested strong anxiety related behavior and hyper locomotor activity. The inhibition of SNAP-25 phosphorylation at Ser187 has also been reported to be involved in the chronic morphine-induced down-regulaion of SNARE complex formation and presents a potential molecular mechanism for the alteration of exocytotic process and neural plasticity during opiate abuse (Xu et al., 2004).

5.1.3 Pharmacological tools that interfere with PKC functions.

The most commonly used pharmacological tools for PKC activation belong to the phorbol-ester family of tumor promoters. Phorbol esters are secondary metabolites of Euphorbiaceae and Thymeleaceae and mimic the action of DAG at C_1 domains. In common with DAG, PEs bind to the C_1 domain of PKCs and induce membrane translocation and activation of the enzyme (Hurley and Meyer., 2001; Newton, 2001; Barry and Kazanietz, 2001). Unfortunately, none of the commonly used PEs is specific for PKCs. In fact, several alternative C_1-domain containing proteins bind PEs with PKC-like affinity (Ron and Kazanietz, 1999; Kazanietz, 2002; Barry and Kazanietz, 2001). Thus, studies of PKC function that rely on the use of PEs as an investigative tool have to be interpreted with caution. This is particularly pertinent for cellular processes that are also regulated by alternative DAG/phorbol-ester receptors (e.g. the regulation of neurotransmitter release by Munc13 isoforms (Betz et al., 1998; Rhee et al., 2002) or ras guanine exchange factors (Dyer et al., 2003). Given the recent data from mammalian cells that PDBu effects on transmitter release can also be mediated by Munc-13 (Rhee et al., 2002), we confirmed that in *Aplysia* neurons the effects of PDBu on synaptic strength are mediated by PKC using a specific PKC inhibitor, Bis. This inhibitor targets the ATP-binding site of PKC and thus do not affect non-kinase DAG-binding proteins such as Munc13 (Morgan et al. 2005). Bis completely blocked the ability of PDBu to increase transmitter release at naïve synapses confirming earlier results (Braha et al., 1990) that this effect is mediated by PKC. The effect of ß-phorbolester in chromaffin cells is also completely blocked by Bis (Gillis et al., 1996). In addition, the increase in synaptic strength seen after PDBu treatment was also

significantly blocked in sensory neurons expressing the non-phosphorylated state mutant of SNAP-25 (Houeland et al., 2006).

The fact that deletion mutations of PKC (Abelovic et al., 1993), PKCß (Leitges et al., 1996), PKCε (Hodge et al., 1999; Khasar et al., 1999) in mice have rather mild phenotypic consequences indicates that there is functional redundancy among the various PKC isoformes. Because of the ubiquitous expression of the known PKC isoforms, the large number of identified PKC regulators and substrates commonly activated by PKC activators, particularly in stimulus secretion coupling the related process of synaptic transmission, the identification of steps at which PKC is involved is difficult. This is because in many studies the physiological readout is a signal several steps downstream of the one modulated by PKC, and, at least until recently, reagents used to stimulate or block PKC have often been quite unspecific.

To account for this problem and ultimately to determine the role of individual PKCs, multiple deletion mutations (e.g. of related PKC isozymes) may also be needed. Such genetic approaches in intact animals could then be ideally complemented with protein overexpression approaches.

5.1.4 PKC and short-term plasticity: rested versus depressed synapses.

Since 5-HT increases synaptic strength at depressed synapses through PKC (Ghirardi et al., 1992; Manseau et al., 2001), why does 5-HT not increase the synaptic strength of naïve synapses through PKC activation, especially since PEs can? One possibility is that the isoforms of PKC activated by PEs and 5-HT may be distinct (Zhao et al., 2006) and phosphorylation of SNAP-25 may be isoform specific. From our result we concluded that homosynaptic depression is reversed by the neurotransmitter 5-HT through activation of a Ca^{2+}-independent form of PKC, PKC Apl II (Ghirardi et al., 1992; Manseau et al., 2001). Whereas activation of PKC by 5-HT increases transmitter release only at depressed synapses, activation of PKC by phorbol esters increases transmitter release at both naïve and depressed synapses (Braha et al., 1990; Ghirardi et al., 1992; Nakhost et al., 2003).

These isoforms probably make up the majority of *Aplysia* PKC isoforms as Sossin et al. (1993) found that antibodies to Apl I and II immunoprecipitated over 90% of the PKC activity in supernatant fractions from crude extracts of nervous tissue. Recently, Bougie et al. (2006) reported cloning and characterization of an additional nervous system isoform of PKC in *Aplysia*, PKC Apl III, homologous to the non-phorbol ester activated ζ and λ isoforms in vertebrates. Hence, the combination of known physiological functions for PKC and a limited number of PKC isoforms makes the *Aplysia* nervous system ideal for determining the specific physiological roles of individual PKC isoforms. Another possibility is that the initial increase in synaptic strength caused by activation of PKA may make the modifications induced by 5-HT irrelevant. Indeed, some data suggest that prolonged applications of 5-HT do result in PKC-mediated increases in synaptic strength at naïve synapses (Jin et al., 2005). PKA-mediated phosphorylation of SNAP-25 at a separate site may also increase synaptic strength (Hepp et al., 2002; Nagy et al., 2004).

Nagy et al. (2002) suggested that because phosphorylation decreases SNAP-25/syntaxin binding, complexes consisting of one SNAP-25 molecule and 2 syntaxin molecules are broken up in preparation to form ternary complexes with VAMP (Nagy et al., 2002). Thus phosphorylation aids in preventing inappropriate SNARE associations. Additional studies by Nagy et al. (2004) showed that PKA controls the size of readily releasable pool of vesicles (Nagy et al., 2004), whereas PKC regulates the refilling of the vesicle pool after they have been depleted. It is possible that PKA could control the size of the readily releasable pool by regulating SNAP-25 binding properties, thus influencing the number of complete complexes and likewise the number of docked vesicles. PKC could regulate the recycling of SNAP-25, thus making it available to form more SNARE complexes and thus more docked vesicles after the preexisting pools have been depleted.

5.2 The role of calcium in transmitter release.

Existence of a prefusion reaction preceding the point of calcium action is suggested not only by the speed of the calcium action but also by the finding that synaptic vesicle

exocytosis can be non-physiologically elicited by hypertonic sucrose in the absence of calcium (Rosemund and Stevens, 1996). Thus calcium is not required for fusion as such, but rather for an added regulatory event. At any given time, 5-10 vesicles are attached to most active zones. All of the attached vesicles are apparently "ready" for release, since they can all be stimulated by hypertonic sucrose to undergo exocytosis (Rosenmund and Stevens, 1996). Nevertheless, a calcium signal during an action potential does not always trigger exocytosis. At most synapses, release is observed with a relatively low probability. Furthermore, when calcium is successful in triggering exocytosis at an active zone, it usually triggers the fusion of a single vesicle, although multiple vesicles are ready to be released at the active zone. Since every action potential normally leads to a fairly uniform flooding of the active zone with calcium, there must be negative regulatory elements involved in addition to the positive regulatory elements, i.e.; there must be mechanisms that inhibit fusion just as there are mechanisms to trigger fusion.

In chapter 3, (Nakhost et al., 2004), we report on a novel alternative spliced form of synaptotagmin I in *Aplysia* neurons. This isoform, synaptotagmin I C2B-ß, is generated by alternative exon usage in the C2B domain leading to nine amino acid changes in the C2B sequence from the previously characterized Syt I, designated as Syt I C2B-α. Quantitative reverse transcriptase-polymerase chain reaction demonstrated that approximately 25% of mRNA encoding Syt I contained the C2B-ß exon in the nervous system. It has been previously shown that binding of calcium to the C2B domain stabilizes it, resulting in the formation of a protease-resistant fragment (Littleton et al., 2001). Many of the C2B interactions with other proteins are regulated by calcium. Therefore, it is possible that the differences that are observed between these two isoforms occur because splicing has increased Syt I C2B-ß's affinity for calcium such that it can be stabilized at relatively low calcium concentrations. Syt I C2B-ß showed greater resistance to digestion by chymotrypsin in the absence of calcium than Syt I C2B-α, although both isoforms required the same amount of calcium to resist chymotrypsin digestion. The source of these changes

in C2B properties was mapped to a single amino acid (threonine 358). The conversion of a glycine in Syt I C2B-α to a threonine in Syt I C2B-ß may cause a structural change. The ability of calcium to stabilize the Syt I C2B domain depended on the residue at position 358. While we did not directly show a confirmational change between the two isoforms, changes in chymotrypsin sensitivity and migration on an SDS gel are consistent with this interpretation. Moreover, the C2B-ß splice also affected *Aplysia* Syt I ability to undergo homodimerization. In general Syts can oligomerize in two different ways. One is calcium independent and is mediated by the N-terminus (Fukuda at al., 1999) the other is calcium dependent and is mediated by the C-terminus (Desai et al., 2000; Chapman et al., 1998; Chapman et al., 1996). A number of C2B–effector interactions have been identified including the following: AP-2 (Zhang et al., 1994; Jorgensen et al., 1995), SV2 (Schivell et al., 1996), ß-SNAP (Schiavo et al., 1995), Ca^{2+} channels (Kim and Catterall 1997; Sheng et al., 1997), inositol polyphosphates (Fukuda et al., 1995) and, finally, homo-oligomerization (Chapman et al., 1996; Sugita et al., 1996). Of these interactions, only oligomerization was promoted by Ca^{2+}.

Interestingly, based on Syt Is 3-D structure (Fernandez et al., 2001), residue 358 is in close proximity to the polybasic region (part of the ß-4 sheet) that is implicated in this type of homo-dimerization (Chapman et al., 1998). Although, some studies have shown that the calcium dependent oligomerization of recombinant Syt I may be due to bacterial contaminants that bind thightly to the C2B domain (Garcia et al., 2000; Ubach et al., 2001), a more recent study shows that highly purified cytoplasmic domains of C2A-C2B assembles into heptameric barrel-like structures on the surface of lipid monolayers and that Ca^{2+} and weak interactions with anionic lipids (Earles et al., 2001; Bai et al., 2002; Fernandez et al., 2001) drive this oligomerization. Assembly of Syt oligomers was abolished by mutations in the C2B domain, suggesting that C2B drives multimerization. Together with the fact that native synaptotagmin can undergo calcium dependent

oligomerization (Osborne et al., 1999) these results suggest that the bacterial contaminants may replace necessary cofactors that are present *in vivo* (Wu et al., 2003).

How is it possible that proteins that clearly identify as orthologs may have markedly different functional properties? Dai et al. (2004) showed that, unexpectedly, both C2 domains of *Drosophila* Syt IV exhibit Ca^{2+}-dependent phospholipid binding, whereas neither C2 domain of rat synaptotagmin IV binds Ca^{2+} or phospholipids efficiently. Crystallography reveals that changes in the orientations of critical Ca^{2+} ligands, and perhaps their flexibility, render the rat synaptotagmin IV C2B domain unable to form full Ca^{2+} -binding sites. These results indicate that synaptotagmin IV is a Ca^{2+} sensor in the fly but not in the rat and that the Ca^{2+} -binding properties of C2 domains cannot be reliably predicted from sequence analyses.

We also showed that SNAP-25 binds synaptotagmin I C2B-ß with a higher affinity than synaptotagmin I C2B-α. Previously it has been shown that SNAP-25 binds Syt I in a calcium dependent manner (Gerona et al., 2000; Zhang et al., 2002), however, the exact binding site on Syt I has not yet been identified. While one study claims that this binding site is exclusively located on the C2B domain (Sciavo et al., 1997), another one shows that C2B does not bind SNAP-25 (Gerona et al., 2000). Gerona et al., (2000) showed that although C2A can bind to SNAP-25 to some extent on its own, high affinity interactions requires both C2A and C2B. Our data is consistent with an important role for the C2B domain of *Aplysia* Syt I in its binding with SNAP-25 given that the C2B-ß splice affects this interaction. Since the properties of C2B domain lead to differences in the efficiency of transmitter release, this suggest that there could be subtle differences in the fusion abilities of induvidual synaptic vesicles based on the relative amount of the C2B splice forms in these vesicles and synaptotagmin I C2B-ß and C2B-α might play an important role as alternative calcium-sensors in the regulation of the efficiency of synaptic vesicle exocytosis. Further studies are needed to determine the physiological impact exerted by the C2B-ß splice form.

5.2.1 Does SNAP-25 modulation of calcium dynamics play a role in depression?

During HSD the depression of transmitter release occurs downstream of calcium influx (Armitage and Siegelbaum, 1998) and probably affects calcium-secretion coupling. A recent study in hippocampal GABAergic synapses shows that SNAP-25 negatively regulates neuronal calcium responsiveness to depolarization (Verderio et al., 2004). How can SNAP-25 mediate this inhibition? These neurons naturally lack SNAP-25, but contain the related SNAP-23. Exogenously expressing SNAP-25 into GABAergic neurons was sufficient to decrease the sensitivity of release to depolarization. Most of the above effect was localized to the 180-197 residues in the C-terminal portion of SNAP-25 containing the PKC phosphorylation site. Therefore it is probably likely that SNAP-25 phosphorylation plays a regulatory role only in specific types of neurons. In chromaffin cells the role of PKC is to increase the proportion of vesicles that are highly sensitive to calcium (Yang et al., 2002). These results together suggest a model where SNAP-25 naturally inhibits a step in calcium secretion coupling and that PKC activation can reverse this step. The carboxy-terminal region is also required for Ca^{2+}-dependent interaction of the calcium sensor Syt I and SNAP-25 during Ca^{2+}-triggered exocytosis (Gerona et al., 2000; Zhang et al., 2002). The interaction of Syt I with the SNARE complex is a leading candidate for the calcium sensitive step regulating exocytosis (Bai et al., 2004; Tucker et al., 2004) and thus regulation of this interaction by phosphorylation could regulate calcium-secretion coupling. In previous work we showed that Syt I binding to SNAP-25 increases about 2-4 fold in the presence of calcium (Nakhost et al., 2004). However, we did not observe any differences in Syt I-SNAP-25 interactions with any of the mutations in the phosphorylation site (Nakhost et al., 2004). It should be noted though that these experiments only investigated the low affinity interaction of Syt I with SNAP-25 compared to the high affinity interaction of Syt I with the entire SNARE complex, since SNAP-25 is largely unstructured as a monomer but acquires α-helicity in binary and ternary SNARE complexes (Fasshauer et al., 1997). G-protein $\beta\gamma$ inhibits exocytosis downstream of priming through binding to the C-terminus of

SNAP-25 (Gerachshenko et al., 2005), perhaps interfering with the Ca^{2+}-dependent binding to Syt I (Blackmer et al., 2005). Removal of this inhibition by 5-HT could be blocked by the EGFP-SNAP-25 dominant negative construct. We provide suggestive evidence that SNAP-25 may play an important role in the block of calcium-secretion coupling that is important for HSD (Houeland et al., 2006).

5.3. Localization of Syt Is in Aplysia.

Overexpression of *Aplysia* sensory neurons with fluorescently labeled Syt I and Syt I_{VQ} revealed that about 5% of punctae contained one or the other isoform. From these results we propose that the linker domain might regulate sorting in the trans-Golgi network and thus affect trafficking of serotonin receptors. When we fluorescently labeled Syt I C2B-ß – and C2B-α splice forms, a subset of puncta, 22% showed a substantial preference for one of the fusion proteins. The higher quantity of vesicles exhibiting differences in the levels of C2B spliced isoforms suggests that there could be subtle differences in the fusion abilities of individual synaptic vesicles. In these experiments we cannot differentiate vesicles originated from the cell body (transport vesicles) and synaptic vesicles that are formed by endocytosis.

