Regulation of an *Aplysia* Trk-like Receptor by Serotonin and Identification of Serotonin G Protein-Coupled Receptors that Can Activate Protein Kinase C Apl II

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ABSTRACT

ApTrkl, an *Aplysia* Trk-like receptor, is required for serotonin (5-HT)-induced activation of ERK and LTF, which underlies behavioral sensitization. We observed constitutive activation of ApTrkl by overexpression, which is dependent on kinase activity. Two modes of internalization were revealed; kinase activity-dependent constitutive internalization and kinase activity-independent internalization induced by 5-HT. Both modes of internalization were independent of a ligand, and the action of 5-HT was mediated through G protein-coupled receptors (GPCRs). Surprisingly, methiothepin, an antagonist to 5-HT GPCRs, increased activation of endogenous ApTrkl to the same level as 5-HT, suggesting a transactivation mechanism due to a novel coupling of GPCRs to receptor tyrosine kinase activation. The neuropeptide sensorin could transiently activate ApTrkl but was not required for 5-HT-induced ApTrkl activation.

Protein kinase C (PKC) Apl II, a novel calcium-independent PKC in *Aplysia*, is required for reversal of synaptic depression, which is thought to underlie behavioral dishabituation. PKC Apl II is translocated to the plasma membrane by 5-HT in sensory neurons. We isolated three 5-HT GPCRs from *Aplysia Californica*; 5-HT_{2Ap}, 5-HT_{4Ap} and 5-HT_{7Ap}, and demonstrated that 5-HT_{2Ap} and 5-HT_{7Ap} were both able to translocate PKC Apl II in a heterologous system, SF9 cells. Translocation by 5-HT_{2Ap} required PLC activation, while that by 5-HT_{7Ap} required both PLC and PLD activation. However, blocking 5-HT_{2Ap} with the effective antagonist pirenperone did not block the translocation of PKC Apl II nor reversal of synaptic depression in neurons. On the other hand,

genistein, a general tyrosine kinase inhibitor, decreased both the translocation and reversal of synaptic depression. These results suggest that there are multiple pathways leading to the activation of PKC Apl II through PLC and PLD as well as tyrosine kianses, which might give a flexibility to the system.

RÉSUMÉ

Le récepteur ApTrkl, qui est un récepteur de type Trk-like chez l'*Aplysie*, est essentiel pour l'induction de 5-HT et l'activation subséquente de ERK ainsi que pour LTF, deux processus responsables de la sensibilisation behaviorale. Nous avons observé que la surexpression de ApTrkl engendre son activation et que cette activation constitutive est tributaire de l'activité kinase. Deux modes d'internalisation furent démontrés; l'internalisation constitutive tributaire de l'activité kinase et l'internalisation non-tributaire de l'activité kinase induite par 5-HT. Les deux modes d'internalisation peuvent survenir en l'absence d'un ligand et l'action de 5-HT dépend des récepteurs couplés à la protéine G (GPCRs). Fait intéressant, la méthiothépine, un antagoniste de 5-HT GPCRs, augmente l'activation des récepteurs ApTrkl endogènes au même niveau que le fait 5-HT, suggérant ainsi la présence d'un nouveau mécanisme de transactivation qui couplerait les GPCRs à l'activation du récepteur tyrosine kinase (RTK). Le neuropeptide sensorine, pourrait activer transitoirement ApTrkl mais ne serait pas essentiel pour l'activation de ApTrkl tributaire de 5-HT.

Protéine kinase C (PKC) Apl II, une nouvelle PKC non-tributaire du calcium chez l'Aplysie, est requise pour contrecarrer la dépression synaptique qui croit-on, est le mécanisme responsable de la désensibilisation behaviorale. PKC Apl II est relocalisée à la membrane plasmique par 5-HT dans les neurones sensoriels. Nous avons isolé trois 5-HT GPCRs à partir de l'*Aplysie Californica*; 5-HT_{2Ap}, 5-HT_{4Ap} et 5-HT_{7Ap}. Nous avons par la suite démontré que 5-HT_{2Ap} et 5-HT_{7Ap} étaient capable de relocaliser PKC Apl II dans les cellules SF9. La

relocalisation de 5-HT_{2Ap} requiert l'activation de PLC tandis que la relocalisation de 5-HT_{7Ap} requiert l'activation de PLC et de PLD. Toutefois, l'inhibition de 5-HT_{2Ap} par l'antagoniste pirenperone n'a pas réussi à bloquer la relocalisation de PKC Apl II ni n'a réussi à contrecarrer la dépression synaptique dans les neurones. Cependant, l'utilisation de la génistéine, un inhibiteur des tyrosine kinases, cause une diminution de la relocalisation et la dépression synaptique. Ces résultats suggèrent que plusieurs mécanismes cellulaires conduisent à l'activation de PKC Apl II par PLC et PLD et des tyrosine kinases qui pourrait contribuer à la flexibilité de ce processus cellulaire.

PUBLICATION ARISING FROM THIS WORK AND CONTRIBUTIONS OF AUTHORS

The chapter three of this thesis is based upon the texts and data that have been presented in the following manuscript:

Mechanisms regulating ApTrkl, a Trk-like receptor in *Aplysia* sensory neurons

Ikue Nagakura, Jake Ormond, and Wayne S. Sossin

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Contributions of Authors

Chapter Three:

I conducted all the experiments throughout the study, made figures, and wrote the draft. However, Jake Ormond collected initial data for sensorin peptide and antibody experiments (Fig. 3. 5. B and C), which was included in the analysis. Dr. Sossin performed all the quantification and edited the draft.

Chapter Four:

I cloned 5-HT_{2Ap} and 5-HT_{4Ap}, supervised the cloning of 5-HT_{7Ap} by Andrew Heppner, and made all the eGFP-tagged constructs for 5-HT receptors. I conducted all the experiments except for the electrophysiology experiments (Fig.

4. 8. B and C, Fig. 4. 9. C and D), which were conducted by Dr. Tyler Dunn. Dr. Carole Abi Farah helped initial experiments in SF9 cells and did quantification for the part of the data. Other quantification was also done by me or by Flora Li. Dr. Sossin performed a phylogenetic analysis (Fig. 4. 3).

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LIST OF ABBREVIATIONS AND SYMBOLS

4E-BP1 = Eukaryotic initiation factor 4E binding protein 1

5-HT = Serotonin

Ab = Antibody

AC = Adenylyl cyclase

AMPA = α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

AP = Action potential

apCAM = *Aplysia* cell adhesion molecule

aPKC = Atypical PKC

ApTrkl = *Aplysia* Trk-like receptor

ASW = Artificial sea water

ATP = Adenosine-5'-triphosphate

BDNF = Brain-derived neurotrophic factor

C/EBP = CCAAT-box enhanced binding protein

Ca^{2+} = Calcium

CAMAP = CAM-associated protein

cAMP = Cyclic adenosine monophosphate

cDNA = Complementary deoxyribonucleic acid

CNS = Central nervous system

CPEB = Cytoplasmic polyadenylation element binding protein

cPKC = Classical PKC

CREB = cAMP response element binding protein

DAG = Diacylglycerol

DNA = Deoxyribonucleic acid

dsRNA = Double-stranded ribonucleic acid

eGFP = Enhanced green fluorescent protein

EGFR = Epidermal growth factor receptor

E-LTP = Early phase long-term potentiation

ER = Endoplasmic reticulum

ERK/MAPK = Extracellular signal-regulated kinase/Mitogen-activated protein kinase

GDP = Guanosine diphosphate

GEF = Guanine exchange factor

GPCR = G protein-coupled receptor

GTP = Hydrolyze guanosine triphosphate

IA = Inhibitory avoidance

IN = Interneuron

IP_3 = Inositol triphosphate

ITF = Intermediate-term facilitation

ITM = Intermediate-term memory

K^+ = Potassium

KR = Kinase-dead

L-LTP = Late phase long-term potentiation

LTD = Long-term depression

LTF = Long-term facilitation

LTM = Long-term memory

LTP = Long-term potentiation

mGluR = Metabotropic glutamate receptor

MN = Motor neuron

mRFP = Monocistronic red fluorescent protein

mRNA = Messenger ribonucleic acid

mTOR = Mammalian target of rapamycin

NMDA = N-methyl-D-aspartic acid

nPKC = Novel PKC

NT = Neurotrophin

ORF = Open reading frame

p75NTR = p75 neurotrophin receptor

PA = Phosphatidic acid

PCR = Polymerase chain reaction

PDGFR = Platelet derived growth factor receptor

PI3K = Phosphoinositide 3-kinase

PICK1 = PRKCA-binding protein

PKA = Protein kinase A (cAMP-dependent protein kinase)

PKC = Protein kinase C

PKM = Protein kinase M

PLC = Phospholipase C

PLD = Phospholipase D

PS = Phosphatidylserine

PSP = Postsynaptic potential

RNA = Ribonucleic acid

RTK = Receptor tyrosine kinase

RT-PCR = Reverse transcription polymerase chain reaction

siRNA = Small interfering ribonucleic acid

SN = Sensory neuron

STEP = Striatal-enriched protein tyrosine phosphatase

STF = Short-term facilitation

STM = Short-term memory

Trk = Troposyosin-related kinase

WT = Wild-type

Y = Tyrosine

YW = Phospho-tyrosine signaling-deficient

α = Alpha

β = Beta

γ = Gamma

δ = Delta

ε = Epsilon

ζ = Zeta

η = Eta

θ = Theta

ι = Iota

CHAPTER ONE

Introduction

An ability to change behavior upon experiences is a fundamental feature of the learning process, and an ability to retain and store newly acquired information as memory and retrieve it when necessary constitutes an important aspect of life. Recent advances in biological techniques and knowledge, especially in cellular and molecular biology, have made it possible to study learning and memory as a biological process. Numerous studies have shown that changes in the strength of the connections between neurons, referred to as synaptic plasticity, underlie how organisms change their behaviors through experience. Activity-dependent changes in synaptic strength as well as changes in excitability are considered to be mechanisms underlying learning and memory. Studies using the marine mollusk *Aplysia* have revealed many basic mechanisms at the molecular level. In particular, signal transduction pathways that are activated after memory-inducing stimuli or serotonin (5-HT) have been identified. Signaling pathways activated by second messengers such as tyrosine kinases, protein kinase A (PKA), and protein kinase C (PKC) play important roles in modulating synaptic plasticity and memory processes in *Aplysia*. Below I review the *Aplysia* system, the involvement of tyrosine kinases and PKC in synaptic plasticity in both vertebrates and *Aplysia*, and 5-HT G protein-coupled receptors (GPCRs) that are thought to be important for the synaptic changes underlying memory.