Recently, overexpression of Syt I in autaptic hippocampal neurons increased the release probability (Han et al., 2004). The interpretation of these data as a gain-of-function effect are exactly the opposite of Nagy et al. (2006), who found that Syt I overexpression increased the size of the RRP in chromaffin cells, without changing the release rate constant. Likewise, in hippocampal neurons, no difference in EPSC shape was found when Syt I nulls were rescued by Syt I or Syt II (Stevens and Sullivan, 2003). Therefore, it appears that, in neurons, a kinetic step independent of calcium binding to Syt I determines the timing of synaptic vesicle fusion from the two pools. Voets et al. (2001) found that the RRP to be missing in chromaffin cells from Syt I KO mice using simultaneous capacitance

and amperometric measurements, however upon Syt I overexpression the RRP is restored (Nagy et al., 2006).

5.3.1 How does our work compare with studies of SNAP-25 in chromaffin cells?

Unlike in endocrine chromaffin cells where exocytosis can be measured directly using capacitance and amperometry measurement, we can only measure exocytosis indirectly using the EPSP in the motor cells. In the *Aplysia* culture system changes in EPSP amplitude are likely to reflect changes in transmitter release because there is neither the complication of multiple synaptic inputs nor that of nervous system development. In addition, since these cells are electrically silent in the absence of extracellular stimulation, we can interpret changes in the size of initial EPSP as changes in exocytosis.

Phosphomimetics of *Aplysia* SNAP-25 decreased the rate of depression. This result is similar to a study in adrenal chromaffin cells where overexpression of phosphomimetic mutants at SNAP-25 Ser187 resulted in enhanced vesicle recruitment (Nagy et al., 2002). In contrast to our results, this study demonstrated that overexpression of non-phosphorylated state mutants at SNAP-25 Ser187 resulted in inhibition of vesicle pool refilling (Nagy et al., 2002). However, in this study the stimulus used for release led to enhanced phosphorylation of SNAP-25. Since PKC is not activated during 0.05 Hz stimulation and there was no effect of the non-phosphorylated state EGFP-SNAP-25 on depression, we concluded that it is unlikely that PKC phosphorylation of SNAP 25 plays a role in depression in *Aplysia* sensory-motor neuron synapses.

5.3.2 Aplysia Syt I has been cloned previously.

Our electrophysiological results showed that in cells expressing EGFP-Syt I 5-HT-induced facilitation of depressed SM synapses was normal. In an earlier paper overexpression of *Aplysia* Syt I decreased the amount of transmitter release at non-depressed synapses (Martin et al., 1995). Martin et al. (1995) proposed that the inhibition of the EPSP size was consistent with Syt I acting as an inhibitory clamp for release. In these experiments Syt I

was expressed after synapse formation and comparisons were done between cells before and after overexpression of Syt I. However, these results are not directly comparable to ours where expression preceded synapse formation, and comparisons were done between different cells. Similarly, as reported by Martin et al. (1995), we also observe a reduction in the initial EPSP size after overexpression of the various Syt I constructs, although, because of the large variability in initial EPSP, this was not significant in an ANOVA.

Whether a particular class of synapse displays an enhancement or depression of transmitter release is likely to depend on the initial state of that synapse. Synapses that start with an initial high probability of release acts as a ceiling, limiting the potential for any further enhancement. In addition, the high probability of release results in a greater rate of depletion of synaptic vesicles upon repetitive stimulation. In contrast, synapses that start with a low probability of release tend to display a larger degree of facilitation. The lack of sensitivity of some synapses to PDBu may indicate that there is a ceiling on the proportion of vesicle pool that can be maintained in the readily releasable condition, and this ceiling had been reached at some synapses prior to PDBu treatment. Furthermore, the heterogeneity in release properties, even among synapses between similar types of neurons, has been correlated with the heterogeneity in active zone morphology. Synapses that show a high probability of facilitation and thus a lower initial probability of release display a lower packing density of synaptic vesicles (Bower and Haberly, 1986) and a smaller active zone area (Schikorski and Stevens, 1997).

Large variabilities in the initial EPSPs are common when using primary neurons and this is true for all studies in primary neuronal cultures of sensory-motor neurons, independent of any expression studies. Since we are only expressing the constructs in the sensory cell and recording EPSPs in the motor neuron, it is most likely we are seeing effects on transmitter release.

5.3.3 How does our work compare with studies of synaptotagmin in central neurons?

In autaptic hippocampal neurons, single EPSCs follow a biphasic time course, and, in the absence of Syt I, the balance is shifted in favor of the asynchronous component, and the total amount of secretion is only slightly reduced (Shin et al., 2003; Nishiki and Augustine, 2004a). Overexpression of Syt I in Syt I null autaptic neurons restored the synchronicity of the EPSCs (Han et al., 2004; Nishiki and Augustine, 2004b). However, closer inspection reveals differences, because in chromaffin cells the Syt I, the C2 charge neutralizing (R233Q) mutation, noticeably slows down fast flash-induced release from the RRP (Sørensen et al., 2003), whereas in neurons the amplitude of a single EPSC is reduced, but the kinetics of the two components are unaffected (Fernández-Chacón et al., 2001; Han et al., 2004). The R233Q mutation also provides to test whether Syt regulates the interconversion of SRP vesicles into RRP vesicles, or that Syt is itself the Ca^{2+} sensor for RRP fusion. In the first case the mutation should have an effect on the relative sizes of RRP and SRP pools, whereas in the latter case properties of the RRP calcium sensor should be changed.

Recently, overexpression of Syt I in autaptic hippocampal neurons increased the release probability (Han et al., 2004). The interpretation of these data as a gain-of-function effect are exactly the opposite of Nagy et al. (2006), who found that Syt I overexpression increased the size of the RRP in chromaffin cells, without changing the release rate constant. Likewise, in hippocampal neurons, no difference in EPSC shape was found when Syt I nulls were rescued by Syt I or Syt II (Stevens and Sullivan, 2003). Therefore, it appears that, in neurons, a kinetic step independent of calcium binding to Syt I determines the timing of synaptic vesicle fusion from the two pools. Voets et al. (2001) found the RRP to be missing in chromaffin cells from Syt I KO mice using simultaneous capacitance and amperometric measurements, however here as well, upon Syt I overexpression the RRP is restored (Nagy et al., 2006).

The apparent calcium affinity of C2 domains depends critically on the local phospholipid composition. This is also a relevant consideration when comparing the triggering rate between different experimental systems, or the different expression of accessory proteins.

Previous studies of the role of Syt I in neurotransmitter release relied on loss-of-function mutations (e.g., see refs. Fernández-Chacón et al., 2001; Robinson et al., 2002 and Nonet et al., 1993; Littleton et al., 1993, DiAntonio et al., 1993 Geppert et al., 1994; Mackler et al., 2002). Although these studies established a central role for synaptotagmin I in the Ca^{2+}-triggering of release, one pitfall of these KOs is that the phenotype may be secondary to developmental changes. Therefore, KO phenotypes should be verified by functional rescue experiments, which, if successful, pave the way for structure-function analysis (Nagy et al., 2006).

5.4 Overexpression studies.

Information to build the brain is coded in the genome, a much simpler structure than the brain itself. Molecular cloning has made it possible to identify the genes and determine the primary structure of many of the proteins that mediate synaptic function and membrane excitability. In *Aplysia* these proteins can be directly overexpressed in individual cells.

Overexpression often gives the most apparent results but is the most difficult to interpret, as cells retain their functional endogenous wild-type protein that could, therefore, contribute to the "normal" kinetic parameters of exocytosis.

In view of the delicate relation between the conformational state of proteins and function, it is difficult to analyze the consequence of large alterations. For example, when the whole SNARE complex does not form, it is impossible to pinpoint a role of a constituent protein (e.g. SNAP-25) in synaptic transmission. The overall functional phenomena will be completely dictated by the lack of the SNARE complex. These studies thus need extra verification studies to confirm that the expression does not interfere with vesicular mobilization. The effect of heterologous expression in *Aplysia* SM synapses critically depends upon a large excess of introduced protein compared to wildtype, to nullify the effect of the endogenous gene expression. Sorensen et al. (2004) over-expressed SNAP-25 constructs in chromaffin cells with high efficiency virus (SFV). They verified the ratio of introduced vs. endogenous expression (25:1) by using quantitative Western Blot analysis.

Since it is impossible to do quantitative western blots in single injected neurons, we were unable to quantitatively determine the level of overexpression. As an alternative indicator of the level of overexpression we used an antibody raised to endogenous SNAP-25. This was less obvious because SNAP-25 immunoreactivity is also present in the motor neuron and the immunolocalization of endogenous protein had more background staining than the fluorescent proteins. Although not quantifiable, these experiments gave us a good indication that the levels of overexpressed EGFP-SNAP-25 are much greater than those of the endogenous protein.

Other ways to get around the prescence of the endogenous protein, is to 1) overexpress the protein in question in cells that do naturally lack the protein or 2) to create a clean genetic background by wiping out the endogenous protein, creating a toxin resistant mutant of a SNARE that allows endogenous SNARES to be inactivated by a toxin after cell permeabilization. This system can then be exploited to examine the functional effect of other mutations. By the exogenous expression of SNAP-25, Verderio et al. (2004) showed that SNAP-25 negatively regulates neuronal calcium responsiveness to stimuli. These results demonstrate that SNAP-25 not only plays key roles in exocytosis, but also controls calcium responsiveness to depolarization, being involved in the regulation of neuronal excitability. In a similar approach Nagy et al. (2006) investigated the often-cited hypothesis that Syt I and Syt II are alternative calcium sensors for fast release and, at the same time, they evaluated the role of the Syt I phosphorylation sites, which might participate in regulatory functions. Because chromaffin cells do not naturally express Syt II (Geppert et al., 1991; Marquèze et al., 1995), expressing either isoform in Syt I null chromaffin cells creates a situation in which only one or the other of these two isoforms is present. Similarly, the effect of mutating Syt I in a phosphorylation site can be investigated without the complication of phosphorylation of wildtype protein. Finley and coworkers used the latter approach when they sought to test directly that phorbol ester enhancement of synaptic transmission was mediated by phosphorylation of SNAP-25 by PKC in hippocampal synaptic transmission. In this study they used a combination of pharmacology and

phosphorylation mutants of SNAP-25 to show that PKC-dependent phosphorylation does not mediate the observed enhancement of neurotransmission observed by PEs at hippocampal synapses They created a clean genetic background by wiping out the endogenous SNAP-25 with botullin toxin, such that only the overexpressed botullin toxin resistant SNAP-25 remained. Unfortunately, this approach does not work in an *Aplysia* cell culture environment. However, it is not clear that the cleaved endogeous *Aplysia* SNAP-25 is completely inert. Apland and coworkers got around this problem by using peptides that mimic the SNAP-25 C-terminal end, corresponding to the ones cleaved by BoNT/A and E to block acetylcholine release from the buccal ganglia of *Aplysia* (Apland et al., 1999).

Overexpression of *Aplysia* Syt I and Syt I_{VQ} in cultured sensorimotor cells suggested a novel role for the juxtamembrane domain. Interestingly, whereas PKC activity is required for the reversal of synaptic depression, overexpression of active PKC actually inhibited the ability of 5-HT to reverse synaptic depression (Manseau et al., 2001). This inhibition appeared kinetically similar to the block by Syt I_{VQ} and may be attributable to an effect of PKC on regulating the availability of 5-HT receptors (Manseau et al., 2001). Our results would be consistent with Syt I_{VQ} being the PKC substrate involved in this phenomenon because Syt $I_{VQ;\ S\text{-}A}$ showed reduced inhibition compared with Syt I_{VQ}. Overexpression of Syt $I_{VQ,}$ but not of Syt I blocked the ability of serotonin to reverse synaptic depression. This effect is upstream of PKC activation, because neither Syt I_{VQ} nor Syt I blocked the effects of PE on reversing synaptic depression or the effects of serotonin on facilitating nondepressed synapses.

5.4.1 The use of EGFP-fusion proteins in Aplysia.

A potential problem in labelling synapses with EGFP-fusion proteins is that such proteins may have functional effects on synapses. In our experiments, overexpression of EGFP-coupled SNAP-25 acted as a dominant negative for the 5-HT mediated reversal of depression, but not for initial synaptic strength or PDBu-mediated increases in synaptic

strength. The dominant negative effect is not simply due to the overexpression of SNAP-25, because this effect is not observed when EGFP and SNAP-25 are encoded on separate plasmids and coinjected. The specificity of the dominant negative effect suggests a distinct role of SNAP-25 in regulating depression independent of its role in transmitter release and vesicle availability. The fact that the EGFP-SNAP-25 S-D mutant can still act as a dominant negative does not address whether PKC phosphorylation of SNAP-25 plays a direct role, since the wild-type protein acts as a dominant negative as well, despite the likelihood that it is still phosphorylated after serotonin addition. Indeed, in chromaffin cells both S187A and S187D mutants had decreases in the fast burst of release most likely due to sensitivity for some SNAP-25 interaction dependent upon the exact conformation of the amino acid at this position (Nagy et al., 2002). Presumably, due to some steric restraint from the EGFP-tag, inhibition mediated by SNAP-25 cannot be released through PKC phosphorylation. This may be due to phosphorylation of SNAP-25 by PKC, or due to PKC phosphorylation of an interacting partner. Thus, although somewhat speculative, one model that is consistent with our data posits that during depression SNAP-25 acts to inhibit release at a post-priming step and thus blocking calcium-secretion coupling. This inhibition by SNAP-25 is sensitive to the amino acid at the phosphorylation site and therefore many of the mutants do not compete with endogenous SNAP-25 for this interaction. In our experiments, overexpression of EGFP-coupled SNAP-25 acted as a dominant negative for the 5-HT mediated reversal of depression, but not for initial synaptic strength or PDBu-mediated increases in synaptic strength. The specificity of the dominant negative effect suggests a distinct role of SNAP-25 in regulating depression independent of its role in transmitter release and vesicle availability.

How could SNAP-25 mediate this inhibition? The carboxy-terminal region is also required for Ca^{2+}-dependent interaction of the calcium sensor Syt I and SNAP-25 during Ca^{2+}-triggered exocytosis (Gerona et al., 2000; Zhang et al., 2002). The interaction of Syt I with the SNARE complex is a leading candidate for the calcium sensitive step regulating exocytosis (Bai et al., 2004; Tucker et al., 2004) and thus regulation of this interaction by

phosphorylation could regulate calcium-secretion coupling. In previous work we showed that Syt I binding to SNAP-25 increases about 2-4 fold in the presence of calcium (Nakhost et al., 2004). However, we did not observe any differences in Syt I-SNAP-25 interactions with any of the mutations in the phosphorylation site (Nakhost et al., 2004). It should be noted that these experiments only investigated the low affinity interaction of Syt I with SNAP-25 compared to the high affinity interaction of Syt I with the entire SNARE complex. In a recent study by Han et al. (2005) Syt I-ECFP fusion proteins inactivated Syt I function while retaining its normal localization. Here, transgenically expressed synaptotagmin I-ECFP failed to rescue the lethal Syt I KO phenotype. The failure of Syt I-ECFP to rescue was confirmed by overexpression of wild type and ECFP-fused Syt I in cultured neurons from Syt I KO mice.

We showed that SNAP-25 S-E could inhibit depression. Conversely, SNAP-25 S-A had no effect, presumably because PKC was not activated. We used phorbol esters to verify whether PKC could inhibit depression. Our results show that this activation of PKC did indeed reduce the steady-state level of depression to a similar level as the SNAP-25 S-E and this was not seen when SNAP-25 S-A is expressed. Moreover, we found that SNAP 25 S-A also blocked the initial facilitations seen with PDBu.

5.5 Pretranslational modifications.

An idea that is emerging from recent work is that average neuronal activity levels are maintained by a set of homeostatic plasticity mechanisms that dynamically adjust synaptic strengths in the correct direction to promote stability (Turrigiano and Nelson, 2004). When a neuron in the mammalian brain receive up to 10,000 synapses, the properties of each of these synapses vary; each is dependent on numerous proteins involved in neurotransmitter transmission –and reception. The ability of neurons to maintain constant activity in the face of constantly changing input could underlie homeostatic plasticity. The induction and propagation of action potentials requires matching the needs of connecting cells.

While, posttranslational modifications, RNA editing, alternative polyadenylation, and multiple sites of transcription contribute, alternative splicing is the major mechanism for generating isoform diversity (Grabowski and Black, 2001). Alternative splicing is the process by which different combinations of exons are included in the mature mRNA, thus allowing a single gene to encode multiple protein isoforms with altered or potentially antagonistic properties. Like hormones switching on certain genes in the course of development, splicing could occur in a parallel fashion to design proteins for optimal performance, ideally suited to scale homeostatic plasticity; like the ability to support incremental changes in ion channel and receptor activities that are relatively long-lasting and independent of gene transcription, but what factors link membrane depolarization to pre-mRNA splicing?

Syt IV is a brain-specific isoform of the Syt family, and could be seen as a potential candidate for upregulation following depolarization. Although the levels of Syt IV expression in the adult brain is typically much lower than that of the primary Ca^{2+} sensors Syt I/II, it has been proposed to be rapidly induced in hippocampus and piriform cortex after stimuli that produce strong depolarization, such as seizure (Vician et al., 1995; Tocco et al., 1996). The Syt IV isoform contains a naturally occurring amino acid substitution at a key residue for Ca^{2+} coordination within its C2A domain (S244 in rat). This substitution impairs Ca^{2+} binding, and therefore the Syt IV C2A domain is considered nonfunctional for Ca^{2+}-dependent interactions (von Poser et al., 1997). These findings, in the context of the impaired Ca^{2+} binding ability of Syt IV, led to the hypothesis that Syt IV upregulation after seizure is a protective mechanism to reduce neural activity (von Poser et al., 1997; Ferguson et al., 1999; Littleton et al., 1999). In a very interesting study, synaptotagmin IV was identified as an immediate early gene (Vician et al., 1995). This finding suggests that a switch from Ca^{2+}-dependent to Ca^{2+}-independent synaptotagmins may occur during strong stimulation of neurons. Such a switch could be particularly useful during pathological hyperexcitation that is accompanied by unimpeded Ca^{2+} influx. A switch to a Ca^{2+}-unresponsive synaptotagmin under those conditions would eliminate a Ca^{2+} target and

maybe inhibit excessive neurotransmitter release (von Poser et al., 1997). Consistent with the neuroprotective hypothesis, overexpression of Syt IV at the *Drosophila* neuromuscular junction reduced the peak amplitude of synaptic responses (Littleton et al., 1999). Littleton has refuted himself here, because the original Syt IV antisera was not isoform specific (Littleton et al., 1999) and resulted in cross reactivity with Syt I. With their new isoform-specific antisera to Syt IV, Littleton and coworkers (Adolfsen et al., 2004) showed that in *Drosophila*, Syt IV is post-synaptic, and not expressed on synaptic vesicles; the same is probably true in vertebrates, where effects of Syt IV have been seen in postsynaptic regulation. Postsynaptic Ca^{2+} influx also stimulates local synaptic differentiation and growth through Syt IV-mediated retrograde signals in a synapse-specific manner (Yoshihara et al., 2005). To further test this hypothesis (that Syt IV upregulation is a neuroprotective mechanism for reducing neurotransmitter release), Syt IV was recently overexpressed in cultured mouse hippocampal neurons (Ting et al., 2006) and acute effects on fast excitatory neurotransmission assessed. Ting and colleagues found no alterations in neurotransmission with respect to basal release probability, Ca^{2+} dependence of release, short-term plasticity, and fusion pore kinetics. On the other hand, they found that expression of a mutant Syt I with diminished Ca^{2+} affinity (R233Q) reduced release probability and altered the Ca^{2+} dependence of release, demonstrating the sensitivity of the system to changes in neurotransmission resulting from changes to the Ca^{2+} sensor. Thus, these recent findings refute the dominant model that Syt IV functions as an inhibitor of neurotransmitter release in mammalian neurons and thus as a protective response.

In our studies quantitative reverse transcription (RT-PCR) studies from the *Aplysia* nervous system indicate that mRNAs encoding Syt I and Syt I_{VQ} are present in the nervous system of *Aplysia* and also in sensory neuron clusters alone at approximately a 1:1 ratio. Treatment of sensory clusters with a paradigm that induces long-term facilitation [5x 5min. pulses of 20 mM 5-HT (Montarolo et al., 1986)] did not alter the ratio of Syt I and Syt I_{VQ}, nor did it alter the ratio of Syt I C2B-α -and C2B-ß.

5.5.1 Alternative splicing during development.

Alternative splicing is controlled at the level of individual neurons to design proteins for optimal performance. The expression profiles of splice isoforms are modified during development (Bark et al., 2004; Nagy et al., 2005) and as neural activity changes (Mu et al, 2003). The inclusion or exclusion of as few as one or two amino acids at protein interaction sites can modify protein functions and might act as molecular switches, permitting or preventing association with target proteins. An emerging theme in molecular neurobiology is that alternative splicing may generate cell specific combinations of proteins isoforms that define the functional properties of the cell and underlie complex processes in the nervous system such as synaptic plasticity (Ule and Darnell, 2006). The essential nature of this process is underscored by the finding that its misregulation is a common characteristic of human disease. Although significant progress has been made in identifying links between altenative splicing and neurological diseases, in many cases our comprehension of the significance of these links and the molecular basis for these diseases is far from complete. Splicing abnormalities have been noted in complex polygenic disorders, including schizophrenia and other psychiatric disorders (Black and Grabowski, 2003; Clinton et al., 2003). However, causes of these splicing defects and their contribution to the disease pathogenesis have not yet been determined.

SNAP-25 is regulated during brain growth and synaptogenesis at the level of expression and by alternative splicing between tandem arranged exons (Bark et al., 1995). This results in a developmental switch between 1 and 3 weeks of age from expression of predominantely SNAP-25a to SNAP-25b (Bark et al., 1995; Boschert et al., 1996; Jacobsson et al., 1999). Bark et al. (2004) addressed the role of these two splice-variant isoforms of SNAP-25 with a targeted mouse mutation that impairs the shift from SNAP-25a to SNAP-25b. Most of these mutant mice die between 3 and 5 weeks of age, which coincides with the time when SNAP-25b expression normally reaches mature levels in brain and synapse formation is essentially completed. The switch between SNAP-25

isoforms alters the efficacy of synaptic transmission that helps to guide the transition from immature to mature synaptic connections, as well as synapse regrowth and remodeling after neural injury. The isoforms differ by only 9 of 39 residues encoded by the alternatively spliced exons (Bark and Wilson, 1994; Bark et al., 1995). Altered expression of these SNAP-25 isoforms influences short-term synaptic function by affecting facilitation but not the initial probability of release. This suggests that mechanisms controlling alternative splicing between SNAP-25 isoforms contribute to a molecular switch important for survival. Both isoforms support Ca^{2+}-stimulated insulin secretion (Gonelle-Gispert et al., 1999). Nevertheless, although either SNAP-25 isoform directs Ca^{2+}-evoked catecholamine release in SNAP-25-deficient chromaffin cells, their ability to stabilize the pool of primed vesicles for secretion differs in these cells (Sørensen et al., 2003). To explore the function of SNAP-25 isoforms in central synapses, Sørensen et al. (2003) generated a mutation that perturbs alternative splicing and limits expression of SNAP-25b in mice. Most of the mutants overexpressing SNAP-25a die between postnatal day 25 (PN25) and PN35 when this switch normally occurs. They observed that facilitation of synaptic transmission at hippocampal synapses of mutant mice is increased, but the probability of release is unaltered. This supports the idea that assembly of different SNARE complexes affects properties of membrane fusion and that the developmental switch between SNAP-25 isoforms alters the efficacy of synaptic transmission that may contribute to the solidification of developing neural circuitry.

The effect of incorporating different SNAP-25 isoforms into the SNARE core complex is to regulate the size of the exocytotic burst, which represents the number of release-ready vesicles (Nagy et al., 2005). Importantly, this is achieved without changing the rate of release from the releasable pools (either the SRP or the RRP (Sørensen et al., 2003). This adds to previous findings that alterations of protein kinase C or protein kinase A phosphorylation sites in SNAP-25 also modify upstream priming reactions, without affecting the fusion rate from the primed vesicle pools (Nagy et al., 2002, 2004).

5.5.2 Possible roles of an alternative splice form in the Syt I juxtamembrane domain.

Interestingly, the VQ sequence is also located at the exon start in the Syt I-related Syt IX (also called Syt V) (Craxton et al., 1997). Alternative exon entry has also been described in this exact region of the linker region of rat Syt I where the amino acids ALK are inserted instead of VQ (Perin et al., 1990) Syt I, II, and IX are more closely related to each other than to invertebrate Syt I isoforms suggesting that they have diverged after the vertebrate-invertebrate separation (Marqueze et al., 2000). The juxtamembrane domain is well conserved in all Syt-I like isoforms (vertebrate and invertebrate), but not in other Syts like Syt IV. While Syt I and Syt II are highly conserved in the juxtamembrane domain, it is also striking that in the region of the VQ insertion, Syt II has a sizable deletion. Thus, there is an alternative exon entry site in the linker domain of Syt I that is conserved over evolution. However, the role for this splicing is unknown. The juxtamembrane domain of Syt I, the region that joins the transmembrane region to the C2A domain, is one of the least studied segments of the Syt I protein. This domain is nevertheless well conserved over evolution in a subtype specific manner. This region is also subject to phosphorylation by a number of PKs (Hilfiker et al., 1999). It has been suggested that this domain may form an α-helix and subsequently interact with as yet unidentified target molecules (Perin et al., 1999). It may also play a role in the localization of of Syt I to distinct cellular locations (Fukuda et al., 2001).

Alternative splicing of the linker sequence between the transmembrane region (TMR) and the C2-domains of Syts and of the TMR itself have been reported. Alternative splicing of a short sequence in the linker of Syt I (Perin et al., 1990) probably represents a sliding exon/intron junction. PCR experiments suggested that for many Syts, variants lacking TMRs are produced by "exon skipping" (Fukuda et al., 1999; vonPoser., 1997). However, the gene structures of these Syts show that exon skipping would create out-of-frame junctions between the N- and C-terminal exons. This result together with the fact that no soluble synaptotagmins were observed make the alternative splicing of synaptotagmin TMRs doubtful (Sudhof, 2002).

5.6 Possible molecular mechanisms for changing synaptic strength.

Memory formation likely takes place through the modulation of presynaptic neurotransmitter release and/or postsynaptic receptor organization and requires the intricate regulation of membrane trafficking events. A wide range of mechanisms has been implicated in the induction of the different forms of plasticity. Presynaptic changes in release occur generally through two types of mechanisms. One involve a change in the amplitude of the transient rise in Ca^{2+} in the presynaptic terminal triggered by a presynaptic action potential, due to either direct Ca^{2+} modulation or indirect effect on presynaptic Ca^{2+}excitablility. The second mechanism occurs at some site downstream of presynaptic Ca^{2+} elevation and is due to the modulation of some stage of the synaptic vesicle cycle. Such changes may involve an alternation of some early phase of in the release process, for example the modification of in the size of the synaptic vesicles available for release. Alternatively there could be a direct modification of some late stage in release, perhaps involving the fusion apparatus itself.

VI. Conclusion

In conclusion, our data support that PKC phosphorylation of SNAP-25 is necessary, but probably not sufficient for PDBu-mediated increases in transmitter release. Moreover, we provide suggestive evidence that SNAP-25 may play an important role in the block of calcium-secretion coupling that is important for HSD.

Overexpression of Syt I and Syt I_{VQ} in cultured sensorimotor cells suggested a novel role for the juxtamembrane domain. In these studies overexpression of Syt I_{VQ} blocked the ability of 5-HT to reverse synaptic depression, while overexpression of Syt I did not. This effect was specific for the reversal of depression. Futhermore, boosting the system with a phorbol ester, resulted in a robust reversal of depression, both for Syt I and Syt I_{VQ}. This in turn suggests that overexpression of Syt I_{VQ} affects the pathway that is involved in the reversal of depression upstream of the activation of PKC.

In terms of overall synaptic signaling in the central nervous system, the “unreliability” of synaptic exocytosis is advantageous. The low release probability gives the synapse considerable leeway for regulation. By changing release probability at individual synapses, the properties of synaptic networks can be finely tuned. Previous activity and the reception of neurotransmitter signals, neuropeptide signals, or both from other synaptic terminals can dramatically change the pattern of exocytosis. One is tempted to ask why such regulation is necessary and why so many regulators. One possibility is that, SNARE regulation in the early part of the secretory pathway may restrict promiscuous SNARE pairing upon protein translocation into ER or prevent trans SNARE binding between cognate SNAREs before they reach their appropriate compartments.

A second reason may relate to site of action. As SNAREs tend to be widely dispersed over their resident compartments, only those molecules adjacent to the site of fusion between apposed membranes need to be activated. Thus, some factors ensure that only apposed cognate SNAREs form functional trans-SNARE complexes, while other factors (i.e phosphorylation) may restrict the activity of SNAREs distal to the site of fusion. A third reason is directely related to the catalysis of SNARE assembly. This process consists of the ATPase-mediated disassembly of cis SNARE complexes and association of factors to maintain SNAREs in their active conformation. The *in vivo* occurrence, functional

significance, cell or developmental specificity, and the stimuli that control the phosphorylation of these SNAREs remain largely unknown.

VII. References

Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell* **88**:615-26.

Abeliovich A, Paylor R, Chen C, Kim JJ, Wehner JM, Tonegawa S (1993). PKC gamma mutant mice exhibit mild deficits in spatial and contextual learning. *Cell* **75**:1263-71.

Abrams TW, Castellucci VF, Camardo JS, Kandel ER and LLoyd PE (1984). Two endogenous neuropeptides modulate the gill- and siphon-withdrawal reflex in Aplysia by presynaptic facilitation involving cAMP-dependent closure of a serotonin-sensitive potassium channel. *Proc Natl Acad Sci* **81**:7956-7960.

Aldea M, Jun K, Shin HS, Andres-Mateos E, Solis-Garrido LM, Montiel C, Garcia AG, Albillos A (2002). A perforated patch-clamp study of calcium currents and exocytosis in chromaffin cells of wild-type and alpha(1A) knockout mice. *J Neurochem* **81**:911-21.

Ales E, Tabares L, Poyato JM, Valero V, Lindau M, Alvarez de Toledo G (1999). High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. *Nat Cell Biol* **1**:40-4.

Antzoulatos EG, Byrne JH (2004). Learning insights transmitted by glutamate. Trends Neurosci. Sep;27(9):555-60. Review.

Antonin W, Fasshauer D, Becker S, Jahn R, Schneider TR (2002). Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nat Struct Biol* **9**:107-11.

Apland JP, Biser JA, Adler M, Ferrer-Montiel AV, Montal M, Canaves JM, Filbert MG (1999). Peptides that mimic the carboxy-terminal domain of SNAP-25 block acetylcholine release at an Aplysia synapse.J *Appl Toxicol* **19**:S23-6.