1. 1. Biological study of learning and memory in *Aplysia*

The marine mollusk, *Aplysia* is a powerful model system to reveal molecular events that control synaptic plasticity. *Aplysia* displays several complex forms of associative learning such as classical and operant conditioning as well as simpler forms of non-associative learning such as sensitization, habituation, and dishabituation. The relative simplicity of its central nervous system with smaller number but much larger size of neurons compared to mammals has made *Aplysia* a strong system to work with in order to understand the mechanism of learning and memory at the cellular and molecular levels. *Aplysia* has only around 20,000 central nerve cells (the mammalian brain has almost a trillion nerve cells), of which fewer than 100 cells may be directly involved and modified in the simplest behaviors, and the cells can be as large as 1mm in diameter, which is enough to be seen with the naked eye (Kandel, 2001). The cells can be easily dissected for biochemical and physiological experiments and readily injected with genetic constructs or antibodies, which has paved the way for the molecular studies of signal transduction within individual cells, providing direct evidence that certain forms of learning rely on the plasticity of individual synaptic connections (Kandel, 2001).

The theory of memory storage, postulating that memory is stored in the growth of new connections between neurons, was first proposed by Cajal in late 19th century and later revised by Donal Hebb, who proposed that associative learning occurs when there is a coincident activity at given synaptic junctions, changing the properties of those synapses and increasing their efficiency.

However, it was almost 80 years later that direct evidence was provided that learning results from changes in the efficacy of specific neural connections. It was demonstrated in *Aplysia* that strength and effectiveness of neuronal connections are altered by learning, and chemical reactions that take place between neurons during and after learning are the fundamental mechanism for memory storage (Castellucci et al., 1970).

1. 2. Molecular and cellular mechanisms underlying learning and memory in *Aplysia*

Sensitization is a type of learned fear where a person or experimental animal learns to respond strongly to a stimulus that is originally neutral or innocuous (Kandel, 2001). In *Aplysia*, this is observed as the increase in the gill-withdrawal reflex; a defensive reflex where the gill is retracted when the siphon is stimulated. Sensitization of this reflex is observed when the gill is retracted to even innocuous stimulus to the siphon after receiving an aversive shock to a part of the body such as the tail (Pinsker et al., 1973). Besides the gill-withdrawal reflex in response to the siphon stimulation, there are two other types of sensitization studied in *Aplysia*; the tail-withdrawal reflex and the siphon-withdrawal reflex both in response to tail stimulation (Byrne and Kandel, 1996). Besides sensitization, there are two other forms of non-associative learning. Habituation of the withdrawal reflex takes place when a stimulus is repeatedly presented, which decreases the reflexive response, while dishabituation takes place when a stronger or noxious stimulation is applied to the previously

habituated animal, which restores a stronger response. However, sensitization is the most studied behavior and its molecular mechanisms are relatively well known.

Memory for sensitization relies in part on the increase in sensory neuron (SN) excitability and the facilitation of SN to motor neuron (MN) transmission, which would eventually lead to the strengthening of the SN-MN synaptic connections (Fig. 1. 1) (Barbas et al., 2003). Additionally, SNs make indirect connections with MNs through excitatory and inhibitory interneurons (INs) (Fig. 1. 1) (Hawkins et al., 1981b, a). The L29 and L30 are excitatory and inhibitory INs, respectively, that are involved in the siphon-withdrawal reflex (Cleary et al., 1995). It has been shown that sensitizing stimuli can induce post-tetanic potentiation at the L29 to MN synapse, which lasts several minutes, suggesting the role for the L29 in short-term memory for sensitization of the this form of reflex (Frost et al., 1988). The same sensitizing stimuli can also induce inhibition of the L30 to L29 inhibitory connection, thus creating disinhibition, which is suggested to contribute to sensitization (Frost et al., 1988; Cleary et al., 1995).

There are likely to be multiple facilitating transmitters involved in sensitization. Synaptic facilitation induced by the L29 is mediated by an unknown transmitter of the L29 neuron (Hawkins and Schacher, 1989), and the neuropeptide SCP can enhance presynaptic facilitation as well as the withdrawal reflex (Abrams et al., 1984). However, 5-HT is known to be a major facilitating neurotransmitter released during sensitization training (Glanzman et al., 1989; Mackey et al., 1989; Marinesco and Carew, 2002). 5-HT acts on a transmembrane GPCR at the presynaptic terminal of the SN to enhance transmitter release

(Barbas et al., 2003). This, in turn, activates adenylyl cyclase (AC) that converts ATP to the second messenger cyclic AMP (cAMP), which then recruits PKA by binding to the regulatory subunits and causing them to dissociate from and free the catalytic subunits (Kandel, 2001). After short application of 5-HT, K^+ channels are phosphorylated, which leads to broadening of action potentials (APs) and enhanced transmitter release by reducing K^+ current and allowing greater Ca^{2+} influx into the presynaptic terminal (Klein and Kandel, 1980). This was thought to be one mechanism for short-term facilitation (STF) and underlies short-term memory (STM) for behavioral sensitization (lasting less than 30 min). However, it was later demonstrated that facilitation induced by 5-HT is largely independent of AP broadening (Klein, 1994). Furthermore, recent experiments have shown that presynaptic Ca^{2+} influx by 5-HT is independent of AP broadening and there is direct modulation of Ca^{2+} influx that contributes to presynaptic facilitation underlying STM for sensitization (Leal and Klein, 2009). Interestingly, molecular mechanisms for sensitization and dishabituation are known to be distinct. A SN-MN synapse undergoes synaptic depression when stimulated at low frequencies, which underlies the mechanism for habituation (Castellucci et al., 1970; Pinsker et al., 1970). This synaptic depression can be reversed by 5-HT application, which is called reversal of synaptic depression and thought to underlie dishabituation (Hochner et al., 1986). While the facilitation at a naïve synapse (underlying sensitization) is dependent on PKA, reversal of synaptic depression is mediated by PKC (Braha et al., 1990; Goldsmith and Abrams, 1991; Ghirardi et al., 1992).

Intermediate-term facilitation (ITF), underlying intermediate-term memory (ITM) lasting up to 3 hr, is achieved by several different protocols, which engage distinct molecular pathways. Five spaced applications of 5-HT induce activity-independent form of ITF that requires PKA and protein synthesis (Sutton and Carew, 2000). Similarly, ITM for sensitization induced by five spaced applications of tail shocks requires PKA and protein synthesis (Sutton et al., 2001). On the other hand, single application of 5-HT coupled with SN firing induces activity-dependent form of ITF that requires PKC but not protein synthesis (Sutton and Carew, 2000). This form of ITF might underlie site specific ITM, tested at the site where training is administered, since site specific ITM does not require PKA nor protein synthesis but instead requires calpain-dependent proteolysis of PKC, which leads to persistent activation of PKC (Sutton et al., 2004). These studies thus imply the flexibility of the system and suggest that multiple forms of memory with distinct molecular requirements exist within a given temporal phase, and the same end point can be achieved through multiple molecular mechanisms, rather than converging on a common molecular mechanism for maintaining the memory (Sutton et al., 2004).

Long-term facilitation (LTF), underlying long-term memory (LTM) lasting more than 24 hours, is produced by repeated (e.g. five) applications of 5HT to SN-MN synapses and requires new mRNA and protein synthesis (Montarolo et al., 1986). Persistent activation of PKA and extracellular signal related kinase (ERK), also known as mitogen-activated protein kinase (MAPK), is required for LTF and both of them have to translocate into the nucleus to induce long-term changes in synaptic strength (Bacskai et al., 1993; Martin et al., 1997a).

Activation of the transcription factor, cAMP-responsive element binding protein (CREB1), which starts the transcription process, and removal of the transcription factor CREB2, which is normally acting to repress CREB1-mediated transcription, have been shown to be critical to induce LTF (Dash et al., 1990; Bartsch et al., 1995). It is suggested that CREB1 and CREB2 are phosphorylated by PKA and ERK after translocation (Martin et al., 1997a; Bailey et al., 2004). Activated CREB1 induces the downstream transcription factor CCAAT-box enhanced binding protein (C/EBP), acting on downstream genes that are important for synaptic growth and stable form of LTF (Alberini et al., 1994). Down-regulation and endocytocis of *Aplysia* cell adhesion molecules (apCAMs) from the plasma membrane of SNs have been implicated in the formation and growth of new synaptic connections that accompany LTF (Bailey et al., 1992; Mayford et al., 1992). CAM-associated protein (CAMAP), a protein that binds to the cytoplasmic tail of apCAM, is dissociated from apCAM and translocated into the nucleus of sensory neurons in a PKA-dependent manner by repeated 5-HT applications either in a cell-wide or synapse-specific manner (Lee et al., 2007). CAMAP acts as a transcriptional coactivator for CREB1 and C/EBP and required for induction of both cell-wide and synapse-specific LTF, suggesting that CAMAP is one of the retrograde signals that translocates from activated synapses to the nucleus during synspse-specific LTF (Lee et al., 2007).

Phosphoinositide-3 kinase (PI3K) pathway, possibly activated downstream of 5-HT GPCRs and/or receptor tyrosine kinases, has been also shown to be critical for LTF, and in particular, for long-term morphological changes that accompany LTF. Cytoplasmic polyadenylation element binding

protein (CPEB) is an RNA-binding protein that activates dormant mRNAs by facilitating polyadenylation through elongation of poly (A) tail (Si et al., 2003). CPEB is induced by 1 x 5HT, and PI3K activities are necessary for 5-HT-dependent increase in CPEB (Si et al., 2003). CPEB is required at the synapses not for the initiation but for the stable maintenance of LTF, suggesting its role in stabilizing activity-dependent synaptic growth through the regulation of the synthesis of molecules that are required for structural synaptic changes (Si et al., 2003). Reorganization of the actin cytoskeleton is required for neurite outgrowth and formation of new SN varicosities induced by 5-HT (Hatada et al., 2000), and actin polymerization in response to exogenous stimuli is often regulated by the Rho family of GTPases (Etienne-Manneville and Hall, 2002). ApCdc42, a member of the Rho family of GTPases, is required for LTF and formation of new varicosities induced by 5-HT (Udo et al., 2005). 5-HT-induced activation of ApCdc42 is dependent on both the PI3K and phospholipase C (PLC) pathways, which in turn recruits the downstream effectors neuronal Wiskott-Aldrich syndrome protein (N-WASP) and p21-Cdc42/Rac-activated kinase (PAK) to regulate reorganization of the presynaptic actin network, leading to the outgrowth of filopodia (Udo et al., 2005).

1. 3. Role for tyrosine kinases in synaptic plasticity

Tyrosine kinases have been known to play important roles in neuronal survival and differentiation. Recently, there has been accumulating evidence demonstrating that they are also involved in regulating synaptic plasticity

underlying learning and memory. The following sections discuss the role of tyrosine kinases, in particular, receptor tyrosine kinases (RTKs) and their signal transduction pathways, in synaptic plasticity underlying learning and memory in mammals as well as in *Aplysia*.

1. 3. 1. Trk-mediated signal transduction

The troposyosin-related kinase (Trk) family of RTKs is involved in the regulation of the growth, development, survival, and repair of the nervous system, and neurotrophic factors mediate this process by binding to Trk receptors and inducing downstream signal transduction (Kaplan and Miller, 2000). The structure of Trk receptors shows unique combination of motif in their extracellular domains with three tandem leucine-rich motifs flanked by two cysteine clusters in their amino termini and two immunoglobulin-like domains in the more membrane-proximal region, usually having a tyrosine kinase catalytic domain in the cytoplasmic domain (Barbacid, 1995; Patapoutian and Reichardt, 2001). So far, three types of Trk receptors have been identified in mammals; TrkA, TrkB, and TrkC (Barbacid, 1995). Normally, a Trk receptor responds to a specific neurotrophin although different protein expression in their extracellular domain, or the presence or absence of short amino acid sequences in the juxtamembrane domains of each receptor, affects the ability of some neurotrophins to activate these receptors (Patapoutian and Reichardt, 2001).