Aravamudan B, Fergestad T, Davis WS, Rodesch CK and Broadie K (1999). Drosophila UNC-13 is essential for synaptic transmission. *Nat Neurosci* **2**:965-971.

Armitage BA and Siegelbaum SA (1998). Presynaptic induction and expression of homosynaptic depression at Aplysia sensorimotor neuron synapses. *J Neurosci* **18**:8770-8779.

Arvanitaki A and Chalazonitis N (1958) Distinct electrical reactions of two definite neurons to the same modifier. *J Physiol* **50**:125-8.

Augustin I, Rosenmund C, Südhof TC and Brose N (1999). Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. *Nature* **400**:457 -461.

Augustine GJ (2001). How does calcium trigger neurotransmitter release? *Curr Opin Neurobiol* **11**:320-6. Review.

Bai J, Wang P and Chapman ER (2002). C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I. *Proc Natl Acad Sci* **99**:1665-1670.

Bailey CH and Chen M (1983). Morphological basis of long-term habituation and sensitization in Aplysia. *Science* **220**:91-3.

Bailey CH and Chen M (1988). Long-term memory in Aplysia modulates the total number of varicosities of single identified sensory neurons. *Proc Natl Acad Sci* **85**:2373-2377.

Bailey CH, Bartsch D, Kandel ER (1996). Toward a molecular definition of long-term memory storage. *Proc Natl Acad Sci* **93**:13445-52. Review.

Bailey CH, Giustetto M, Huang YY, Hawkins RD, Kandel ER (2000). Is heterosynaptic modulation essential for stabilizing Hebbian plasticity and memory? *Nat Rev Neurosci* **1**:11-20. Review.

Bao JX, Kandel ER and Hawkins RD (1997). Involvement of pre- and postsynaptic mechanisms in posttetanic potentiation at Aplysia synapses. *Science* **275**:969-973.

Bao JX, Kandel ER and Hawkins RD (1997). Involvement of presynaptic and postsynaptic mechanisms in a cellular analog of classical conditioning at Aplysia sensory-motor neuron synapses in isolated cell culture. *J Neurosci* **18**:458-66.

Bark C, Bellinger FP, Kaushal A, Matthews JR, Partridge LD, Wilson C (2004). Developmental regulated switch in alternatively spliced SNAP-25 isoforms alters facilitation of synaptic transmission. *J Neurosci* **24**:8796-8805.

Barry OP, Kazanietz MG (2001). Protein kinase C isozymes, novel phorbol ester receptors and cancer chemotherapy. *Curr Pharm* **7**:1725-44. Review.

Baynes K, Eliassen JC, Lutsep HL, Gazzaniga MS (1998). Modular organization of cognitive systems masked by interhemispheric integration. *Science* **280**:902-5.

Bennett MK, Calakos N, Scheller RH (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257**:255-9.

Bennett MVL (2000). A comparison of electrically and chemically mediated transmission. In: Pappas GD and Purpura DP, Editors, Structure and Function of Synapses. Raven Press, New York. 221-256.

Betz A, Ashery U, Rickmann M, Augustin I, Neher E, Südhof TC, Rettig J and Brose N (1998). Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. *Neuron* **21**:123 -136.

Bliss TV and Collingridge GL (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**:31-9. Review.

Boakes R (1984). From Darwin to behaviourism: Psychology and the minds of animals. Cambridge University Press, Cambridge.

Bommert K, Charlton MP, DeBello WM, Chin GJ, Betz H, Augustine GJ (1993). Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature* **363**:163-5.

Bonner PH, Friedli AF, Baker RS (1994). Botulinum A toxin stimulates neurite branching in nerve-muscle cocultures. *Brain Res Dev Brain Res* **79**:39-46.

Bougie J, Lim T, Ferraro G, Manjunath V, Scott D, Sossin WS (2006). Cloning and characterization of protein kinase C (PKC) Apl III, a homologue of atypical PKCs in Aplysia. *Soc Neurosci Abst* **669.10**

Bower JM, Haberly LB (1986). Facilitating and nonfacilitating synapses on pyramidal cells: a correlation between physiology and morphology. *Proc Natl Acad Sci* **83**:1115-9.

Braha O, Dale N, Hochner B, Klein M, Abrams TW, Kandel ER (1990). Second messengers involved in the two processes of presynaptic facilitation that contribute to sensitization and dishabituation in Aplysia sensory neurons. *Proc Natl Acad Sci* **87**:2040-2044.

Broadie K, Bellen HJ, DiAntonio A, Littleton JT, Schwarz TL (1994). Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission. *Proc Natl Acad Sci* **91**:10727-31.

Brose N, Hofmann K, Hata Y and Sudhof TC (1995) Mammalian homologues of Caenorhabditis elegans unc-13 gene define novel family of C2-domain proteins. *J Biol Chem* **270**:25273-25280

Brunger AT (2000). Structural insights into the molecular mechanism of Ca(2+)-dependent exocytosis. Curr Opin Neurobiol **10**:293-302. Review.

Burgoyne RD. Fast exocytosis and endocytosis triggered by depolarisation in single adrenal chromaffin cells before rapid Ca2+ current run-down (1995). *Pflugers Arch* **430**:213–219.

Burrell BD and Sahley CL (2001). Learning in simple systems. *Curr Opin Neurobiol* **11**:757-764.

Butz S, Fernandez-Chacon R, Schmitz F, Jahn R, Sudhof TC (1999). The subcellular localizations of atypical synaptotagmins III and VI. Synaptotagmin III is enriched in synapses and synaptic plasma membranes but not in synaptic vesicles. *J Biol Chem* **274**:18290-6.

Byrne J Castellucci VF, Hawkins RD and Kandel ER (1974). Receptive field and response properties of mechanoreceptor neurons innervating skin and mantle shelf of Aplysia. *J Neurophysiol* **37**:1041-1064.

Byrne JH Castellucci VF, Carew TJ and Kandel ER (1978). Stimulus response relations and stability of mechanoreceptor and motor neurons mediating defensive gill withdrawalreflex in Aplysia. *J Neurophysiol* **41**:402-417.

Byrne JH, Zwartjes R, Homayouni R, Critz SD, Eskin A (1993). Roles of second messenger pathways in neuronal plasticity and in learning and memory. Insights gained from Aplysia. *Adv Second Messenger Phosphoprotein Res* **27**:47-108.

Byrne JH and Kandel ER (1996). Presynaptic facilitation revisited: state and time dependence. *J Neurosci* **16**:425-35. Review.

Carew TJ, Castellucci VF, Kandel ER (1971). An analysis of dishabituation and sensitization of the gill-withdrawal reflex in Aplysia. *Int J Neurosci* **2**:79-98.

Carew TJ and Kandel ER (1973). Acquisition and retention of long-term habituation in Aplysia: correlation of behavioral and cellular processes. *Science* **182**:1158-60.

Carew TJ, Walters ET and Kandel ER (1983). Associative learning in Aplysia cellular correlates supporting a conditioned fear hypothesis *Science* **211**:501-504

Carew TJ Walters ET and Kandel ER (1981). Classical conditioning in simple withdrawal reflex in Aplysia californica. *J Neurosci* **1**:1426-37

Carew TJ, Hawkins RD, Abrams TW and Kandel ER (1984). A test of Hebb's postulate at identified synapses which mediate classical conditioning in Aplysia. *J Neurosci* **4**:1217-1224.

Carew TJ (1996). Molecular enhancement of memory formation. *Neuron* **16**:5-8. Review.

Castellucci V, Pinsker H, Kupferman I and Kandel, ER (1970). Neuronal mechanisms of habituation and dishabituation of the gill withdrawal reflex in Aplysia. *Science* **167**:1745-1748.

Castellucci V and Kandel ER (1974). A quantal analysis of the synaptic depression underlying habituation of the gill-withdrawal reflex in Aplysia. *Proc Natl Acad Sci* **71**:5004-8.

Castellucci V and Kandel ER (1976). Presynaptic facilitation as a mechanism for behavioral sensitization in Aplysia. *Science* **194**:1176-78.

Castillo J del and Katz B (1954). Quantal components of the end-plate-potential. *J Physiol* **124**:560-573.

Catterall WA (2000). Structure and regulation of voltage-gated Ca2+ channels. *Annu Rev Cell Dev Biol* **16**:521-55. Review.

Ceccarelli B, Hurlbut WP, Mauro A (1973). Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J Cell Biol* **57**:499-524.

Chapman ER and Jahn R (1994). Calcium-dependent interaction of the cytoplasmic region of synaptotagmin with membranes. Autonomous function of a single C2-homologous domain. *J Biol Chem* **269**:5735-41.

Chapman ER, An S, Edwardson JM, Jahn R (1996). A novel function for the second C2 domain of synaptotagmin. Ca2+-triggered dimerization. *J Biol Chem* **271**:5844-9.

Chapman ER, Davis AF (1998).Direct interaction of a Ca2+-binding loop of synaptotagmin with lipid bilayers. *J Biol Chem* **273**:13995-4001.

Charvin N, L'Eveque C, Walker D, Berton F, Raymond C, Kataoka M, Shoji-Kasai Y, Takahashi M, De Waard M, Seagar MJ (1997). Direct interaction of the calcium sensor protein synaptotagmin I with a cytoplasmic domain of the alpha1A subunit of the P/Q-type calcium channel. *EMBO J* **16**:4591-6.

Chen YA, Scales SJ, Patel SM, Doung YC, Scheller RH (1999). SNARE complex formation is triggered by Ca2+ and drives membrane fusion. *Cell* **97**:165-74.

Chow RH, von Ruden L, Neher E (1992). Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. *Nature* **356**:60-3.

Cochilla AJ, Angleson JK, Betz WJ (1999). Monitoring secretory membrane with FM1-43 fluorescence. *Annu Rev Neurosci* **22**:1-10. Review.

Cohen JE, Onyike CU, McElroy VL, Lin AH, Abrams TW (2003). Pharmacological Characterization of an Adenylyl CyclaseCoupled 5-HT Receptor in Aplysia: Comparison With Mammalian 5-HT Receptors. *J Neurophysiol* **89**:1440-1455.

Cousin MA and Robinson PJ (2001). The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. *Trends Neurosci* **24**:659-65.

Coussens L, Parker P J, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Francke, U, and Ullrich A (1986). Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. *Science* **233**:859-866.

Craxton M, Olsen A, Goedert M (1997). Human synaptotagmin V (SYT5): sequence, genomic structure, and chromosomal location. *Genomics* **42**:165-9.

Dai H, Shin OH, Machius M, Tomchick DR, Sudhof TC, Rizo J (2004). Structural basis for the evolutionary inactivation of Ca2+ binding to synaptotagmin 4. *Nat Struct Mol Biol* **11**:844-9.

Dale N and Kandel ER (1990). Facilitatory and inhibitory transmitters modulate spontaneous transmitter release at cultured Aplysia sensorimotor synapses. *J Physiol* **421**:203-22.

Damasio AR (1990). Category-related recognition defects as a clue to the neural substrates of knowledge. *Trends Neurosci* **13**:95-8. Review.

Daveltov BA and Sudhof TC (1993). A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding. *J Biol Chem* **268**:26386-90.

Davis R (1993). Mushroom bodies and Drosophila learning. *Neuron* **11**:1-14.

Day LM and Bentley M (1911). "A Note on Learning in Paramecium". *Animal Behavior* **1**:67-73.

Debus K and Lindau M (2000). Resolution of patch capacitance recordings and of fusion pore conductances in small vesicles. *Biophys J* **78**:2983-97.

Dennison SM, Bowen ME, Brunger AT, Lentz BR (2006). Neuronal SNAREs do not trigger fusion between synthetic membranes but do promote PEG-mediated membrane fusion. *Biophys J* **90**:1661-75.

Desai RC, Vyas B, Earles CA, Littleton JT, Kowalchyck JA, Martin TF, Chapman ER (2000). The C2B domain of synaptotagmin is a Ca(2+)-sensing module essential for exocytosis. *J Cell Biol* **150**:1125-36.

DiAntonio A, Parfitt KD, Schwarz TL (1993). Synaptic transmission persists in synaptotagmin mutants of Drosophila. *Cell* **73**:1281-90.

DiAntonio A, Schwarz TL (1994). The effect on synaptic physiology of synaptotagmin mutations in Drosophila. *Neuron* **12**:909-20.

Dodge, FA Jr and Rahamimoff, R (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J Physiol* **193**:419-432.

Dobrunz LE and Stevens CF (1999). Response of hippocampal synapses to natural stimulation patterns. *Neuron* **22**:157-66.

Earles CA, Bai J, Wang P and Chapman ER (2001). The tandem C2 domains of synaptotagmin contain redundant Ca2+ binding sites that cooperate to engage t-SNAREs and trigger exocytosis. *J Cell Biol* **154**:1117-1123.

Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ and Stone JC (1998) RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science* **280:**1082-1086.

Eliot LS, Kandel ER, Hawkins RD (1994a). Modulation of spontaneous transmitter release during depression and posttetanic potentiation of Aplysia sensory-motor neuron synapses isolated in culture. *J Neurosci* **14**:3280-92.

Eliot LS, Hawkins RD, Kandel ER, Schacher S (1994b). Pairing-specific, activity-dependent presynaptic facilitation at Aplysia sensory-motor neuron synapses in isolated cell culture. *J Neurosci* **14**:368-83.

Fasshauer D, Bruns D, Shen B, Jahn, and Brunger AT (1997). A structural change occurs upon binding of syntaxin to SNAP-25. *J Biol Chem* **272**: 4582-4590.

Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci* **95**:15781-6.

Fasshauer D, Antonin W, Subramaniam V, Jahn R (2002). SNARE assembly and disassembly exhibit a pronounced hysteresis. *Nat Struct Biol* **9**:144-51.

Fatt P amd Katz B (1951). Spontaneous subthreshold activity at motor nerve endings. *J Physiol* **117**:109-128.

Ferguson GD, Thomas DM, Elferink LA, Herschman HR (1999). Synthesis degradation, and subcellular localization of synaptotagmin IV, a neuronal immediate early gene product. *J Neurochem* **72**:1821-31.

Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Sudhof TC (2001). *Nature* **410**:41-49.

Fernandez I Arac, D Ubach J, Gerber S H, Shin O, Gao Y, Anderson RGW, Südhof TC, Rizo J (2001). Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine. *Neuron* **32**:1057-1069.

Ferro-Novick S, Jahn R (1994).Vesicle fusion from yeast to man. *Nature* **370**:191-3. Review.

Fesce R, Grohovaz F, Valtorta F & Meldolesi J (1994). Neurotransmitter release: fusion or 'kiss-and-run'? *Trends Cell Biol* **4**:1-4.

Fesce R and Meldolesi J (1999). Peeping at the vesicle kiss. *Nat Cell Biol* **1**:E3-4.

Frost WN and Kandel ER (1995). Structure of the network mediating siphon-elicited siphon-withdrawal in Aplysia. *J Neurophysiol* **73**: 2413-2427.

Frost L, Kaplan SW, Cohen TE, Henzi V, Kandel ER and Hawkins RD (1997). A simplified preparation for relating cellular events to behavior - contribution of LE and unidentified siphon sensory neurons to mediation and habituation of the Aplysia gill- and siphon-withdrawal reflex. *J Neurosci* **17**: 2900-2913.

Fujita Y, Sasaki T, Fukui K, Kotani H, Kimura T, Hata Y, Sudhof TC, Scheller RH, Takai Y (1996). Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C: its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. *J Biol Chem* **271**:7265-8.

Fukuda M, Moreira JE, Lewis FM, Sugimori M, Niinobe M, Mikoshiba K, Llinas R (1995). Role of the C2B domain of synaptotagmin in vesicular release and recycling as determined by specific antibody injection into the squid giant synapse preterminal. *Proc Natl Acad Sci* **92**:10708-12.