Neurotrophins, or growth factors, act on their corresponding Trk receptor by binding to them and causing the dimerization of the receptor, which then leads

to the activation of the kinase domain and autophosphorylation of the tyrosine residues of the receptor (Fig. 1. 2) (Barbacid, 1995; Patapoutian and Reichardt, 2001). There are four identified neurotrophins in mammals that bind to and activate Trk receptors; nerve growth factor (NGF) that activates TrkA, brain-derived neurotrophic factor (BDNF) that activates TrkB, neurotrophin (NT)-3 that activates TrkC, and NT-4 that activates TrkB (Patapoutian and Reichardt, 2001). Besides Trk receptors, neurotrophins bind to another type of a cell surface receptor called p75 neurotrophin receptor (p75NTR), which acts together with Trk receptors to coordinate and modulate the responses of neurons to neurotrophins (Kaplan and Miller, 2000). Trk receptors and p75NTR act in a paradoxical way, in which the signals generated by both receptors can either collaborate with or inhibit each other's action: Trk receptors transmit positive signals such as enhanced survival and growth and p75NTR transmits both positive and negative signals (Kaplan and Miller, 2000).

After neurotrophin binding to Trk receptors, autophosphorylation of the tyrosine residues occurs in the cytoplasmic domain of the receptors. Two residues, Y490 and Y785, are thought to be involved in signal transduction, interacting with the adaptor protein Shc and PLC-gamma, respectively (Fig. 1. 2) (Patapoutian and Reichardt, 2001). Phosphorylation of Shc results in the recruitment of a complex of the adaptor growth factor receptor-bound protein 2 (Grb2) and the Ras exchange factor son of Sevenless (SOS), which then activates Ras (Patapoutian and Reichardt, 2001). Fibroblast growth factor receptor substrate-2 (FRS-2) is another adaptor protein that binds to the phosphorylated Trk receptor (Patapoutian and Reichardt, 2001). Ras leads to the activation of two

major signaling pathways; ERK and PI3K signaling pathways, which are involved in the regulation of neuronal survival and differentiation as well as in synaptic plasticity (Fig. 1. 2) (Kaplan and Miller, 2000). However, the signaling pathway induced by PLC-gamma was shown to be more important in some forms of synaptic plasticity (Minichiello et al., 2002; Gruart et al., 2007).

1. 3. 2. Role for TrkB-mediated signal transduction in synaptic plasticity in vertebrates

Recently, evidence has emerged for an important role for TrkB and its ligand BDNF in long-term potentiation (LTP) and behavioral learning and memory in mammals (Minichiello, 2009). LTP is an abrupt and sustained increase in the efficacy of synaptic transmission and the main experimental model of synaptic plasticity underlying learning and memory (Bliss and Collingridge, 1993). LTP can be divided into at least two temporally distinct phases that are fundamentally different in their underlying mechanisms. Early phase LTP (E-LTP), lasting for 30-60 min, is induced by a weak high frequency tetanus (e.g. single train of 100 pulses at 100 Hz) and does not require de novo protein synthesis, while late phase LTP (L-LTP), lasting hours, days, or even weeks, is induced by repeated, strong high frequency stimulations (e.g. multiple trains of 100 pulses at 100 Hz) and requires de novo protein synthesis (Malenka and Bear, 2004). Similarly, hippocampus-dependent memory can be divided into at least two phases, short-term memory (STM) and long-term memory (LTM). STM and LTM share some similarities to E-LTP and L-LTP, respectively. STM, lasting

from seconds to a few hours, does not require new protein nor mRNA synthesis, while LTM, lasting from many hours to weeks or even longer, requires new protein and mRNA synthesis (McGaugh, 2000; Dudai, 2004). As with LTF in *Aplysia*, induction of L-LTP results in the activation and nuclear translocation of PKA and ERK, which activate transcription factors such as CREB and induce transcription of downstream genes that are important for the structural changes and function of the synapses (Impey et al., 1998; Davis et al., 2000; Malenka and Bear, 2004; Lu et al., 2008). One of the genes induced by the L-LTP-inducing tetanus is BDNF (Lu et al., 2008).

Substantial evidence supports for a role for BDNF and TrkB in both E-LTP and L-LTP in the hippocampus (Lu et al., 2008; Minichiello, 2009). BDNF knockout mice show impaired induction of E-LTP (Korte et al., 1995; Pozzo-Miller et al., 1999), which is rescued by the application of recombinant BDNF (Patterson et al., 1996). Moreover, L-LTP is attenuated without BDNF binding to TrkB or in BDNF knockout mice, suggesting the role for BDNF in maintaining LTP (Kang et al., 1997; Korte et al., 1998). Studies using mutant mice revealed that the effect of BDNF and LTP triggered at the CA3-CA1 hippocampal synapses is mediated through the PLC-gamma site, but not the Shc site, of TrkB (Minichiello et al., 2002; Gruart et al., 2007). BDNF acts both pre- and postsynaptically to modulate synaptic plasticity. The BDNF knockout mice reveal a significant reduction in the number of vesicles docked at presynaptic active zones in the CA1 region of the hippocampus and show impairments in presynaptic transmitter release during high-frequency stimulation, which is rescued by the treatment with recombinant BDNF (Pozzo-Miller et al., 1999).

Consistently, the *trkB* mutant mice exhibit impaired presynaptic function and reduced ability of tetanic stimulation to induce LTP, suggesting the role of BDNF acting through TrkB presynaptically to modulate LTP (Xu et al., 2000). On the other hand, induction of LTP requires postsynaptic activation of Ca^{2+} channels and NMDA receptors, and BDNF enhances synaptic transmission in the hippocampus through postsynaptic tyrosine phosphorylation, which increases the phosphorylation of NMDA receptor subunits NR1 and NR2B (Levine et al., 1995; Suen et al., 1997; Lin et al., 1998; Kovalchuk et al., 2002). It has been shown that BDNF is released presynaptically and transferred to postsynaptic neurons in an activity-dependent manner (Kohara et al., 2001). It is thus proposed that BDNF, released presynaptically, binds to and phosphorylates TrkB located on both pre- and postsynaptic neurons, which leads to the increased transmitter release from the presynaptic sites and the postsynaptic Ca^{2+} influx through the phosphorylation of NMDA receptors in the postsynaptic sites (Yamada and Nabeshima, 2003). In particular, it is suggested that long-term maintenance of L-LTP is sustained by BDNF through activity-dependent transcription and translation in the postsynaptic neurons downstream of increased intracellular Ca^{2+} concentration (Lu et al., 2008).

BDNF also has a crucial role in hippocampus-dependent STM and LTM. An increase in BDNF mRNA expression in the hippocampus is associated with performance in hippocampus-dependent learning tasks such as contextual and spatial learning (Hall et al., 2000). Using antisense oligonucleotide or antibodies to BDNF, it has been shown that BDNF is required for the formation as well as the maintenance of hippocampus-dependent memory (Mu et al., 1999; Mizuno et

al., 2000; Alonso et al., 2002). Treatment with anti-BDNF antibodies at 1-4 h but not 6 hr after inhibitory avoidance (IA) training impaired LTM tested 24 hr later (Alonso et al., 2002). Moreover, intra-hippocampal infusion of BDNF antisense oligonucleotide 12 hr after IA training impaired memory retention 7 days but not 2 days later (Bekinschtein et al., 2007). Therefore, there are at least two time windows for the requirement of BDNF for LTM: one at 1-4 hr after learning, which is critical for LTM lasting for 1-2 days, whereas the other at around 12 hr after memory formation, which is important for persistence of LTM 7 days later. Indeed, recent experiments have shown that infusion of BDNF can rescue the deficit in memory persistence 7 days after IA training caused by the protein synthesis inhibitor, anisomycin, applied 12 hr after the training, suggesting that BDNF is a key protein synthesized during this late protein synthesis-dependent phase necessary for persistence of LTM (Bekinschtein et al., 2008). It was shown that the levels of phosphorylated ERK were increased 12 hr after training, the effect of which was blocked by the infusion of BDNF antisense oligonucleotide (Bekinschtein et al., 2008). Moreover, an ERK inhibitor applied 12 hr after training hindered memory retention 7 days, but not 2 days, later, implicating the involvement of ERK activated downstream of BDNF in LTM storage (Bekinschtein et al., 2008).

There is also other evidence implicating ERK in BDNF-dependent synaptic plasticity. Exogenously added BDNF can induce LTP at medial perforant path-granule cell synapses in the hippocampal dentate gyrus (Messaoudi et al., 1998). This BDNF-induced LTP was shown to be coupled to increased ERK and CREB phosphorylation and blocked by ERK inhibitors applied during the

induction but not after LTP was established, suggesting the role for ERK in the induction but not the maintenance for this type of LTP (Ying et al., 2002). Also, increased level of cAMP rapidly activates TrkB and can induce both BDNF- and ERK-dependent LTP in the hippocampus (Patterson et al., 2001). Interestingly, activation of TrkB was not critical for the ERK activation but important for the nuclear translocation of the activated ERK, which suggests a model where during the induction of LTP, cAMP causes the activation of TrkB signaling cascades through the release of BDNF acting as a ligand to TrkB, which induces nuclear translocation of the activated ERK leading to the phosphorylation of transcription factors such as CREB, although TrkB is not the primary upstream activator of ERK (Patterson et al., 2001). In addition to the hippocampus, TrkB signaling is also implicated in synaptic plasticity in the amygdala. The levels of BDNF and phosphorylated TrkB were increased in the amygdala after fear conditioning, and BDNF was able to enhance fear memory through the activation of ERK and PI3K (Ou and Gean, 2006).

Although experimental evidence supporting for the role of TrkB-PI3K signaling is not well substantiated, there is also another study showing that the PI3K signaling is involved in the memory formation in a BDNF-dependent manner. Spatial learning was impaired when a PI3K inhibitor was injected, and phosphorylation of TrkB, PI3K, and Akt, a downstream target of PI3K, increased in parallel with spatial memory formation, which was inhibited by the injection of antisense BDNF oligodeoxynucleotide (Mizuno et al., 2003). Interestingly, reactivation of spatial memory in well-trained rats caused an increase in phosphorylated eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and a

decrease in phosphorylated eukaryotic elongation factor-2 (eEF-2), which is a negative regulator of mRNA translation (Mizuno et al., 2003). 4E-BP1 is a negative regulator for eukaryotic initiation factor 4E (eIF4E) that has a suppressive effect on translational processes and is inactivated by mammalian target of rapamycin (mTOR)-dependent phosphorylation (Hara et al., 1997). Therefore, it is suggested that the TrkB/PI3K/Akt/mTOR signaling pathway is involved the processes underlying learning and memory through the regulation of mRNA translation, or increase in protein synthesis.

1. 3. 3. Role for tyrosine kinases in synaptic plasticity in *Aplysia*

Tyrosine kinase signaling cascades also play a crucial role in synaptic plasticity in *Aplysia*. Activation and nuclear translocation of ERK are necessary for 5-HT-induced LTF (Martin et al., 1997a). Purcell et al. (2003) have shown that a tyrosine kinase-ERK cascade is involved in 5-HT-induced LTF as well as LTM for sensitization. LTF was blocked by the application of a general tyrosine kinase inhibitor, genistein, and increasing endogenous tyrosine kinase function by the application of a general tyrosine phosphatase inhibitor, pbV, converted a single pulse of 5-HT, which usually induces only STF and not LTF, into a stimulus sufficient to induce LTF (Purcell et al., 2003). Activation of tyrosine kinases induced phosphorylation of ERK in SNs, and enhanced induction of LTF and LTM was blocked by an ERK inhibitor, U0126 (Purcell et al., 2003). Moreover, LTF induced by paring of pbV and single 5-HT application was mimicked by the application of mammalian BDNF, the effect of which was also

dependent on ERK, suggesting that activation of neurotrophin and its downstream tyrosine kinase cascades play a role in synaptic plasticity in *Aplysia* (Purcell et al., 2003). Later, they demonstrated that TrkB function is required for 5-HT-induced activation of ERK, LTF, and LTM for sensitization by using an extracellular fusion protein that sequesters secreted TrkB ligands (Sharma et al., 2006).

Sensorin is a sensory neuron-specific neuropeptide that is released presynaptically only when contacted with appropriate postsynaptic targets (L7 motor neurons), and regulates both increase and maintenance of synaptic strength, presynaptic varicosities, and mRNA distribution (Hu et al., 2004b). Hu et al. (2004b) found that sensorin was released following five applications of 5-HT to the SN-MN co-culture. Sensorin incubation after a single application of 5-HT led to the activation and translocation of ERK into the SN nuclei, and induced LTF and long-term synaptic changes such as growth of new presynaptic varicosities, which are not normally caused by a signle 5-HT application (Hu et al., 2004a). Sensorin activated ERK only after the activation of PKA by 5-HT since inhibition of PKA during but not after 5-HT application blocked sensorin-induced LTF, while inhibition of ERK after but not during 5-HT application blocked the effect of sensorin (Hu et al., 2004a). An inhibitor of Trk-like receptor tyrosine kinases, K252a, blocked LTF induced by 5 x 5-HT and sensorin-induced LTF as well as ERK activation and translocation when it was applied after 5-HT (Hu et al., 2004a). These results thus indicate the sequential activation of kinases where PKA activity is required before sensorin can activate receptor tyrosine kinases and ERK. The effects of sensorin were similar to those of BDNF where the application of single pulse of 5-HT and BDNF caused ERK activation and LTF

(Purcell et al., 2003). It is suggested that sensorin, released by 5-HT, acts on Trk-like receptor tyrosine kinases, leading to the activation of ERK that eventually leads to LTF and long-term synaptic changes (Hu et al., 2004a).

Elucidating the molecular mechanisms for sensorin synthesis and release, Hu et al. (2006) found that interaction of type II PKA, which is concentrated at synaptic sites, with A-kinase anchoring proteins (AKAPs) was required for the secretion of the newly synthesized sensorin from the varicosities and inducing sensorin-mediated LTF. Moreover, PI3K inhibitor and rapamycin blocked LTF induced by 5 x 5-HT and rapid synthesis of sensorin at varicosities without sensory neuron cell bodies (Hu et al., 2006). These results suggest that 5-HT induces two distinct pathways, which lead to the synthesis and secretion of sensorin by PI3K and type II PKA, respectively, in order to activate additional pathways to induce LTF (Hu et al., 2006). While activity-independent LTF is induced by 5 x 5-HT, activity-dependent LTF is induced by a single pairing of a tetanus with 5-HT application and was shown to require PKC- and rapamycin-sensitive increase in local sensorin synthesis (Hu et al., 2007b). Unlike for activity-independent LTF, PI3K was not required for sensorin synthesis for activity-dependent LTF (Hu et al., 2007b). Moreover, PKC, although required for activity-dependent LTF, was not required for activity-independent LTF, while PKA was required for both activity-dependent and –independent forms of LTF (Hu et al., 2006; Hu et al., 2007b). These results suggest the flexibility in the system and that different stimulation protocols activate different signaling pathways that converge to regulate synthesis and release of sensorin to produce long-term synaptic changes.

Ormond et al. (2004) succeeded in cloning of an *Aplysia* Trk-like receptor, ApTrkl, and provided evidence that ApTrkl mediates 5-HT-induced activation of ERK and LTF. ApTrkl is expressed in SNs and has a conserved intracellular domain while the extracellular domain is not conserved compared to the mammalian Trks (Ormond et al., 2004). Recent evolutionary analysis suggests that Trk and Trk-like receptors are conserved in most species (Wilson, 2009), and ApTrkl is likely to have arisen from a duplication of the ancestral Trk after its divergence from other Trk-related receptors such as receptor tyrosine kinase-like orphan receptors (Rors) and muscle specific kinases (Musks) (Sossin, 2006). Either pV, a tyrosine phosphatase inhibitor, or 5-HT application (either 5 min, 90 min, or 5 pulses) caused activation of ApTrkl, and activation of ERK observed after activating ApTrkl with pV or by 5-HT application was inhibited by K252a or by injecting SNs with ApTrkl dsRNA, confirming the requirement of endogenous ApTrkl in activating ERK (Ormond et al., 2004). LTF was blocked by the application of K252a or injection of ApTrkl dsRNA into SNs only when 5-HT is applied to the cell body but not when 5-HT is bath applied (Ormond et al., 2004). There are two components for LTF induced by bath application of 5-HT. One component is induced by 5-HT acting at the SN-MN synapse and synapse specific, lasting 72 hr and accompanied by growth of new varicosities, while the other component is induced by 5-HT acting at the cell body, lasting no more than 48 hr and not accompanied by growth of new varicosities (Ormond et al., 2004). Therefore, it suggests that ApTrkl has a role in changes occurring in the cell body rather than at the synapses.

Taken together, these studies show that tyrosine kinase activities are involved in changes in long-term synaptic plasticity such as LTF and LTM for sensitization in *Aplysia*. Sequential activation of kinases is suggested, where 5-HT activates PKA, which is required for sensorin, released by 5-HT, to activate Trk-like receptor tyrosine kinases and induce downstream activation and nuclear translocation of ERK, which eventually leads to gene transcription and expression to induce long-term changes in synaptic plasticity. ApTrkl, activated by 5-HT, is likely mediating these processes by activating ERK, although it remains unknown if neurotrophin-like molecules such as sensorin are involved in these processes. It is important to understand how ApTrkl is regulated by 5-HT.

1. 3. 4. Proposed mechanisms for ApTrkl regulation

There are several possibilities regarding how ApTrkl is regulated by 5-HT to induce downstream activation of ERK and LTF. One possibility is that 5-HT may cause release of one or more neurotrophin-like molecules from SNs, possibly sensorin, which acts as a ligand to ApTrkl and initiates tyrosine kinase signaling cascades. This model is consistent with mammalian cases where LTP is induced by a neurotrophin-binding to its Trk receptor, like BDNF-binding to TrkB, which initiates downstream tyrosine kinase cascades such as ERK and PI3K activation. However, sensorin is released after 5 pulses of 5-HT and activates tyrosine kinases and ERK only after PKA activation by 1 pulse of 5-HT (Hu et al., 2004a), whereas ApTrkl is activated by 5 min (1 pulse) of 5-HT (Ormond et al., 2004). Fast action of 5-HT on ApTrkl suggests that 5-HT might be able to activate

ApTrkl without sensorin, although it is possible that sensorin itself can still activate ApTrkl. Later involvement of sensorin cannot be ruled out because ApTrkl is still activated after 5 pulses of 5-HT (Ormond et al., 2004).

Second, ApTrkl might have to be translocated to the plama membrane in order to further activate downstream cascades such as ERK and induce LTF, and repeated or prolonged application of 5-HT might induce this translocation of ApTrkl. This is consistent with evidence in mammals that Trk receptors are largely sequestered in intracellular vesicles in some CNS neurons and second signals such as elevation of cAMP and Ca^{2+} are required for the receptors to be inserted into the plasma membrane (Meyer-Franke et al., 1998; Patapoutian and Reichardt, 2001; Howe and Mobley, 2005). It is also possible that internalization, or endocytosis, of ApTrkl is important to induce downstream cascades. Trk receptors signal to ERK only after internalization (York et al., 2000). Thus, additional signals, which are not stimulated by short bouts of receptor activation but require prolonged activation of the receptor, might be necessary for the activation of further signaling cascades. Consistently, although 1 pulse of 5-HT is enough to activate ApTrkl, it is not enough to activate ERK, suggesting that prolonged application of 5-HT might recruit additional signaling components that are required to activate ERK in addition to ApTrkl (Ormond et al., 2004). Sensorin, instead of acting as a ligand to ApTrkl, might mediate this translocation of ApTrkl either from the cytoplasm to the plasma membrane or vice versa downstream of ApTrkl activation.

A third possibility is that 5-HT causes removal of inhibitory tyrosine phosphatase activity that acts to oppose the induction of long-term changes in

synaptic plasticity under basal conditions (Purcell et al., 2003). Striatal-enriched protein tyrosine phosphatase (STEP), which is a component of the NMDA receptor complex, acts to inhibit the induction of LTP in the hippocampus (Pelkey et al., 2002). Also, the neurotransmitter dopamine leads to the phosphorylation and inactivation of STEP through a PKA-dependent pathway (Paul et al., 2000). It is thus possible that PKA acts to relieve the inhibitory constraint of STEP on long-term synaptic plasticity. In *Aplysia*, PKA is persistently activated by different patterns of 5-HT application from 1 to 5 pulses, and it is possible that PKA has a role in regulating tyrosine phosphatase activities to remove inhibitory constraint on ApTrkl.

Finally, 5-HT might activate ApTrkl through an intracellular transactivation mechanism by 5-HT-binding to GPCRs. In mammalian cells, transactivation of RTKs by GPCRs is reported for epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and TrkA (Daub et al., 1996; Lee and Chao, 2001; Kotecha et al., 2002). In EGFR, GPCR-triggered signals are enough to activate the receptor and eventually lead to ERK activation, mimicking the role of neurotrophins (Daub et al., 1996; Daub et al., 1997; Luttrell et al., 1997; Prenzel et al., 1999; Maudsley et al., 2000; Pierce et al., 2001). TrkA receptors are also activated in the absence of neurotrophins by the application of a neuropeptide that acts through GPCRs (Lee and Chao, 2001; Lee et al., 2002). One mechanism for transactivation is for the GPCR to increase the levels of ligand for the RTK. EGFR is activated by the metalloproteinase-dependent cleavage of proHB-EGF upon GPCR activation (Prenzel et al., 1999). In other cases, transactivation is ligand-independent, and Src tyrosine kinases are

involved in the transactivation of EGFR and TrkA (Daub et al., 1997; Luttrell et al., 1997; Maudsley et al., 2000; Lee and Chao, 2001). D4 dopamine receptor transactivates PDGFR and leads to the activation of ERK possibly through the recruitment of β-arrestin and Src or through other components of the ERK signaling pathway (Ferguson, 2003). These studies thus raise a possibility that ApTrkl is transactivated by 5-HT GPCRs, either by the recruitment of a ligand or by the activation of intracellular tyrosine kinases such as Src. Sensorin is a candidate if transactivation of ApTrkl through 5-HT GPCRs requires a ligand.