Fukuda M, Kanno E, Mikoshiba K (1999). Conserved N-terminal cysteine motif is essential for homo- and heterodimer formation of synaptotagmins III, V, VI, and X. *J Biol Chem* **274**:31421.

Fukuda M, Kowalchyk, JA Zhang X, Martin TF and Mikoshiba K. (2002). Synaptotagmin IX regulates Ca2+-dependent secretion in PC12 cells. Journal of *Biological Chemistry* **277**:4601-4604.

Fuster JM (1997). Network memory. Trends Neurosci 20:451-459.

Genoud S, Pralong W, Riederer BM, Eder L, Catsicas S, Muller D (1999). Activity-dependent phosphorylation of SNAP-25 in hippocampal organotypic cultures. *J Neurochem* **72**:1699-1706.

Garcia RA, Forde CE, Godwin HA (2000).Calcium triggers an intramolecular association of the C2 domains in synaptotagmin. *Proc Natl Acad Sci* **97**:5883-8.

Gerona RR, Larsen EC, Kowalchyk JA and Martin TF. (2000). The C terminus of SNAP-25 is essential for Ca^{2+}-dependent binding of synaptotagmin to SNARE complexes. *J Biol Chem* **275**:6328-6336.

Garcia-Blanco MA, Baraniak AP, Lasda EL (2004). Alternative splicing in disease and therapy. *Nat Biotechnol* **22**:535-46.

Geppert M, Archer BT 3rd, Sudhof TC (1991). Synaptotagmin II. A novel differentially distributed form of synaptotagmin. *J Biol Chem* **266**:13548-52.

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, and Sudhof TC (1994). Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. *Cell* **79**:717-727.

Geppert M and Sudhof TC (1998). RAB3 and synaptotagmin: the yin and yang of synaptic membrane fusion. *Annu Rev Neurosci* **21**:75-95. Review.

Ghirardi M, Braha O, Hochner B, Montarolo PG, Kandel ER, Dale N (1992) Roles of PKA and PKC in facilitation of evoked and spontaneous transmitter release at depressed and nondepressed synapses in Aplysia sensory neurons. *Neuron* **9**:479-489.

Ghirardi M, Montarolo PG and Kandel ER (1995). A novel intermediate stage in the transition between short- and long-term facilitation in the sensory to motor neuron synapse of Aplysia. *Neuron* **14**: 413-420.

Gillis KD, Mossner R, Neher E (1996). Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. *Neuron* **16**:1209-20.

Gingrich KJ, Byrne JH (1985). Simulation of synaptic depression, posttetanic potentiation, and presynaptic facilitation of synaptic potentials from sensory neurons mediating gill-withdrawal reflex in Aplysia. *J Neurophysiol* **53**:652-69.

Glanzman DL, Mackey SL, Hawkins RD, Dyke AM, Lloyd PE and Kandel ER (1989). Depletion of serotonin in the nervous system of Aplysia reduces the behavioral enhancement of gill withdrawal as well as the heterosynaptic facilitation produced by tail shock. *J Neurosci* **9**:4200-4213.

Glanzman DL 1995 The cellular basis of classical conditioning in Aplysia californica – it's less simple than you think. *TINS* **18**:30-36.

Goelet P, Castellucci VF, Schacher S, Kandel ER (1986). The long and the short of long-term memory--a molecular framework. *Nature* **322**:419-22. Review.

Graham ME, Washbourne P, Wilson MC, Burgoyne RD (2002). Molecular analysis of SNAP-25 function in exocytosis. *Ann N Y Acad Sci* **971**:210-21.

Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science* **258**:1903-10.

Gray EG and Willis RA (1970). On synaptic vesicles, complex vesicles and dense projections. *Brain Res* **24**:149-168.

Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**:780-5.

Gross CG (2000) Neurogenesis in the adult brain: death of a dogma. *Nat Rev Neurosci* **1**:67-73.

Han W, Rhee JS, Maximov A, Lin W, Hammer RE, Rosenmund C, Sudhof TC (2005). C-terminal ECFP fusion impairs synaptotagmin 1 function: crowding out synaptotagmin 1. *J Biol Chem* **280**:5089-100.

Hanks SK and Hunter T (1995). Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* **9**:576-96. Review.

Hanson PI, Roth R, Morisaki H, Jahn R, Heuser JE (1997). Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* **90**:523-35.

Haucke V, Wenk MR, Chapman ER, Farsad K, De Camilli P (2000). Dual interaction of synaptotagmin with mu2- and alpha-adaptin facilitates clathrin-coated pit nucleation. *EMBO J* **19**:6011-9.

Hay JC and Martin TF (1992). Resolution of regulated secretion into sequential MgATP-dependent and calcium-dependent stages mediated by distinct cytosolic proteins. *J Cell Biol* **119**:139-51.

Hawkins RD, Castellucci VF and Kandel ER (1981). Interneurons involved in mediation and modulation of the gill-withdrawal reflex in Aplysia I. Identification and characterization. *J Neurophysiol* **45**:304-314.

Hawkins RD, Castellucci VF and Kandel ER (1981). Interneurons involved in mediation and modulation of the gill-withdrawal reflex in Aplysia II. Identified neurons producec heterosynaptic facilitation contributing to behavioural sensitisation and characterization. *J Neurophysiol* **45**:315-326.

Hawkins RD and Schacher S (1989). Identified facilitator neurons L29 and L28 are excited by cutaneous stimuli used in dishabituation, sensitization, and classical conditioning of Aplysia. *J Neurosci* **9**:4236-4245.

Hawkins RD, Kandel ER and Siegelbaum SA (1993). Learning to modulate transmitter release: Themes and variations in synaptic plasticity. *Ann Rev Neurosci* **16**:625-665.

Hawkins RD, Cohen TE, Kandel ER (2006). Dishabituation in Aplysia can involve either reversal of habituation or superimposed sensitization. *Learn Mem* **13**:397-403.

Hebb, DO (1949). The organization of behavior, Wiley.

Hepp R, Cabaniols JP, Roche PA (2002). Differential phosphorylation of SNAP-25 in vivo by protein kinase C and protein kinase A. *FEBS Lett* **532**:52-6.

Hertz L, Hansson E, and Ronnback L (2001). "Signaling and gene expression in the neuron-glia unit during brain function and dysfunction: Holger Hyden in memoriam." *Neurochem Int* **39**:227-52. Review.

Hess DT, Slater TM, Wilson MC, Skene JH (1992). The 25 kDa synaptosomal-associated protein SNAP-25 is the major methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. *J Neurosci* **12**:4634-41.

Heuser JE and Reese TS (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J Cell Biol* **57**:315-44.

Hilfiker S, Greengard P, Augustine GJ(1999). Coupling calcium to SNARE-mediated synaptic vesicle fusion. *Nat Neurosci* **2**:104-6.

Hilfiker S and Augustine GJ (1999). Regulation of synaptic vesicle fusion by protein kinase C. *J Physiol* **515**:1.

Hirling H and Scheller RH (1996). Phosphorylation of synaptic vesicle proteins: modulation of the alpha SNAP interaction with the core complex. *Proc Natl Acad Sci* **93**:11945-9.

Hochner B, Klein M, Schacher S, Kandel ER (1986a). Action-potential duration and the modulation of transmitter release from the sensory neurons of Aplysia in presynaptic facilitation and behavioral sensitization. *Proc Natl Acad Sci* **83**:8410-8414.

Hochner B, Klein M, Schacher S, Kandel ER (1986b). Additional component in the cellular mechanism of presynaptic facilitation contributes to behavioral dishabituation in Aplysia. *Proc Natl Acad Sci* **83**:8794-8.

Hodge CW, Mehmert KK, Kelley SP, McMahon T, Haywood A, Olive MF, Wang D, Sanchez-Perez AM, Messing RO (1999). Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon. *Nat Neurosci* **2**:997-1002.

Hodgkin AL (1937). Evidence for electrical transmission in nerve. Part I and II. *J Physiol* **90**:183-232.

Hodgkin AL and Katz B (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J Physiol* **108**:37-77.

Hodgkin AL, Huxley AF and Katz B (1952). Measurement of current voltage relations in the membrane of the giant axon of Loligo. *J Physiol* **116**:424-448.

Houeland G, Nakhost A, Sossin WS, Castellucci VF (2007). PKC modulation of transmitter release by SNAP-25 at sensory to motor synapses in Aplysia. *J Neurophysiol* 97:134-143.

Hsu SF, Jackson MB (1996). Rapid exocytosis and endocytosis in nerve terminals of the rat posterior pituitary. *J Physiol* **494**:539-53.

Jahn R, Lang T, Sudhof TC. (2003). Membrane fusion. *Cell* **112**:519-33. Review.

Jaskolski F, Coussen F, Nagarajan N, Normand E, Rosenmund C, Mulle C (2004). Subunit composition and alternative splicing regulate membrane delivery of kainate receptors. *J Neurosci* **24**:2506-15.

Jennings, HS (1906). Behavior of the Lower Organisms. Columbia University Press, New York.

Jeremic A, Cho WJ, Jena BP (2004). Membrane fusion: what may transpire at the atomic level. *J Biol Phys Chem* **4**:139-142.

Ji J, Tsuk S, Salapatek AM, Huang X, Chikvashvili D, Pasyk EA, Kang Y, Sheu L, Tsushima R, Diamant N, Trimble WS, Lotan I, Gaisano HY (2002). The 25-kDa synaptosome-associated protein (SNAP-25) binds and inhibits delayed rectifier potassium channels in secretory cells. *J Biol Chem* **277**:20195-204.

Jin I, Hawkins RD (2003). Presynaptic and postsynaptic mechanisms of a novel form of homosynaptic potentiation at aplysia sensory-motor neuron synapses. *J Neurosci* **23**:7288-97.

Jin I, Kandel ER, Hawkins RD (2005). The roles of PKA, PKC and CAMKII in facilitation at the *Aplysia* sensory motor neuron synapses depend on the duration of the exposure of 5-HT. *Soc Neurosci Abs* **540.5**.

Jorgensen EM, Hartwieg E, Schuske K, Nonet ML, Jin Y, Horvitz HR (1995). Defective recycling of synaptic vesicles in synaptotagmin mutants of Caenorhabditis elegans. Nature **378**:196-9.

Kanashiro CA and Khalil RA(1998). Signal transduction by protein kinase C in mammalian cells. *Clin Exp Pharmacol Physiol* 25:974-85. Review.

Kandel ER and Tauc L (1965a). Mechanisms of heterosynaptic facilitation in the giant cell of the abdominal ganglion of Aplysia depilans. *J Physiol* **181**:1-27.

Kandel ER and Tauc L (1965b). Heterosynaptic facilitation in neurones of the abdominal ganglion of Aplysia depilans. *J Physiol* **181**:28-47.

Kandel ER (1976) Cellular basis of behaviour: An introduction to behavioural biology. San Francisco; Freeman.

Kandel ER, Schwartz JH, Jessell TM (2000). Principles of Neural Science, 4th ed. McGraw-Hill, New York.

Kandel ER (2000). The Molecular Biology of Memory Storage: A Dialog between Genes and Synapses. Nobel lecture 3.

Kandel ER (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**:1030-1038.

Kataoka M, Kuwahara R, Matsuo R, Sekiguchi M, Inokuchi K, Takahashi M (2006). Development- and activity-dependent regulation of SNAP-25 phosphorylation in rat brain. *Neurosci Lett* **407**:258-62.

Katz B and Miledi R (1965). The effect of calcium. on acetylcholine release from motor nerve. terminals. *Proc R Soc Lond B Biol Sci* **161**:496-503.

Katz B (1969). The release of neuronal transmitter substances. Liverpool ,UK, Liverpool University press.

Katz B (1970). On the Quantal Mechanism of Neural Transmitter Release Nobel lecture 2.

Kawasaki F, Zou B, Xu X, Ordway RW (2004). Active zone localization of presynaptic calcium channels encoded by the cacophony locus of Drosophila. *J Neurosci* **24**:282-5.

Kazanietz MG (2002). Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol Pharmacol* **61**:759-67. Review.

Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO (1999). A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron* **24**:253-60.

Klein M and Kandel ER (1980). Mechanism of calcium current modulation underlying presynaptic facilitation and behavioral sensitization in Aplysia. *Proc Natl Acad Sci* **77**:6912-6.

Kim DK, Catterall WA (1997). Ca2+-dependent and -independent interactions of the isoforms of the alpha1A subunit of brain Ca2+ channels with presynaptic SNARE proteins. *Proc Natl Acad Sci* **94**:14782-6.

Klein M, Hochner B, Kandel ER (1986). Facilitatory transmitters and cAMP can modulate accommodation as well as transmitter release in Aplysia sensory neurons: Evidence for parallel processing in a single cell. *Proc Natl Acad Sci* **83**:7994-7998.

Klenchin VA, Martin TF (2000). Priming in exocytosis: attaining fusion-competence after vesicle docking. *Biochimie* **82**:399-407. Review.

Klingauf J, Kavalali ET, Tsien RW (1998). Kinetics and regulation of fast endocytosis at hippocampal synapses. *Nature* **394**:581-5.

Krasne FB (1978). Extrinsic control of intrinsic neuronal plasticity: an hypothesis from work on simple systems. *Brain Res* **140**:197-216.

Kupferman I, Castellucci V, Pinsker H, and Kandel, ER (1970) Neuronal correlates of habituation and dishabituation of the gill withdrawal reflex in Aplysia. *Science* **167**:1745-1748.

Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennett MV, Zukin RS (2001). Protein kinase C modulates NMDA receptor trafficking and gating. *Nat Neurosci* **4**:382-90.

Lashley K (1950)."In search of the engram". Lashley KS Symp Soc Exp Biol **4**:454-482.

Leal K, Abi-Farah C, Zhao Y, Martin KC, Sossin WS, Klein M (2005). Mobilization of calcium dependent protein kinase C by concurrent activity and serotonin in Aplysia sensory neurons. *Soc Neurosci Abst* **610.20**.

Lechner HA and Byrne JH (1998). New perspectives on classical conditioning - a synthesis of hebbian and non-hebbian mechanisms. *Neuron* **20**: 355-358.

Leitges M, Schmedt C, Guinamard R, Davoust J, Schaal S, Stabel S, Tarakhovsky A (1996). Immunodeficiency in protein kinase cbeta-deficient mice. *Science* **273**:788-91.

Levenson J, Sherry DM, Dryer L, Chin J, Byrne JH, Eskin A (2000). Localization of glutamate and glutamate transporters in the sensory neurons of Aplysia. *J Comp Neurol* **423**:121-131.

Li Q, Roberts AC, Glanzman DL (2005). Synaptic facilitation and behavioural dishabituation in Aplysia: dependence on release of Ca^{2+} from postsynaptic intracellular stores, postsynaptic exocytosis and modulation of postsynaptic AMPA receptor efficacy. *J Neurosci* **25**:5623-5637.

Lin RC and Scheller RH (1997). Structural organization of the synaptic exocytosis core complex. *Neuron* **19**:1087-94.

Lin XY and Glanzman DL (1994a). Hebbian induction of long-term potentiation of Aplysia sensorymotor synapses: partial requirement for activation of a NMDA related receptor. *Proc R Soc Lond B Biol Sci* **255**:215-221.

Lin XY and Glanzman DL (1994b). Long-term potentiation of Aplysia sensorymotor synapses: regulation by postsynaptic voltage. *Proc R Soc Lond B Biol Sci* **255**:113-118.

Lin XY and Glanzman DL (1997) Effect of interstimulus interval on pairing induced LTP of Aplysia sesory-motor synapses in cell culture. *J Neurophysiol* **77**:667-674.