These possibilities are not mutually exclusive and other mechanisms might also be involved. It is important to elucidate a mechanism of ApTrkl regulation by 5-HT. In particular, it is interesting to see if sensorin is involved in the activation of ApTrkl. However, since ApTrkl does not have a known extracellular ligand-binding site, ApTrkl might not be activated by ligand-binding. Instead, ApTrkl might be transactivated by 5-HT through GPCRs.

1. 4. Role for PKC in synaptic plasticity

PKC is a family of serine/threonine kinases that mediate a wide range of cellular processes including the regulation of synaptic strength in the nervous system. In vertebrates, there are four families of PKC isoforms: classical PKCs (cPKCs; α, β, and γ), novel type I PKCs (nPKCs; ε and η), novel type II PKCs (δ and θ), and atypical PKCs (aPKCs; ζ and ι) (Sossin, 2007). All PKCs share a kinase domain, a carboxy-terminal domain and a pseudosubstrate that inhibits kinase activity (Fig. 1. 3). The regulatory domain of cPKCs and nPKCs consists

of two C1 domains and a C2 domain although the C2 domain of nPKCs is in N-terminal to the C1 domains (Fig. 1. 3) Activation of cPKCs requires both Ca^{2+}, which binds to the C2 domain, and diacylglycerol (DAG), which binds to the C1 domains. On the other hand, nPKCs are activated by DAG alone and their C2 domain does not bind to Ca^{2+}. aPKCs have a single C1 domain and a PB1 domain, which is involved in protein-protein interactions, and activation of aPKCs requires neither Ca^{2+} nor DAG (Fig. 3) (Sossin, 2007). Although PKCs are implicated in many forms of synaptic plasticity, specific isoform(s) of PKC that underlie(s) these events are not clear. In *Aplysia*, it is known that the same neurotransmitter, 5-HT, induces distinct types of synaptic plasticity mediated by distinct isoforms of PKC, suggesting the isoform specificity for understanding the roles for PKCs in regulating synaptic plasticity.

1. 4. 1. Role for different PKC isoforms in synaptic plasticity in vertebrates

The role for different isoforms of PKCs has been reported in various types of synaptic plasticity in vertebrates. In cerebellar Purkinje cells, the simultaneous activation of the glutamate receptor mGLUR1 and voltage-gated calcium channels induces long-term depression (LTD) of parallel fiber-Purkinje synapses that underlies behavioral plasticity such as associative eye-blink conditioning (Linden, 2003). The requirement for the conjunction of DAG activation, induced by mGLUR, and calcium entry suggests a requirement for cPKC (Sossin, 2007). In fact, cerebellar LTD is impaired in PKCα knockout mice, which is rescued by the expression of either PKCα or by its unique PDZ binding site to PICK1

inserted into a nonpermissive isoform PKCγ (Leitges et al., 2004). It is suggested that PICK1-bound PKC then phosphorylates the glutamate receptor subunit, GluR2, which leads to the internalization of GluR2 and induction of LTD (Leitges et al., 2004). PKCβ knockout mice have a learning deficit specifically in fear conditioning, suggesting a role for the PKCβ isoform in learning-related signal transduction especially in the amygdala (Weeber et al., 2000). PKCγ regulates LTP and LTD, and PKCγ knockout mice have impaired motor coordination and mild deficits in hippocampus-dependent tasks (Saito and Shirai, 2002). The limited deficits in the cPKC knockout mice suggest that there is a redundancy among these closely related kinases (Sossin, 2007).

As for nPKCs, PKCε is known to regulate nociceptor function and pain sensation. The cardinal symptom of inflammation is increased sensitivity to mechanical stimuli causing hyperalgesia, and PKCε is a critical second messenger in nociceptor sensitization caused by inflammation, chronic alcohol consumption, diabetes, and cancer chemotherapy (Khasar et al., 1999; Aley et al., 2000; Dina et al., 2000; Hucho et al., 2005). Although the role for PKCδ in synaptic plasticity has yet to be determined, PKCδ is known to regulate apoptosis outside the CNS, and apoptosis in salivary glands is suppressed in PKCδ knockout mice (Humphries et al., 2006). PKCθ has a role in activity-dependent synaptic elimination at the neuromuscular junction, which is deficient in PKCθ knockout mice (Li et al., 2004).

An enzyme known as protein kinase M (PKM) is formed by cleavage in the hinge domain of the atypical PKCs or, in the case of PKMζ, by a distinct mRNA from an alternative transcriptional start site generated by a promoter found

in an intron of PKCζ (Hernandez et al., 2003). PKM is constitutively active because it lacks the pseudosubstrate from the regulatory domain and known to have a critical role in persistent forms of synaptic plasticity and memory (Sossin, 2007). In particular, PKMζ was shown to be necessary and sufficient for LTP maintenance and inhibiting PKMζ with a pseudosubstrate-based inhibitor (ZIP) blocked the late-phase, but not the early-phase, of LTP (Ling et al., 2002; Serrano et al., 2005). Interestingly, ZIP could reverse both previously established LTP and hippocampus-dependent spatial memory when ZIP was injected 1 day after learning without any recall, suggesting that constitutive activation of PKCζ is required for maintaining LTP and spatial memory (Pastalkova et al., 2006).

1. 4. 2. Role for different isoforms of PKCs in synaptic plasticity in *Aplysia*

In *Aplysia*, three isoforms of PKCs, which are enriched in the nervous system, have been cloned; PKC Apl I (cPKC), PKC Apl II (nPKC), and PKC Apl III (aPKC) (Kruger et al., 1991; Sossin et al., 1993; Bougie et al., 2009). Unlike vertebrate atypical PKCζ, which can form PKMζ from an alternative transcription, PKC Apl III does not have a transcript encoding PKMζ (Bougie et al., 2009). Instead, a PKM fragment of PKC Apl III is formed by cleavage, which is dependent on Ca^{2+} and sensitive to calpain inhibitors (Bougie et al., 2009). Although the mechanism for the PKM formation may have changed over evolution, it is suggested that PKM forms of atypical PKCs may have a conserved role in regulating AMPA receptor trafficking (Bougie et al., 2009). Recent experiments have shown that maintenance of 5-HT-induced postsynaptic

facilitation, measured as enhancement of the glutamate-evoked potential in isolated MNs, is mediated specifically by PKC Apl III (Villareal et al., 2009).

PKC Apl I and PKC Apl II are known to translocate to the plasma membrane under different conditions to mediate distinct types of synaptic plasticity. PKC Apl II is translocated by 5-HT application while PKC Apl I is not (Zhao et al., 2006), and dominant-negative PKC Apl II, but not dominant-negative PKC Apl I, blocks 5-HT-induced reversal of synaptic depression (Manseau et al., 2001). On the other hand, when 5-HT is coupled to SN firing, a form of ITF is induced that lasts more than 1 hr after stimulation, which requires activation of PKC for its expression (Sutton and Carew, 2000). PKC Apl I is translocated when SN is fired in the presence of 5-HT although it is not translocated by 5-HT alone (Zhao et al., 2006). Moreover, dominant-negative PKC Apl I, but not dominant-negative PKC Apl II, blocks ITF that is induced by coupling of 5-HT and SN firing (Zhao et al., 2006). These results suggest that both activation of DAG through 5-HT GPCRs and Ca^{2+} entry via voltage-gated channels are required to activate PKC Apl I and mediate ITF. In addition, PKC Apl I is implicated in operant conditioning. Operant conditioning is a form of associative learning where an animal learns the consequences of its behavior. PKC Apl I was shown to be required for a single-cell analog of operant conditioning of feeding behavior in *Aplysia* neuron B51, which is critical for the expression of ingestive behavior reinforced by dopamine (Lorenzetti et al., 2008). Two measures of an increase in fictive ingestion, a decrease in burst threshold of B51 and an increase in its input resistance, were used to examine the expression of conditioning, which was demonstrated to be blocked by an inhibitor of PKA,

PKC, or the expression of dominant-negative PKC Apl I, but not dominant-negative PKC Apl II (Lorenzetti et al., 2008).

PKC also plays an important role in long-term synaptic changes. PKC is required for activity-dependent form of LTF, produced by a single paring of a tetanus with 5-HT, through the regulation of synthesis and secretion of sensorin (Hu et al., 2007b), as well as for the formation and maturation of synapses (Hu et al., 2007a). PKC-dependent initial formation of synapses is, however, not through the regulation of sensorin, whereas maturation of synapses accompanied by increased neurite outgrowth and varicosities is regulated by sensorin as well as ERK and PI3K (Hu et al., 2007a). Since these studies used the PKC inhibitor chelerythrine to examine the role of PKC, it is not known which isoform of PKC is involved in the synapse formation and maturation as well as sensorin regulation. It will be an important next step to examine isoform specificity for these long-term effects.

1. 4. 3. Molecular mechanism for PKC Apl II activation

Both cPKCs and nPKCs contain two C1 domains and one C2 domain, but the C2 domain of nPKCs is located N-terminal to the C1 domains and lacks critical aspartic acid residues important for coordinating Ca^{2+} ions in cPKCs (Nalefski and Falke, 1996). In cPKCs, the C2 domain binds to the membrane lipid phosphatidylserine (PS) in a Ca^{2+}-dependent manner (Newton, 1997). In conjunction with the C1 domain interacting with DAG, the binding of the C2 domain to PS results in pseudosubstrate release, which induces a conformational

change that activates the enzyme (Newton, 1997). However, the function of the C2 domain of nPKCs is less clear. It has been reported that the C2 domain of PKCε is able to bind to phospholipids, especially phosphatidic acid (PA), this PA binding to the C2 domain is required for translocation of PKCε (Jose Lopez-Andreo et al., 2003). In PKC Apl II, an epsilon family of PKC, phosphorylation of the C2 domain increases translocation of PKC Apl II (Pepio and Sossin, 2001). In contrast to the positive role of the C2 domain in activating PKC Apl II, it has been shown that the removal of the C2 domain allows for the activation of the enzyme at lower concentrations of PS, suggesting that the C2 domain is a negative regulator of PKC Apl II (Sossin et al., 1996).