Littleton JT, Stern M, Schulze K, Perin M, Bellen HJ (1993). Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca(2+)-activated neurotransmitter release. *Cell* **74**:1125-34.

Littleton JT, Stern M, Perin M, Bellen HJ (1994). Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in Drosophila synaptotagmin mutants. *Proc Natl Acad Sci* **91**:10888-92.

Littleton JT and Bellen HJ (1995). Presynaptic proteins involved in exocytosis in Drosophila melanogaster: a genetic analysis. *Invert Neurosci* 1:3-13. Review.

Littleton JT, Serano TL, Rubin GM, Ganetzky B, Chapman ER (1999). Synaptic function modulated by changes in the ratio of synaptotagmin I and IV. *Nature* **400**:757-60.

Liu J, Hu JY, Schacher S, Schwartz JH (2004). The two regulatory subunits of aplysia cAMP-dependent protein kinase mediate distinct functions in producing synaptic plasticity. *J Neurosci* **24**:2465-2474.

Liu Y and Edwards RH (1997). The role of vesicular transport proteins in synaptic transmission and neural degradation. *Annu Rev Neurosci* **20**:125-156.

Llinas R, Steinberg IZ, Walton K (1981). Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys J* **33**:323-51.

MacDonald PE, Wang G, Tsuk S, Dodo C, Kang Y, Tang L, Wheeler MB, Cattral MS, Lakey JR, Salapatek AM, Lotan I, Gaisano HY (2002). Synaptosome-associated protein of 25 kilodaltons modulates Kv2.1 voltage-dependent K(+) channels in neuroendocrine islet beta-cells through an interaction with the channel N terminus. *Mol Endocrinol* **16**:2452-61.

Mackler JM, Drummond JA, Loewen CA, Robinson IM, Reist NE (2002) The C2B Ca2+-binding motif of synaptotagmin is required for synaptic transmission in vivo. *Nature* **418**:340-344.

Majewski H and Iannazzo L (1998). Protein kinase C: a physiological mediator of enhanced transmitter output. *Prog Neurobiol* **55**:463-75.

Manseau F, Fan X, Hueftlein T, Sossin WS, Castellucci VF (2001). Ca $^{2+}$-independent PKC Apl II mediates the serotonin induced facilitation at depressed synapses in Aplysia. *J Neurosci* **21**:1247–1256.

Marqueze B, Berton F, Seagar M (2000). Synaptotagmins in membrane traffic: which vesicles do the tagmins tag? *Biochimie* **82**:409-20.

Marqueze B, Boudier JA, Mizuta M, Inagaki N, Seino S, Seagar M (1995). Cellular localization of synaptotagmin I, II, and III mRNAs in the central nervous system and pituitary and adrenal glands of the rat. *J Neurosci* **15**:4906-17.

Martin KC and Kandel ER (1996). Cell adhesion molecules, CREB, and the formation of new synaptic connections. *Neuron* **17**:567-70. Review.

Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER (1996). Control of memory formation through regulated expression of a CaMKII transgene. *Science* **274**:1678-83.

Milner B, Squire LR, Kandel ER (1998). Cognitive neuroscience and the study of memory. *Neuron* **20**:445-468.

McNew JA, Parlati F, Fukuda R, Johnston RJ, Paz K, Paumet F, Sollner TH, Rothman JE (2000). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* **407**:153-9.

Montarolo PG, Goelet P, Castellucci VF, Morgan J, Kandel ER and Schacher SA (1986). critical period for macromolecular synthesis in long-term heterosynaptic facilitation in Aplysia. *Science* **234**:1249-1254.

Montecucco C, Schiavo G, Pantano S (2005).SNARE complexes and neuroexocytosis: how many, how close? *Trends Biochem Sci* **30**:367-72. Review.

Morgan A, Burgoyne RD, Barclay JW, Craig TJ, Prescott GR, Ciufo LF, Evans GJ, Graham ME (2005).Regulation of exocytosis by protein kinase C. *Biochem Soc Trans* **33**:1341-4. Review.

Mu Y, Otsuka T, Horton A, Scott D, Ehlers M. (2003). Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. *Neuron* **40**:581-94.

Muller U and Carew TJ (1998). Serotonin induces temporally and mechanistically distinct phases of persistent PKA activity in Aplysia sensory neurons. *Neuron* **21**:1423-1434.

Murphy GG and Glanzman DL (1997). Mediation of classical conditioning in Aplysia californica by long-term potentiation of sensorimotor synapses. *Science* **278**:467-471.

Nagy G, Matti U, Nehring RB, Binz T, Rettig J, Neher E, Sorensen JB (2002). Protein kinase C-dependent phosphorylation of synaptosome-associated protein of 25 kDa at Ser187 potentiates vesicle recruitment. *J Neurosci* **22**:9278-86.

Nagy G, Reim K, Matti U, Brose N, Binz T, Rettig J, Neher E, Sorensen JB (2004). Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. *Neuron* **5**:417-29.

Nagy G, Milosevic I, Fasshauer D, Muller EM, de Groot BL, Lang T, Wilson MC, Sorensen JB (2005). Alternative splicing of SNAP-25 regulates secretion through nonconservative substitutions in the SNARE domain. *Mol Biol Cell* **16**:5675-85.

Naraghi M, Neher E (1997). Linearized buffered Ca2+ diffusion in microdomains and its implications for calculation of [Ca2+] at the mouth of a calcium channel. *J Neurosci* **17**:6961-73.

Neves G, Gomis A and Lagnado L (2001). Calcium influx selects the fast mode of endocytosis in the synaptic terminal of retinal bipolar cells. Proc Natl Acad Sci **98**:15282-7.

Newton AC (2001). Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* **101**:2353-64. Review.

Nichols RA, Haycock JW, Wang JK and Greengard P (1987). Phorbol ester enhancement of neurotransmitter release from rat brain synaptosomes. *J Neurochem* **48**:615-21.

Nielander HB, Onofri F, Valtorta F, Schiavo G, Montecucco C, Greengard P, Benfenati F (1995). Phosphorylation of VAMP/synaptobrevin in synaptic vesicles by endogenous kinases. *J Neurochem* **65**:1712-20.

Nishiki T, Augustine GJ (2004a). Synaptotagmin I synchronizes transmitter release in mouse hippocampal neurons. *J Neurosci* **24**:6127-32.

Nishiki T, Augustine GJ (2004b). Dual roles of the C2B domain of synaptotagmin I in synchronizing Ca2+-dependent neurotransmitter release. *J Neurosci* **24**:8542-50.

Nishizuka Y (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**:607-614.

de Nó RL (1938) Analysis of the activity of the chains of internuncial neurons. *J Neurophysiol* **1**:207-244.

Osborne SL, Herreros J, Bastiaens PI, Schiavo G (1999). Calcium-dependent oligomerization of synaptotagmins I and II. Synaptotagmins I and II are localized on the same synaptic vesicle and heterodimerize in the presence of calcium. *J Biol Chem* **274**:59-66.

Osen-Sand A, Catsicas M, Staple JK, Jones KA, Ayala G, Knowles J, Grenningloh G, Catsicas S (1993). Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. *Nature* **364**:445-8.

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE, Wilson MC (1989). The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. *J Cell Biol* **109**:3039-52.

Pavlov IP (1927) Conditioned reflexes of the physiological activity of the cerebral cortex, Oxford university press.

Peckham GW and EG Peckham (1887). Some observations on the mental powers of spiders. *J Morphol* **1**:383-419.

Perin MS, Fried VA, Mignery GA, Jahn R, Sudhof TC (1990). Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature* **17**:345260-3.

Perin MS, Johnston PA, Ozcelik T, Jahn R, Francke U and Sudhof TC (1991). Structural and functional conservation of Synaptotagmin (p65) in Drosophila and humans. *J Biol Chem* **266**:615-622.

Penfield, W (1951). Memory mechanisms. *Trans Am Neurol Assoc* **56**:15-39.

Pinsker H, Kupfermann I, Castellucci V, Kandel E (1970). Habituation and dishabituation of the gill-withdrawal reflex in Aplysia. *Science* **167**:1740-1742.

Poirier MA, Xiao W, Macosko JC, Chan C, Shin YK, Bennett MK (1998). The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nat Struct Biol* **5**:765-9.

Poskanzer KE, Marek KW, Sweeney ST, Davis GW (2003). Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. *Nature* **426**:559-63.

Poskanzer KE, Fetter RD, Davis GW (2006). Discrete residues in the c(2)b domain of synaptotagmin I independently specify endocytic rate and synaptic vesicle size. *Neuron* **50**:49-62.

Powell K (2005). Curbside recycling at the synapse. *J Cell Biol* 170:166.

Rayport SG and Schacher S (1986). Synaptic plasticity in vitro: cell culture of identified Aplysia neurons mediating short-term habituation and sensitization. *J Neurosci* **6**:759-63.

Reim K, Mansour M, Varoqueaux F, McMahon HT, Sudhof TC, Brose N and Rosenmund C (2001). Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release. *Cell* **104**:71-81.

Reist NE, Buchanan J, Li J, DiAntonio A, Buxton EM, Schwarz TL (1998). Morphologically docked synaptic vesicles are reduced in synaptotagmin mutants of Drosophila. *J Neurosci* **18**:7662-73.

Rettig J, Sheng ZH, Kim DK, Hodson CD, Snutch TP, Catterall WA (1996). Isoform-specific interaction of the alpha1A subunits of brain Ca2+ channels with the presynaptic proteins syntaxin and SNAP-25. *Proc Natl Acad Sci* **93**:7363-8.

Richmond JE, Weimer RM and Jorgensen EM (2001). An open form of syntaxin bypasses the requirement for unc-13 in vesicle priming. *Nature* **412**:338-341.

Richmond JE, Davis WS and Jorgensen EM (1999). UNC-13 is required for synaptic vesicle fusion in C. elegans. *Nat Neurosci* **2**:959 -964

Risinger C and Bennett MK (1999). Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. *J Neurochem* **72**:614-24.

Rizo J (2003). SNARE function revisited. *Nat Struct Biol* **10**:417-9.

Robinson IM, Ranjan R, Schwarz TL (2002). Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain. *Nature* **418**:336-40.

Robitaille R, Adler EM, Charlton MP (1990). Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. *Neuron* **5**:773-9.

Ron D, Kazanietz MG (1999). New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J* **13**:1658-76. Review.

Rosenmund C, Stevens CF (1996). Definition of the readily releasable pool of vesicles at hippocampal synapses. *Neuron* **16**:1197-207.

Rosenmund C, Rettig J, Brose N (2003). Molecular mechanisms of active zone function. *Curr Opin Neurobiol* **13**:509-19. Review.

Rothman JE (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**:318-24.

Rothman JE (1994). Intracellular membrane fusion. Adv Second Messenger *Phosphoprotein Res* **29**:81-96. Review.

Sabatini BL and Regher WG (1999). Timing of synaptic transmission. *Ann Rev Physiol* **61**:521-542.

Sacks, O (1985). The man who mistook his wife for a hat. London: Picador. Chapter 2

Sacktor TC and Schwartz JH (1990). Sensitizing stimuli cause translocation of protein kinase C in Aplysia sensory neurons. *Proc Natl Acad Sci* **87**:2036-9.

Sakmann B (1992). Elementary steps in synaptic transmission revealed by currents through singe ion channels. Nobel lecture

Sheng ZH, Yokoyama CT, Catterall WA (1997). Interaction of the synprint site of N-type Ca2+ channels with the C2B domain of synaptotagmin I. *Proc Natl Acad Sci* **94**:5405-10.

Schiavo G, Santucci A, Dasgupta BR, Mehta PP, Jontes J, Benfenati F, Wilson MC, Montecucco C (1993). Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Lett* **335**:99-103.

Schiavo G, Stenbeck G, Rothman JE, Sollner TH (1997). Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. *Proc Natl Acad Sci* **94**:997-1001.

Schiavo G, Matteoli M, Montecucco C (2000). Neurotoxins affecting neuroexocytosis. *Physiol Rev* **80**:717-66. Review.

Schikorski T and Stevens CF (1997). Quantitative ultrastructural analysis of hippocampal excitatory synapses. *J Neurosci* **17**:5858-67.

Schikorski T and Stevens CF (2001). Morphological correlates of functionally defined synaptic vesicle populations. *Nat Neurosci* **4**:391-5.

Schiller F (1992). Paul Broca: Explorer of the brain. Oxford University Press.

Schivell AE, Batchelor RH, Bajjalieh SM (2000). Phosphorylation of synaptic vesicle protein 2 modulates binding to synaptotagmin. *J Biol Chem* **275**:17195-200.

Schoch S, Deak F, Konigstorfer A, Mozhayeva M, Sara Y, Sudhof, TC and Kavalali ET (2001). SNARE function analyzed in synaptobrevin/VAMP knockout mice. *Science* **294**:1117-1122.

Seagar M, Leveque C, Charvin N, Marqueze B, Martin-Moutot N, Boudier JA, Boudier JL, Shoji-Kasai Y, Sato K, Takahashi M (1999). Interactions between proteins implicated in exocytosis and voltage-gated calcium channels. *Philos Trans R Soc Lond B Biol* Sci **354**:289-97. Review.

Sheng ZH, Rettig J, Cook T, Catterall WA (1994). Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* **13**:1303-13.

Sheng ZH, Yokoyama CT, Catterall WA (1997). Interaction of the synprint site of N-type Ca2+ channels with the C2B domain of synaptotagmin I. *Proc Natl Acad Sci* **94**:5405-10.

Sherrington CS (1897). The central nervous system. In a textbook of physiology, 7th ed., Part 3, Foster M ed. London: Macmillan.

Sherrington C (1941). Man on his Nature. Cambridge UK: Cambridge University Press; New York: Macmillan.

Shimazaki Y, Nishiki T, Omori A, Sekiguchi M, Kamata Y, Kozaki S, Takahashi M (1996). Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. *J Biol Chem* **271**:14548-53.

Shimizu E, Tang YP, Rampon C, Tsien JZ (2000). NMDA receptor-dependent synaptic reinforcement as a crucial process for memory consolidation. *Science* **290**:1170-4.

Shin OH, Rhee JS, Tang J, Sugita S, Rosenmund C, Sudhof TC (2003). Sr2+ binding to the Ca2+ binding site of the synaptotagmin 1 C2B domain triggers fast exocytosis without stimulating SNARE interactions. *Neuron* **37**:99-108.

Simon SM and Llinas RR (1985). Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys J* **48**:485-98.

Snyder DA, Kelly ML, Woodbury DJ (2006). SNARE complex regulation by phosphorylation. *Cell Biochem Biophys* **45**:111-23. Review.

Sollner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (1993). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75**:409-18.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Sossin WS, Diaz AR, Schwartz JH (1993) Chararterization of two isoforms of protein kinase C in the nervous system of Aplysia californica *J Biol Chem* **268**:5763-5768.

Sossin WS, Diaz-Arrastia R, Schwartz JH (1993).Characterization of two isoforms of protein kinase C in the nervous system of Aplysia californica. *J Biol Chem* **268**:5763-8.

Sorensen JB, Matti U, Wei SH, Nehring RB, Voets T, Ashery U, Binz T, Neher E, Rettig J (2002). The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. *Proc Natl Acad Sci* **99**:1627-32.

Spafford JD, Munno DW, Van Nierop P, Feng ZP, Jarvis SE, Gallin WJ, Smit AB, Zamponi GW, Syed NI (2003). Calcium channel structural determinants of synaptic transmission between identified invertebrate neurons. *J Biol Chem* **278**:4258-67.

Stevens, CF and Sullivan JM (2003). The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission. *Neuron* **39**:299-308.

Stevens CF and Sullivan JM (1998). Regulation of the readily releasable vesicle pool by protein kinase C. *Neuron* **21**:885-93.

Stevens CF and Williams JH (2000)."Kiss and run" exocytosis at hippocampal synapses. *Proc Natl Acad Sci* **97**:12828-33.

Stewart BA, Mohtashami M, Trimble WS and Boulianne GL (2000). SNARE proteins contribute to calcium cooperativity of synaptic transmission. *Proc Natl Acad Sci* **97**:13955-13960.

Storozhuk M and Castellucci VF (1999). Modulation of cholinergic transmission in the neuronal network of the gill and siphon withdrawal reflex in Aplysia. *Neuroscience* **90**: 291-301.

Sudhof TC, Baumert M, Perin MS, Jahn R (1989). A synaptic vesicle membrane protein is conserved from mammals to Drosophila. *Neuron* **2**:1475-81.

Sudhof TC, De Camilli P, Niemann H, Jahn R (1993). Membrane fusion machinery: insights from synaptic proteins. *Cell* **75**: 1-4.

Sudhof TC (1995). The synaptic vesicle cycle: a cacade of protein-protein interactions. *Nature* **375**:645-653.

Südhof TC and Rizo J. (1996). Synaptotagmins: C2-domain proteins that regulate membrane traffic. *Neuron* **17**:379-388.

Sudhof TC (2002). Synaptotagmins: why so many? *J Biol Chem* **277**:7629-7632.

Sugita S, Hata Y, Sudhof TC (1996). Distinct Ca(2+)-dependent properties of the first and second C2-domains of synaptotagmin I. *J Biol Chem* **271**:1262-5.

Sugita S, Shin OH, Han W, Lao Y, Sudhof TC (2002). Synaptotagmins form a hierarchy of exocytotic Ca(2+) sensors with distinct Ca(2+) affinities. *EMBO J* **21**:270-80.

Sutton RB, Davletov BA, Berghuis AM, Sudhof TC, Sprang SR (1995). Structure of the first C2 domain of synaptotagmin I: a novel Ca2+/phospholipid-binding fold. *Cell* **80**:929-938.

Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* **395**:347-53.

Tauc L and Gerschenfeld HM (1961). Cholinergic transmission mechanisms for both excitation and inhibition in molluscan central synapses. *Nature* **192**:366-7.

Tadié J-Y and Tadié M (1999). Le sens de la mémoire. Folio essays, Gallimard p21

Takahashi M, Itakura M, Kataoka M (2003). New aspects of neurotransmitter release and exocytosis: regulation of neurotransmitter release by phosphorylation. *J Pharmacol Sci* **93**:41-5. Review.

Takai Y, Kishimoto A, Inoue M, and Nishizuka Y (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum *J Biol Chem* **252**:7603-7609.

Tardin C, Cognet L, Bats C, Lounis B, Choquet D (2003). Direct imaging of lateral movements of AMPA receptors inside synapses. *EMBO J* **22**:4656-65.

Thorndike EL (1965). Animal intelligence. Experimental studies. Hafner NewYork.

Ting JT, Kelley BG, Sullivan JM (2006). Synaptotagmin IV does not alter excitatory fast synaptic transmission or fusion pore kinetics in mammalian CNS neurons. *J Neurosci* **26**:372-80.

Tocco G, Bi X, Vician L, Lim IK, Herschman H, Baudry M (1996). Two synaptotagmin genes, Syt1 and Syt4, are differentially regulated in adult brain and during postnatal development following kainic acid-induced seizures. *Mol Brain Res* **40**:229-39.

Trimble WS, Cowan DM, Scheller RH (1988).VAMP-1: a synaptic vesicle-associated integral membrane protein. *Proc Natl Acad Sci* **85**:4538-42.

Trudeau L-E and Castellucci VF (1993). Functional uncoupling of inhibitory interneurons plays an important role in short-term sensitization of Aplysia gill and siphon withdrawal reflex. *J Neurosci* **13**:2126-2135.

Trudeau L-E and Castellucci VF (1993). Sensitization of the gill and siphon withdrawal reflex of Aplysia: mulitple sites of changes in the neuronal network. *J Neurophysiol* **70**:1210-1220.

Tsien JZ, Huerta PT and Tonegawa S (1996a). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* **87**:1327-38.

Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, Tonegawa S (1996b). Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* 87:1317-26.

Tucker, WC, Edwardson, JM, Bai, J, Kim, HJ, Martin, TF and Chapman, ER (2003). Identification of synaptotagmin effectors via acute inhibition of secretion from cracked PC12 cells. *J Cell Biol* **162**, 199-209.

Turner KM, Burgoyne RD, Morgan A (1999). Protein phosphorylation and the regulation of synaptic membrane traffic. *Trends Neurosci* **22**:459-64. Review.

Turrigiano GG and Nelson SB (2004). Homeostatic plasticity in the developing nervous system. *Nature Reviews Neuroscience* **5**:97-107.

Ubach J, Zhang X, Shao X, Südhof TC and Rizo J (1998). Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain? *EMBO J* **17**: 3921-3930.

Ubach J, Garcia J, Nittler MP, Südhof TC, and Rizo J (1999). Structure of the Janus-faced C2B domain of rabphilin. *Nat Cell Biol* **1**:106-112.

Ule J, Darnell RB (2006). RNA binding proteins and the regulation of neuronal synaptic plasticity. *Curr Opin Neurobiol* **16**:102-10. Review.

Varoqueaux F, Sigler A, Rhee JS, Brose N, Enk C, Reim K and Rosenmund C (2002). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc 13-mediated vesicle priming. *Proc Natl Acad Sci* **99**:9037-9042.

Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C, Matteoli M (2004). SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron* 41:599-610.

Vician L, Lim IK, Ferguson G, Tocco G, Baudry M, Herschman HR (1995). Synaptotagmin IV is an immediate early gene induced by depolarization in PC12 cells and in brain. *Proc Natl Acad Sci* **92**:2164-8.

von Gersdorff H and Matthews G (1994). Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic terminals. *Nature* **367**:735–739.

Walters ET, Byrne JH, Carew TJ and Kandel ER (1983). Mechanoafferent neurons innervating tail of Aplysia. I. Responses properties and synaptic connections. *J Neurophysiol* **50**:1522-1542.

Walters ET, Byrne JH, Carew TJ and Kandel ER (1983). Mechanoafferent neurons innervating tail of Aplysia. II. Modulation by sensitizing stimulation. *J Neurophysiol* **50**:1543-1559.

Walters ET and Cohen LB (1997). Functions of the LE sensory neurons in Aplysia. *Invert Neurosci* **3**:15-25. Review.

Wang CT, Lu JC, Bai J, Chang PY, Martin TF, Chapman ER, Jackson MB (2003). Different domains of synaptotagmin control the choice between kiss-and-run and full fusion. *Nature* **424**:943-7.

Wang P, Wang CT, Bai J, Jackson MB, Chapman ER (2003). Mutations in the effector binding loops in the C2A and C2B domains of synaptotagmin I disrupt exocytosis in a nonadditive manner *J Biol Chem* **278**:47030-7.

Washbourne P, Bortoletto N, Graham ME, Wilson MC, Burgoyne RD, Montecucco C (1999). Botulinum neurotoxin E-insensitive mutants of SNAP-25 fail to bind VAMP but support exocytosis. *J Neurochem* **73**:2424-33.

Washbourne P, Cansino V, Mathews JR, Graham M, Burgoyne RD, Wilson MC (2001). Cysteine residues of SNAP-25 are required for SNARE disassembly and exocytosis, but not for membrane targeting. *Biochem J* **357**:625-34.

Washbourne P, Thompson PM, Carta M, Costa ET, Mathews JR, Lopez-Bendito G, Molnar Z, Becher MW, Valenzuela CF, Partridge LD, Wilson MC (2002). Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nat Neurosci* **5**:19-26.

Waters J and Smith SJ (2002). Vesicle pool partitioning influences presynaptic diversity and weighting in rat hippocampal synapses. *J Physiol* **541**:811-823.

Way KJ, Chou E, King GL (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* **21**:181-7. Review.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* **92**:759-772.

Weimbs T, Low SH, Chapin SJ, Mostov KE, Bucher P, Hoffman K (1997). A conserved domain is present on different families of vesicular fusion proteins: a new superfamily. *Proc Natl Acad Sci* **94**:3046-511.

Wightman RM and Haynes CL (2004). Synaptic vesicles really do kiss and run. *Nat Neurosci* **7**:321-322.

Wiser O, Bennett MK, Atlas D (1996). Functional interaction of syntaxin and SNAP-25 with voltage-sensitive L- and N-type Ca2+ channels. *EMBO J* **15**:4100-10.

Wu Y, He Y, Bai J, Ji SR, Tucker WC, Chapman ER, Sui SF (2003). Visualization of synaptotagmin I oligomers assembled onto lipid monolayers. *Proc Natl Acad Sci* **100**:2082-7.

Yamada WM and Zucker RS (1992). Time course of transmitter release calculated from simulations of a calcium diffusion model. *Biophys J* **61**:671-82.

Yao Y, Ferrer-Montiel AV, Montal M, Tsien RY (1999). Activation of store-operated Ca2+ current in Xenopus oocytes requires SNAP-25 but not a diffusible messenger. *Cell* **98**:475-85.

Yoshihara M, Littleton JT (2002). Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. *Neuron* **36**:897-908.

Yoshihara M, Adolfsen B, Littleton JT (2003) Is synaptotagmin the calcium sensor? *Curr Opin Neurobiol* **13**:315-23. Review.

Yoshihara M and Montana ES (2004).The Synaptotagmins: Calcium Sensors for Vesicular Trafficking. *Neuroscientist* **10**:566-574.

Yoshihara M, Adolfsen B, Galle KT, Littleton JT (2005). Retrograde signaling by Syt 4 induces presynaptic release and synapse-specific growth. *Science* **310**:858-63.

Xu NJ, Yu YX, Zhu JM, Liu H, Shen L, Zeng R, Zhang X, Pei G (2004). Inhibition of SNAP-25 phosphorylation at Ser187 is involved in chronic morphine-induced down-regulation of SNARE complex formation. *J Biol Chem* **279**:40601-8.

Xu T, Rammner B, Margittai M, Artalejo AR, Neher E, Jahn R (1999). Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis. *Cell* **99**:713-22.

Zhang JZ, Davletov BA, Sudhof TC, Anderson RG (1994). Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. *Cell* **78**:751-60.

Zhao Y, Klein M (2002). Modulation of the readily releasable pool of transmitter and of excitation-secretion coupling by activity and by serotonin at Aplysia sensorimotor synapses in culture. J Neurosci. 22:10671-9.

Zhao Y, Leal K, Abi-Farah C, Sossin WS, Martin KC, Klein M (2006). Isoform specificity of PKC translocation in living Aplysia sensory neurons and a role for Ca2+-dependent PKC APL I in the induction of intermediate-term facilitation. *J Neurosci* **26**:8847-885.

VIII. Annex

8.1 Personal contribution to the articles

Article 1

Methods:

Electrophysiology: I used intracellular electrophysiology to study the role of protein kinase C (PKC) phosphorolation of Syt I and a Syt I splice form in the synaptic transmission in *Aplysia* californica.

Confocal imaging: I overexpressed the fluorescently tagged SytI constructs (Syt I and Syt I_{VQ}) in *Aplysia* sensory neurons. I used confocal studies to examine if the Syt I constructs were localized in synaptic vesicles (+AplVAMP) and whether the different Syt I splice forms wee co-localized at the synapse.

Contribution: First author (shared first author with Arash Nakhost)

Article 2

Confocal Imaging: I overexpressed the fluorescently tagged Syt I constructs (C2B-α and C2B-ß) in *Aplysia* sensory neurons. I used confocal studies to examine if the Syt I constructs were localized in synaptic vesicles (+AplVAMP) and whether the different Syt I splice forms were co-localized at the synapse.

Contribution: second author

Article 3

Electrophysiology: I used intracellular electrophysiology to study the role of protein kinase C (PKC) phosphorolation of SNAP-25 in the synaptic transmission in *Aplysia californica.*

Molecular Biology: I cloned AplSNAP-25 using degenerate PCR. I also raised an antibody to *Apl*SNAP-25

Confocal Imaging: I used confocal studies to examine if the SNAP-25 constructs were localized in synaptic vasicles (+AplVAMP). I also used confocal imaging and immunohistochemistry to verify the co-localisation of overexpresed fluorescently tagged SNAP-25 constructs: (SNAP-25 wildtype and SNAP-25s with a mutated PKC phosphorylation site) and the endogenous antibody labelled SNAP-25.

Contribution: first author

Gry Helen Houeland
Département de Physiologie
Faculté de médecine,
Université de Montréal
Programme: Ph.D. (Sciences neurologiques) (3-530-1-0)

Article :

Nakhost A, **Houeland G**, Blandford VE, Castellucci VF, Sossin WS (2004) Identification and characterization of a novel C2B splice variant of synaptotagmin I. J Neurochem 89:354-63

Imagerie confocale
Contribution : deuxième auteur

Nous avons aussi cloné une autre isoforme de synaptotagmine, Syt I C2B-β avec 39 changements dans la région C2B. Une mesure de protéolyse avec chymotrypsine démontre que cet isoforme peut, dans l'absence de calcium supporter un changement de conformation normalement déclenché par calcium. J'ai co-exprimé ECFP- Syt I C2B-β et ECFP- Syt I C2B-α, et, avec un microscope confocale, vérifié si cette épissure affectait leur localisation. Parallèlement j'ai examiné la co-localisation des SytI-C2Bs avec VAMP, une protéine qui marque les connections synaptiques. Nous avons également montré que Syt I C2B-β avait une plus grande affinité pour SNAP-25 que Syt I C2B-α. En conclusion, ces résultats suggèrent que l'épissage change la structure du domaine C2B et puisse ainsi affecter la fonction de la protéine.

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Gry Houeland inclut cet article dans sa thèse de doctorat, qui a pour titre « Kinase C substrates and synaptic plasticity in Aplysia »

Coauteur	Signature	Date
Arash Nakhost		March 19/06
	Vincent Castellucci	Jan 12/07
Wayne Sossin		Jan. 24 07
		Jan 24.07

Gry Helen Houeland,
Département de Physiologie
Faculté de médecine,
Université de Montréal
Programme: Ph.D. (Sciences neurologiques) (3-530-1-0)

Article :
G. Houeland; A. Nakhost; V.F. Castellucci; W.S Sossin (2006)
PKC modulation of transmitter release by SNAP-25 at sensory to motor synapses in *Aplysia*.
soumise à journal of neurophysiology

Méthodes :
Électrophysiologie,
Biologie moléculaire
Contribution : premier auteur

J'ai étudié la fonction de SNAP-25, une protéine impliquée dans la relâche de neurotransmetteur. Les sites qui sont phosphorylées par PKC *in vivo* ont été conservés à travers l'évolution des espèces et il a été démontré que l'action des PKCs régule cette protéine. Dans ce projet, j'ai cloné l'Apl-SNAP-25 (la forme présente chez l'aplysie) à l'aide de la méthode de PCR dégénéré. J'ai également exprimé les formes sauvages et mutantes d'Apl-SNAP-25 dans des neurones sensoriels de l'*Aplysie* afin de déterminer si la phosphorylation de cette protéine par PKC joue un rôle dans la transmission synaptique et sa modulation. La surexpression de SNAP-25 n'avait aucun effet sur les cinétiques de dépression et de facilitation. Cependant, la surexpression d'EGFP-SNAP-25 réduit la facilitation des synapses déprimées d'une façon significative comparées avec l'EGFP seule et la forme mutée EGFP-SNAP-25 (S-A). Cependant quand injecté séparément cet effet est annulé. Nous avons construit une forme mutée S-E ou S-D, imitant la charge négative de la phosphorylation. Ces mutants ralentissaient les cinétiques de la dépression. En présence de PDBu, un phorbol ester qui active le PKC indépendamment de 5-HT, pour ainsi imiter la phosphorylation naturelle de la protéine wild-type pendant toute la période de dépression, j'ai observé la même tendance qu'avec les protéines phosphomimétiques. Cet effet a été bloqué dans les cellules exprimant un site de phosphorylation PKC non-phosphorylable.