In order to clarify the role of PA and the C2 domain in the translocation of PKC Apl II, we have previously examined the translocation of several PKC Apl II constructs and mutants. PA at relatively high concentrations (10-50 μg/mL) was able to translocate PKC Apl II in SF9 cells, which did not require nor was inhibited by the C2 domain since the construct without the C2 domain, PKC Apl II ΔC2, was translocated to the same extent (Farah et al., 2008). Moreover, low concentrations of DAG and PA, which do not normally cause the translocation of PKC Apl II on their own, could synergistically translocate PKC Apl II when applied together (Farah et al., 2008). PA binds to the C1b domain to induce the translocation since expression of the C1b domain of PKC Apl II, PKC Apl II-C1b, was enough to translocate this construct, and a mutation in the C1b domain (arginine 273 to histidine; PKC Apl II-R273H) removed the effects of exogenous PA (Farah et al., 2008). At low concentrations of DAG, PKC Apl II ΔC2 translocated better than PKC Apl II, which was not observed when combined with

PA, indicating that the role for PA in the translocation of PKC Apl II is to remove the C2 domain-mediated inhibition and decrease the concentration of DAG required for the translocation (Farah et al., 2008). In *Aplysia* SNs, translocation of PKC Apl II induced by 5-HT was partially blocked by the inhibitor to either PLC (U-73122, which impedes DAG synthesis from phosphatidylinositol) or PLD (1-butanol, which impedes the production of PA), suggesting that both DAG and PA are required for the translocation of PKC Apl II in response to 5-HT (Farah et al., 2008).

In vertebrates, the orthologue of PKC Apl II, PKC-epsilon can be activated by a cAMP-activated guanine exchange factor (GEF) Epac through the downstream activation of PLC and PLD, identifying a novel signaling from cAMP to PKC that involves PLC and PLD (Hucho et al., 2005). Epac is a cAMP-activated GEF for Ras and Rap, and one of the PLC isoforms, PLC-epsilon has a Ras binding domain and is activated by Ras (Sossin and Abrams, 2009). Recent experiments demonstrated that Epac activates PKC-epsilon through PLC-epsilon (Oestreich et al., 2009). Therefore, it is possible that PKC Apl II is activated by cAMP through the activation of PLC-epsilon and PLD by Epac. As another mechanism, PLC-epsilon might be activated through Ras activation induced by transactivation of ApTrkl by 5-HT. Involvement of tyrosine kinase activity has been reported for the activation of PKC Apl I during ITF and ITM for sensitization, although it appeared to be required for the activation of ERK rather than PLC (Shobe et al., 2009).

However, there is a critical missing part for understanding the mechanism for the translocation of PKC Apl II induced by 5-HT: Which 5-HT GPCR is

coupled to PKC Apl II? The finding that activation of both the PLC and PLD pathways is required for PKC Apl II translocation brings into question whether multiple GPCRs are required to activate PKC Apl II. Interestingly, 5-HT translocates PKC Apl II in SNs but not in MNs, which is not due to an inability of PKC Apl II to translocate in MNs since a nonspecific activator of PKCs, PDBu, can translocate PKC Apl II in MNs (Zhao et al., 2006). The differential translocation of PKC Apl II by 5-HT in SNs and MNs may be a result of differential expression of 5-HT GPCRs. Therefore, identifying 5-HT GPCRs and their role(s) in activating PKC Apl II is an important step to understand the mechanism for PKC Apl II activation.

1. 5. 5-HT GPCRs in *Aplysia*

5-HT is a major facilitatory neutortansmitter in *Aplysia*, acting as an activator for many important signaling cascades including ApTrkl and PKC Apl II. In spite of its critical role in synaptic plasticity, the receptors for 5-HT in *Aplysia* have not been studied well. In particular, 5-HT GPCRs are known to be critically involved in facilitating synaptic plasticity by inducing activation of second messengers such as PKA and PKC. In vertebrates, 5-HT receptors are composed of seven families ($5\text{-HT}_{1\text{-}7}$), of which 5-HT_3 are ionotropic receptors and all others are GPCRs, which couple to heterotrimeric GTP binding proteins (G proteins). G proteins are composed of an α, β, and γ subunit, and upon agonist binding to the receptor, the α subunit releases GDP and binds to GTP, leading to

the dissociation of the α subunit from the β and γ subunits, which are tightly bound to each other and only function as the βγ complex (Downes and Gautam, 1999). The GTP-bound Gα and Gβγ subunits are then freed to interact with other proteins and activate distinct intracellular effector molecules (Clapham and Neer, 1997). 5-HT_1 and 5-HT_5 inhibit AC through G_i family of G proteins, whereas 5-HT_4, 5-HT_6 and 5-HT_7 activate AC through G_s family of G proteins, resulting in the increased production of cAMP and increased activity of protein kinase A (PKA) (Raymond et al., 2001; Barbas et al., 2003). 5-HT_2 activates PLC through $G_{q/11}$ family of G proteins, resulting in PLC-mediated phosphatidylinositol (PI) hydrolysis, which generates the second messengers DAG and inositol trisphosphate (IP_3) (Conn and Sanders-Bush, 1986; Raymond et al., 2001). DAG is bound to the plasma membrane and activates PKC, and IP_3 leads to the release of Ca^{2+} from intracellular stores (Nichols, 2004).

In *Aplysia*, two G_i family of 5-HT GPCRs that are negatively coupled to AC have been cloned, which are called 5-HT_{ap1} and 5-HT_{ap2} (Angers et al., 1998; Barbas et al., 2002). Their amino acid sequences are similar to 5-HT_1-like receptors and the phylogenetic analysis of these receptors put them within the family of mammalian 5-HT_1 receptors (Angers et al., 1998; Barbas et al., 2002). 5-HT_{ap1} is expressed in every tissue and every ganglia of the nervous system (Angers et al., 1998), while expression of 5-HT_{ap2} is restricted to the CNS with the abdominal ganglion and the bag cells having high expression, suggesting the role for 5-HT_{ap2} in modulating afterdischarge during the egg-laying behavior (Barbas et al., 2002).

A 5-HT receptor that activates AC has been pharmacologically characterized. Cohen et al. (2003) identified methiothepin as a most potent antagonist for this class of receptor, called 5-HT_{apAC}. Methiothepin was most effective among different 5-HT receptor antagonists in blocking 5-HT stimulation of AC in *Aplysia* CNS, and it also blocked AC stimulation in SN membranes (Cohen et al., 2003). 5-HT stimulation of AC acted through PKA to increase SN excitability and spike duration, which are mediated by reduction in S-K^+ current, and methiothepin inhibited these two effects of 5-HT on SN firing properties; increased SN excitability and spike broadening (Cohen et al., 2003). Furthermore, Dumitriu et al. (2006) demonstrated that PKA- and PKC-mediated forms of synaptic plasticity were discriminated by two broad-spectrum 5-HT receptor antagonists; methiothepin and spiperone. Methiothepin was highly effective in blocking AC-coupled 5-HT receptors in *Aplysia*, while spiperone, an antagonist for PLC-coupled 5-HT receptors in mammals, did not block AC-coupled 5-HT receptors in *Aplysia* (Cohen et al., 2003). In electrophysiological studies, methiothepin, but not spiperone, blocked 5-HT-induced broadening of SN action potential in the presence of TEA/nifedipine, which is mediated by modulation of the S-K^+ currents and an assay for PKA-mediated plasticity (Dumitriu et al., 2006). On the other hand, spiperone, but not methiothepin, blocked 5-HT-induced facilitation of highly depressed synapses, which is an assay for PKC-mediated plasticity (Dumitriu et al., 2006).

However, it was only until recent that a full-length 5-HT receptor that is positively coupled to AC was reported in *Aplysia*. Lee et al. (2009) have identified a 5-HT receptor that activates AC in *Aplysia* neurons. The cloned

receptor, 5-HT_{apAC1}, was expressed in the CNS and in both SNs and MNs of *Aplysia* and able to stimulate cAMP production in heterologous cells, which was blocked by methiothepin consistent with the previous study (Cohen et al., 2003; Lee et al., 2009). Also consistent with the known role for the cAMP-PKA pathway in the SN firing, blockade of 5-HT_{apAC1} by injecting dsRNA for the receptor (ds5-HT_{apAC1}) impaired 5-HT-induced membrane excitability and spike broadening (Lee et al., 2009). Furthermore, short-term facilitation of nondepressed and moderately depressed synapses was impaired by ds5-HT_{apAC1}, although the facilitation of highly depressed synapses, which is known to be mediated by PKC, was normal (Lee et al., 2009).

1. 6. Rational and Objectives

Aplysia is a strong model system to study molecular and cellular mechanisms underlying learning and memory. Sensitization, habituation, and dishabituation of the gill-withdrawal reflex are the major behavior models for non-associative learning, which are replicated in culture of SN-MN synapses as synaptic facilitation, synaptic depression, and reversal of synaptic depression, respectively. 5-HT is released during sensitization training and mediates synaptic strengthening in synaptic facilitation and reversal of synaptic depression. In vertebrates, Trk-mediated signal transduction is well known to be involved in synaptic plasticity underlying learning and memory. In *Aplysia* SNs, ApTrkl is activated by 5-HT and induces activation of ERK and LTF (Ormond et al., 2004), suggesting the role for ApTrkl in mediating sensitization. However, how 5-HT

leads to the activation of ApTrkl is not clear because ApTrkl is unlikely to be directly activated by 5-HT. In particular, it is not known whether the activation of ApTrkl requires involvement of a ligand, like mammalian Trk receptors. ApTrkl does not have a conserved, or known, extracellular ligand-binding site (Ormond et al., 2004), suggesting the possibility that ApTrkl may not be activated by ligand-binding. The first objective was to elucidate the mechanism of ApTrkl regulation by 5-HT, where we initially hypothesized that ApTrkl is transactivated by 5-HT through 5-HT GPCRs without ligand-binding (Fig. 1. 4).

PKC Apl II is also activated by 5-HT but mediates a different form of synaptic plasticity; reversal of synaptic depression underlying dishabituation (Manseau et al., 2001; Zhao et al., 2006). PKC Apl II is translocated to the plasma membrane in SF9 cells by either DAG or high concentration of PA (Zhao et al., 2006; Farah et al., 2008). Moreover, the combination of low concentrations of DAG and PA can synergistically translocate PKC Apl II (Farah et al., 2008). In *Aplysia* SNs, translocation of PKC Apl II by 5-HT is decreased by the PLC or PLD inhibitor (Farah et al., 2008). Involvement of both the PLC and PLD pathways in PKC Apl II translocation brings into question whether multiple 5-HT GPCRs are required to activate PKC Apl II (Fig. 1. 5). Surprisingly, although signal transduction pathways induced by 5-HT are well known to have important roles in synaptic plasticity, little is known about the receptors for 5-HT. The second objective was to identify all 5-HT GPCRs from *Aplysia* and find the receptor(s) coupled to PKC Apl II activation.

Figure 1. 1

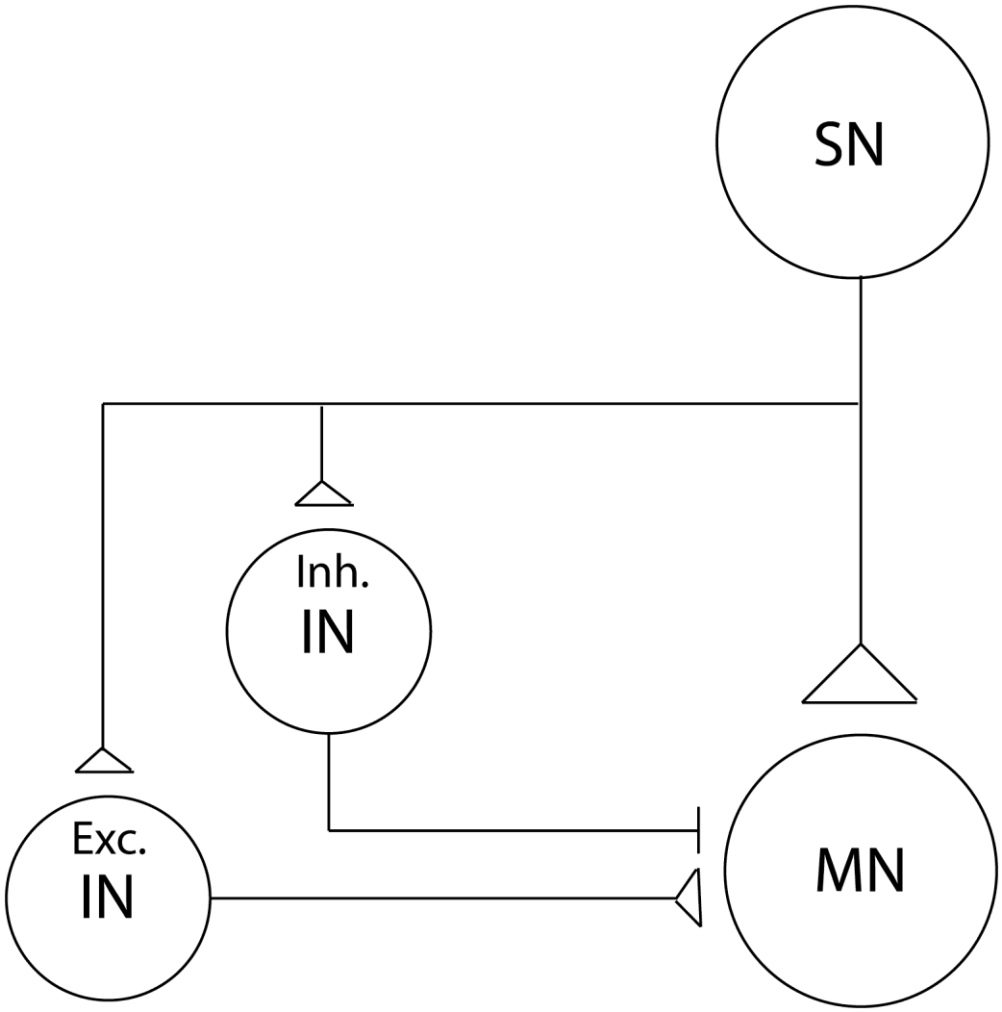


Figure 1. 2

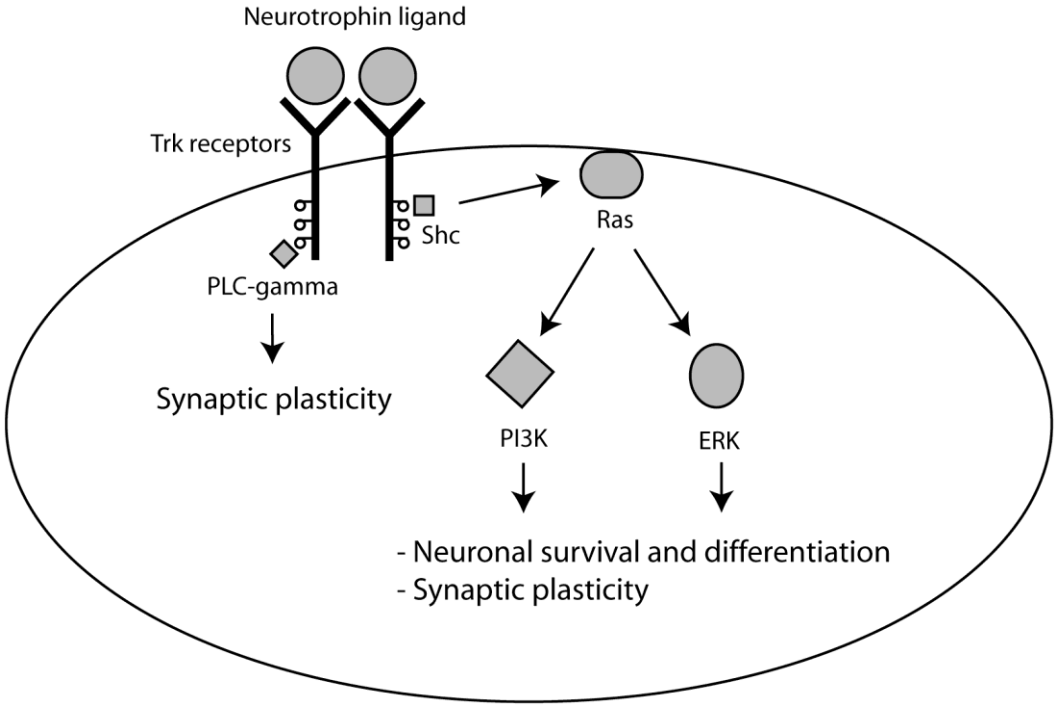

Neurotrophin ligand
Trk receptors
Shc
Ras
PLC-gamma
Synaptic plasticity
PI3K
ERK
- Neuronal survival and differentiation
- Synaptic plasticity

Figure 1. 3

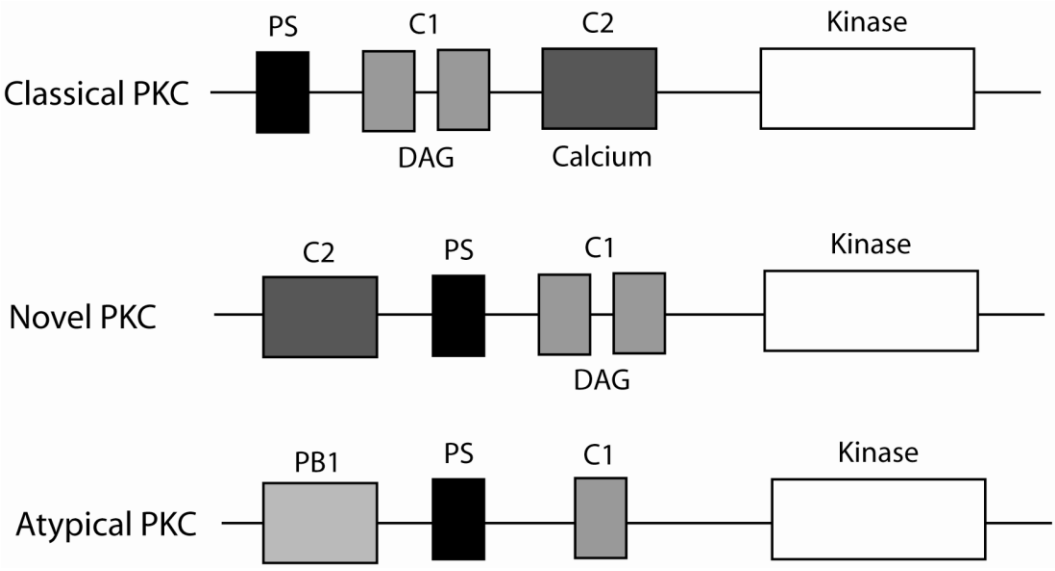

PS
C1
C2
Kinase
Classical PKC
DAG
Calcium
C2
PS
C1
Kinase
Novel PKC
DAG
PB1
PS
C1
Kinase
Atypical PKC

Figure 1. 4

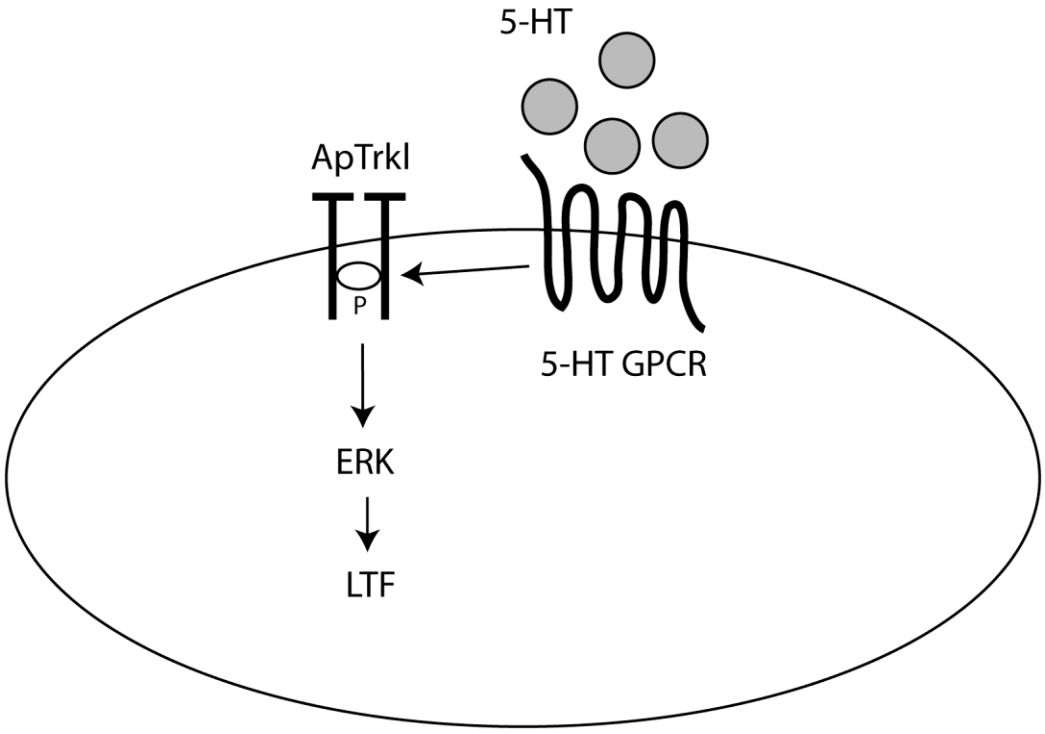


Figure 1. 5

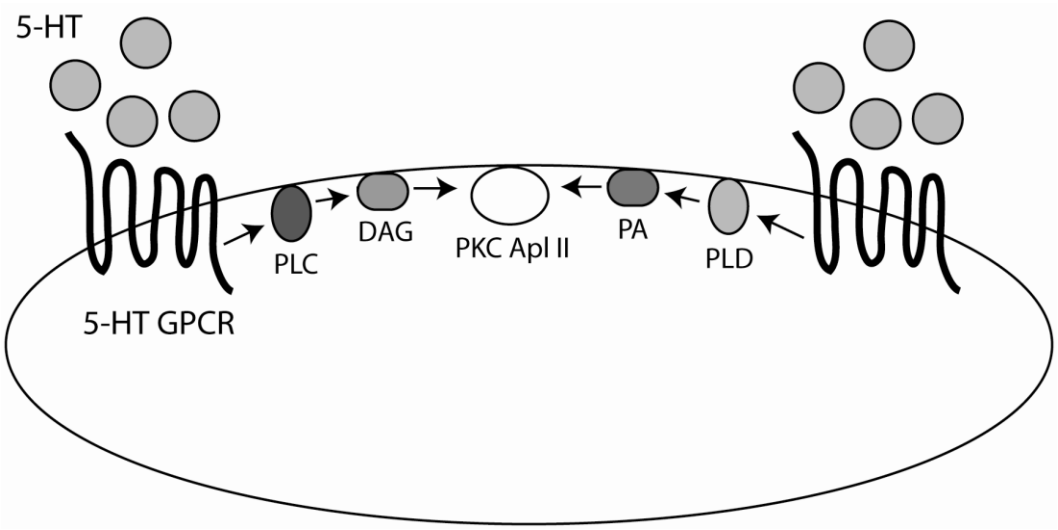


Figure Legends

Figure 1. 1. Simplified neural circuit in *Aplysia*. A sensory neuron (SN) makes a direct connection with a motor neuron (MN) to increase synaptic transmission. SN also makes indirect connections with MN through an excitatory (Exc.) and an inhibitory (Inh.) interneuron (IN).