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Gry Houeland inclut cet article dans sa thèse de doctorat. qui a pour titre « Kinase C substrates and synaptic plasticity in Aplysia »

Arash Nakhost		March 19/06
Coauteur	Signature	Date
Vincent Castellucci		Jan 12/07
Coauteur	Signature	Date
Wayne Sossin		Jan. 20/07
Coauteur	Signature	Date

Gry Helen Houeland,
Département de Physiologie
Faculté de médecine,
Université de Montréal
Programme: Ph.D. (Sciences neurologiques) (3-530-1-0)

Article :
A. Nakhost[†]; G. Houeland[†]; V.F. Castellucci; W S. Sossin (2003)
Differential Regulation of Transmitter Release by Alternatively Spliced Forms of Synaptotagmin I. J. Neurosci 23:6245-6254

Méthodes :
Électrophysiologie,
Imagerie confocale
Contribution . Premier auteur (contribution égale à Arash Nakhost)

Synaptotagmine-I est une protéine vésiculaire qui fonctionne comme un détecteur de calcium et joue un rôle essentiel dans la relâche des neurotransmetteurs. Dans le processus de cloner synaptotagmine-I, nous avons trouvé une nouvelle isoforme avec deux acides aminés +VQ ajoutés dans le domaine N-terminal de C2-A ($SytI_{VQ}$). Un RT-PCR quantitatif démontre un niveau significatif de ARNm des deux isoformes dans le système nerveux J'ai injecté les plasmides codant pour les synaptotagmines sauvages ou mutées (dans le site de phosphorylisation de PKC) dans des cultures sensori-motrices. J'ai examiné les cinétiques de dépression et de facilitation. Toutes les formes avaient une facilitation réduit d'une façon significative, sauf la forme SytI pour laquelle la facilitation est identique aux contrôles. Cependant, les cinétiques de 5-HT restaient normales. Ceci indique que la région VQ de la synaptotagmine joue un rôle important dans la facilitation des synapses déprimées. J'ai ensuite testé si EGFP- $SytI_{VQ}$ pouvait bloquer l'effet de PDBu, un phorbol esther qui active le PKC indépendamment de 5-HT. En effet, le EGFP- $SytI_{VQ}$ n'était plus capable de bloquer le reversement de la facilitation, suggérant que le déficit soit lié à la capacité de 5-HT d'activer PKC. Pour déterminer si cet effet était généralisé j'ai testé la facilitation dans des synapses non-déprimées. Cet effet est provoqué par l'activation de PKA par 5-HT et non par PKC. Ici la facilitation n'était pas bloquée par la sur expression de SytI ou $SytI_{VQ}$. Ceci démontre que la sur expression de $SytI_{VQ}$ interfère spécifiquement avec la capacité de 5-HT d'activer PKC sans affecter la transmission synaptique ou bien la capacité d'activer PKA. Cet effet spécifique de $SytI_{VQ}$ sur la transmission s'explique si cet isoform est la seule qui se trouve dans les vésicules synaptiques. Nous avons également construit des plasmides codant pour $SytI_{VQ}$ et SytI, fusionnés avec des rapporteurs différents (ECFP ou EYFP). J'ai surexprimé ces protéines et, avec un microscope confocale, vérifié si cette épissure affectait leur localisation. Dans certains cas, nous avons observé une différence d'expression dans les cônes de croissance et dans les axones. Nos résultats indiquent que la région VQ de la synaptotagmine joue un rôle important dans la facilitation des synapses déprimées. Parallèlement j'ai examiné la co-localisation des SytIs avec VAMP, une protéine qui marque les connections synaptiques.

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Gry Houeland inclut cet article dans sa thèse de doctorat, qui a pour titre « Kinase C substrates and synaptic plasticity in Aplysia »

Arash Nakhost		March 19/06
Coauteur	Signature	Date
Vincent Castellucci		Jan 12/07
Coauteur	Signature	Date
Wayne Sossin		Jan 24/07
Coauteur	Signature	Date

The American Physiological Society

9650 Rockville Pike, Bethesda, MD 20814-3991, USA
Phone: (301) 634-7243
Fax: (301) 634-7243

January 18, 2007

To Whom It May Concern:

The American Physiological Society grants Gry Houeland permission to use the Journal of Neurophysiology article, PKC Modulation of Transmitter Release by SNAP-25 at Sensory-to-Motor Synapses in Aplysia, 97134-143, 2007 by Houeland C, Nakhost A, Sossin WS, and Catellucci VF, for use in his Ph.D. thesis.

The American Physiological Society publication must be credited as the source with the words "used with permission" added.

Sincerely,

Director of Publications
The American Physiological Society

MR/pr

APPROVED
By PRipka at 10:06 am, Jan 18, 2007

THE AMERICAN PHYSIOLOGICAL SOCIETY
9650 Rockville Pike, Bethesda, MD 20814-3991

Permission is granted for use of the material specified above provided the publication is credited as the source, including the words "used with permission."

Publications Manager & Executive Editor

SOCIETY FOR NEUROSCIENCE

The Journal of Neuroscience

March 17, 2006

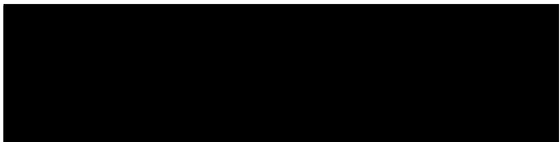
To Whom It May Concern

Gry Houeland is authorized to enclose the article listed below in her Ph.D. thesis.

Article:

Arash Nakhost, Gry Houeland, Vincent F. Castellucci and Wayne S. Sossin. (2003) Differential regulation of transmitter release by alternatively spliced forms of synaptotagmin I. *J Neurosci*, 23, 6238-6244.

Sincerely,



Gary L. Westbrook, M.D.
Editor-In-Chief
The Journal of Neuroscience

1121 14th Street, NW Suite 1010 Washington, DC 20005 Telephone: (202) 962-4000 www.sfn.org

Université de Montréal

Faculté des études supérieures
Vice-décanat

Le 13 octobre 2006

Monsieur Gry Houeland

Objet : Rédaction d'une thèse en anglais

Monsieur,

Suite à votre lettre, la Faculté des études supérieures vous autorise à rédiger votre thèse en anglais.

Veuillez agréer, Monsieur, l'expression de mes sentiments les meilleurs.

Le vice-doyen,

André Ferron

C.c. : M. Vincent Castellucci, directeur de recherche
M. John Kalaska, directeur – Sc. neurologiques

C.P. 6128, succursale Centre-ville
Montréal QC H3C 3J7

Téléphone : (514) 343-5891
(514) 343-6957
(514) 343-6922

Télécopieur : (514) 343-2252

De : "Journals Rights" <JournalsRights@oxon.blackwellpublishing.com>
Date : October 5, 2006 7:56:26 AM EDT (CA)
À : [redacted]
Objet : Rép : Permission Request

Dear Gry Houeland,

Journal of Neurochemistry, 89(2), p354 - 363

Thank you for your request. Permission is granted for you to use the material specified above subject to the usual acknowledgements (author, title of material, title of book/journal, ourselves as publisher) and on the understanding that nowhere in the original text do we acknowledge another source for the requested material. Non-exclusive World English Language, one edition, print and electronic version of publication only. This permission is granted on the condition that you contact the author for consent should you wish to adapt/modify the material. This is not the responsibility of Blackwell Publishing.

Best Wishes
Ali Walker
Temporary Permissions Assistant

Gry Helen Houeland,
Département de Physiologie
Faculté de médecine,
Université de Montréal
Programme: Ph.D. (Sciences neurologiques) (3-530-1-0)

Article :
A. Nakhost[†]; G. Houeland[†]; V.F. Castellucci; W S. Sossin (2003)
Differential Regulation of Transmitter Release by Alternatively Spliced Forms of Synaptotagmin I. J. Neurosci 23:6245-6254

Méthodes :
Électrophysiologie,
Imagerie confocale
Contribution . Premier auteur (contribution égale à Arash Nakhost)

Synaptotagmine-I est une protéine vésiculaire qui fonctionne comme un détecteur de calcium et joue un rôle essentiel dans la relâche des neurotransmetteurs. Dans le processus de cloner synaptotagmine-I, nous avons trouvé une nouvelle isoforme avec deux acides aminés +VQ ajoutés dans le domaine N-terminal de C2-A ($SytI_{VQ}$). Un RT-PCR quantitatif démontre un niveau significatif de ARNm des deux isoformes dans le système nerveux J'ai injecté les plasmides codant pour les synaptotagmines sauvages ou mutées (dans le site de phosphorylisation de PKC) dans des cultures sensori-motrices. J'ai examiné les cinétiques de dépression et de facilitation. Toutes les formes avaient une facilitation réduit d'une façon significative, sauf la forme SytI pour laquelle la facilitation est identique aux contrôles. Cependant, les cinétiques de 5-HT restaient normales. Ceci indique que la région VQ de la synaptotagmine joue un rôle important dans la facilitation des synapses déprimées. J'ai ensuite testé si EGFP- $SytI_{VQ}$ pouvait bloquer l'effet de PDBu, un phorbol esther qui active le PKC indépendamment de 5-HT. En effet, le EGFP- $SytI_{VQ}$ n'était plus capable de bloquer le reversement de la facilitation, suggérant que le déficit soit lié à la capacité de 5-HT d'activer PKC. Pour déterminer si cet effet était généralisé j'ai testé la facilitation dans des synapses non-déprimées. Cet effet est provoqué par l'activation de PKA par 5-HT et non par PKC. Ici la facilitation n'était pas bloquée par la sur expression de SytI ou $SytI_{VQ}$. Ceci démontre que la sur expression de $SytI_{VQ}$ interfère spécifiquement avec la capacité de 5-HT d'activer PKC sans affecter la transmission synaptique ou bien la capacité d'activer PKA. Cet effet spécifique de $SytI_{VQ}$ sur la transmission s'explique si cet isoform est la seule qui se trouve dans les vésicules synaptiques. Nous avons également construit des plasmides codant pour $SytI_{VQ}$ et SytI, fusionnés avec des rapporteurs différents (ECFP ou EYFP). J'ai surexprimé ces protéines et, avec un microscope confocale, vérifié si cette épissure affectait leur localisation. Dans certains cas, nous avons observé une différence d'expression dans les cônes de croissance et dans les axones. Nos résultats indiquent que la région VQ de la synaptotagmine joue un rôle important dans la facilitation des synapses déprimées. Parallèlement j'ai examiné la co-localisation des SytIs avec VAMP, une protéine qui marque les connections synaptiques.

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Gry Houeland inclut cet article dans sa thèse de doctorat, qui a pour titre « Kinase C substrates and synaptic plasticity in Aplysia »

Arash Nakhost — March 19/06
Coauteur — Date
Vincent Castellucci — Jan 12/07
Coauteur — Date
Wayne Sossin — Jan 24/07
Coauteur — Date

Gry Helen Houeland,
Département de Physiologie
Faculté de médecine,
Université de Montréal
Programme: Ph.D. (Sciences neurologiques) (3-530-1-0)

Article :

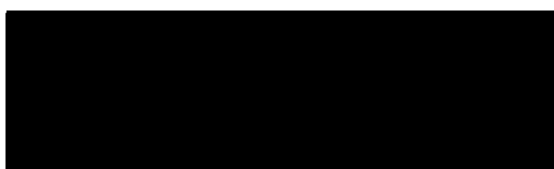
Nakhost A, **Houeland G**, Blandford VE, Castellucci VF, Sossin WS (2004) Identification and characterization of a novel C2B splice variant of synaptotagmin I. J Neurochem 89:354-63

Imagerie confocale
Contribution : deuxième auteur

Nous avons aussi cloné une autre isoforme de synaptotagmine, Syt I C2B-β avec 39 changements dans la région C2B. Une mesure de protéolyse avec chymotrypsine démontre que cet isoforme peut, dans l'absence de calcium supporter un changement de conformation normalement déclenché par calcium. J'ai co-exprimé ECFP- Syt I C2B-β et ECFP- Syt I C2B-α, et, avec un microscope confocale, vérifié si cette épissure affectait leur localisation. Parallèlement j'ai examiné la co-localisation des SytI-C2Bs avec VAMP, une protéine qui marque les connections synaptiques. Nous avons également montré que Syt I C2B-β avait une plus grande affinité pour SNAP-25 que Syt I C2B-α. En conclusion, ces résultats suggèrent que l'épissage change la structure du domaine C2B et puisse ainsi affecter la fonction de la protéine.

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Gry Houeland inclut cet article dans sa thèse de doctoral, qui a pour titre « Kinase C substrates and synaptic plasticity in Aplysia »

Coauteur	Signature	Date
Arash Nakhost		March 19/06
[signature]		Jan 12/07
Wayne Sossin		Jan. 24 07
Vanessa Blandford		Jan 24.07



Gry Helen Houeland
Département de Physiologie
Faculté de médecine,
Université de Montréal
Programme: Ph.D. (Sciences neurologiques) (3-530-1-0)

Article :
G. Houeland; A. Nakhost; V.F. Castellucci; W.S Sossin (2006)
PKC modulation of transmitter release by SNAP-25 at sensory to motor synapses in *Aplysia*.
soumise à journal of neurophysiology

Méthodes :
Électrophysiologie,
Biologie moléculaire
Contribution : premier auteur

J'ai étudié la fonction de SNAP-25, une protéine impliquée dans la relâche de neurotransmetteur. Les sites qui sont phosphorylées par PKC *in vivo* ont été conservés à travers l'évolution des espèces et il a été démontré que l'action des PKCs régule cette protéine. Dans ce projet, j'ai cloné l'Apl-SNAP-25 (la forme présente chez l'aplysie) à l'aide de la méthode de PCR dégénéré. J'ai également exprimé les formes sauvages et mutantes d'Apl-SNAP-25 dans des neurones sensoriels de l'*Aplysie* afin de déterminer si la phosphorylation de cette protéine par PKC joue un rôle dans la transmission synaptique et sa modulation. La surexpression de SNAP-25 n'avait aucun effet sur les cinétiques de dépression et de facilitation. Cependant, la surexpression d'EGFP-SNAP-25 réduit la facilitation des synapses déprimées d'une façon significative comparées avec l'EGFP seule et la forme mutée EGFP-SNAP-25 (S-A). Cependant quand injecté séparément cet effet est annulé. Nous avons construit une forme mutée S-E ou S-D, imitant la charge négative de la phosphorylation. Ces mutants ralentissaient les cinétiques de la dépression. En présence de PDBu, un phorbol ester qui active le PKC indépendamment de 5-HT, pour ainsi imiter la phosphorylation naturelle de la protéine wild-type pendant toute la période de dépression, j'ai observé la même tendance qu'avec les protéines phosphomimétiques. Cet effet a été bloqué dans les cellules exprimant un site de phosphorylation PKC non-phosphorylable.

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Gry Houeland inclut cet article dans sa thèse de doctorat. qui a pour titre « Kinase C substrates and synaptic plasticity in Aplysia »

Arash Nakhost		March 19/06
Coauteur		Date
Vincent		Jan 12 /07
Coauteur		Date
Wayne Sossin		Jan. 20/07
Coauteur	Signature	Date