Figure 1. 2. Trk-mediated signal transduction. Binding of a neurotrophin to Trk receptors causes dimerization of the receptors, kinase activation, and tyrosine autophosphorylation, leading to phosphorylation of the adaptor protein Shc and PLC-gamma. Phosphorylation of Shc leads to activation of Ras, which in turn activates ERK and PI3K. ERK and PI3K signaling pathways are involved in the regulation of neuronal survival and differentiation as well as in synaptic plasticity. The signaling pathway induced by PLC-gamma is also implicated in synaptic plasticity.

Figure 1. 3. Structure of PKC isoforms. All PKCs share a kinase domain and a pseudosubstrate (PS) that inhibits kinase activity. The regulatory domain of the classical and novel PKCs consists of two C1 domains and a C2 domain, although the C2 domain of the novel PKC is located N-terminal to the C1 domains. The atypical PKC has a single C1 domain and a PB1 domain, which is involved in protein-protein interactions.

Figure 1. 4. Model for ApTrkl activation. In our model, 5-HT activates ApTrkl through 5-HT GPCRs, leading to downstream activation of ERK and LTF. This transactivation of ApTrkl by 5-HT does not involve a ligand-binding to ApTrkl and is solely induced by association of ApTrkl to 5-HT GPCRs.

Figure 1. 5. Model for PKC Apl II activation. Activation of PKC Apl II requires both DAG generated by PLC and PA generated by PLD. Different 5-HT GPCRs might induce activation of the PLC and PLD pathways independently. Alternatively, it is possible that same 5-HT GPCRs can induce activation of both pathways.

CHAPTER TWO

Methodology

This chapter describes the materials and methods used in the experiments throughout the following chapters three and four.

2. 1. Animals

Aplysia Californica (50-125g for SNs; 50-250g for LFS MNs) were obtained from Marine Speciments Unlimited (Pacific Palisades, CA), the Mariculture Facility of the University of Miami (Miami, FL), or from Alacrity Marine Supply (Redondo Beach, CA). Animals were mostly used from Miami, FL.

2. 2. Plasmid construction for ApTrkl

Wild-type (WT), kinase-dead (KR), and phospho-tyrosine signaling-deficient (YW) ApTrkl-pNEX3 constructs with and without the extracellular domain of mammalian TrkC (TrkC-WT, TrkC-KR, and TrkC-YW, respectively) were generated previously (Ormond et al., 2004). To make TrkC-WT and WT constructs tagged with monocistronic red fluorescent protein (mRFP), the mRFP sequence was amplified by PCR using 3' primer (GGGTCGCGACTTGTACAGGGCGCCGGT) and 5' primer

(GGGCTCGAGGGATGGCCTCCTCCGAGGACGT) and cloned using a TrueBlue vector (Genomics One). The insert was cut out with the Nru restriction sites and ligated into the unique Nru site in the sequence encoding the carboxy-terminal insertion in TrkC-WT. The mutants were inserted into the mRFP-tagged constructs by cutting them out of the non-tagged constructs at the Kpn and EcoRV restriction sites and ligating into the mRFP constructs at the same sites. TrkC-WT, TrkC-KR, and TrkC-YW refer to the constructs with mRFP (Fig. 3. 1. A).

2. 3. *Aplysia* neuron cultures and plasmid injection

Aplysia dissociated SN cultures were prepared based on a previously described method with some modifications (Farah et al., 2008), and plated on poly-L-lysine coated coverslips for immunocytochemistry or on MatTek glass-bottom culture dishes with a glass surface of 14 mm (MatTek Corporation, Ashland, MA) for live imaging. In experiments on endogenous ApTrkl, cell cultures were grown for 3-4 days before treatments and fixation. 5-HT (Sigma-aldrich) and methiothepin (Sigma-aldrich) were used at the concentrations of 20 and 100 μM, respectively. Sensorin peptide (100 ng/mL) and antibody (400 ng/mL) were kind gifts from Dr. Samuel Schacher.

For plasmid injection, the DNA plasmids were microinjected at a concentration ranging from 100 to 400 ng/μL together with 0.4-0.5% of fast

green. SNs were injected 1-4 days after they were plated on poly-L-lysine-coated coverslips or on MatTek dishes, and fixed or imaged 1-2 days after injection.

For electrophysiological experiments, LFS MNs were isolated from the abdominal ganglion and identified by morphology and plated such that the axon(s) was in contact with the axon of SNs. After 4 days in culture and prior to electrophysiological recordings, the culture media was replaced with artificial sea water (ASW: NaCl 460 mM, $MgCl_2$ 55 mM, $CaCl_2$ 10 mM, KCl 10 mM, HEPES 10 mM, pH 7.6).

2. 4. Immunocytochemistry

After preparation of cell cultures or plasmid injection, the samples were treated with reagents and fixed with 4% paraformaldehyde and 30% sucrose in PBS. Cells were then permeabilized by washing with 0.1% Triton X-100 and 30% sucrose in PBS for 10-15 min. Cells were washed with PBS, washed again in NH_4Cl to quench free aldehydes for 15-20 min, and blocked for 30 min in a blocking solution of 10% normal goat serum and 0.5% Triton X-100 in PBS. The samples were then incubated for at least 1 hr with phospho-ApTrkl (P1) antibody, sequence CINKNPT(pY)FSP, at a 1:500 dilution in the blocking solution. P1 antibody was preincubated with nonphosphorylated peptide. Following primary antibody incubation, the samples were washed 3-4 times with PBS, and secondary antibody incubation was then carried out for 1 hr in the dark with goat anti-rabbit CY3 (Jackson Immunochemicals) for methiothepin and sensorin experiments or with goat anti-rabbit IgG (H + L; Molecular Probes) for injection experiments at a

dilution of 1:800 or 1:500, respectively. Cells were then washed 3-4 times with PBS, and coverslips were mounted on slides and fixed using mounting media.

2. 5. Measurement of change in the activation and localization of ApTrkl

All images were taken at near the center of the cell where the nucleus was well defined and obtained from the Zeiss LSM 510 confocal microscope. The samples were imaged using 488 nm and 543 nm wavelength laser lines, and images were collected at 63× magnification using the Zeiss LSM software. The images were then coded, and quantification was done by a blind observer. To quantify the levels of endogenous P1 staining, the NIH image was used. The cytoplasm excluding the nucleus was manually outlined, and mean pixel intensity for P1 staining was measured. In each experiment, both control and experimental cells were normalized to the average of the control cells.

For quantifying the localization of overexpressed constructs, each cell was automatically outlined using a program, IPLab (Scanalytics, Inc.), and concentric circles were then automatically constructed at 1-pixel intervals until the center of the cell was reached. The area 2-5 pixels away from the outermost part of the neuron was defined as the plasma membrane, 7-12 pixels away as the juxtamembrane, and 15-25 pixels away as the cytoplasm (Fig. 3. 1. B). Because of varied expression levels, we always measured the relative intensity of mRFP expression (receptor localization) or P1 staining (receptor activation) at the plasma membrane to the juxtamembrane and/or to the cytoplasm. In some cells, the nucleus was very close to the plasma membrane (e.g., Fig. 3. 2. A); in these

cases, only the other side of the cell was quantified. Quantification was done blindly, and only experiments with three or more successfully expressed cells in each group were included into the data.

2. 6. PCR cloning of 5-HT_{2Ap}, 5-HT_{4Ap}, and 5-HT_{7Ap}

To start cloning 5-HT GPCRs from *Aplysia Californica*, we first performed bioinformatics search in closer invertebrate species, which gave us *Lymnaea* 5-HT_2, putative *Lottia* 5-HT_4, and *Helisoma* 5-HT_7 sequences. We then performed *Aplysia* trace search and got pieces of *Aplysia* genomic DNA sequences that are homologous to these sequences. For the regions that are not available nor homologous, in particular, for the 5' end, the genomic DNA sequences were used as a template to get the upstream sequences. Finally, we assembled those genomic DNA sequences using the software Lasergene SeqMan (DNASTAR) and obtained a putative *Aplysia* genomic DNA sequence for 5-HT_{2Ap}, 5-HT_{4Ap}, and 5-HT_{7Ap}. Primers based on the putative sequences were used for PCR amplification using a cDNA library prepared from RNAs extracted from the nervous system of *Aplysia Californica* (Table 2. 1). For 5-HT_{2Ap} and 5-HT_{4Ap}, overlap PCR was performed from five and three, respectively, overlapped regions of the separate PCR products. The products of these PCR amplifications were subcloned into pJET1.2 vectors and sequenced. After the sequences were verified, the entire sequence of each protein was subcloned into pNEX3 vectors.

2. 7. Production of eGFP-tagged 5-HT receptors

Signaling sequence of sensorin was PCR amplified using the following sense and antisense primers, 5'-AGCAACATGCCTTCCAGAG-3' and 5'-TGTCGGATATGAAGACCTGC-3', and ligated into enhanced green fluorescent protein (eGFP)-pNEX3 vectors digested with BamHI and NcoI (sen-eGFP). To produce eGFP-5-HT_{7Ap} and eGFP-5-HT_{1Ap}, 5-HT_{7Ap}-pNEX3 and 5-HT_{1Ap}-pNEX3 were cut with SacI and BsiWI and ligated with sen-eGFP cut with SacI and BsrGI. To produce eGFP-5-HT_{2Ap}, both sen-eGFP and 5-HT_{2Ap}-pNEX3 were cut with SacI and BsrGI and ligated, which produced eGFP-5-HT_{2Ap} with 3'end missing (Δ3'eGFP-5-HT_{2Ap}). In order to produce eGFP-5-HT_{2Ap} with an intact 3'end, both Δ3'eGFP-5-HT_{2Ap} and 5-HT_{2Ap}-pNEX3 were cut with FspI and ligated and correct orientation was confirmed.

2. 8. Single cell RT-PCR

RNA was first synthesized from either *Aplysia* sensory or LFS motor neurons using RNAqueous-4PCR (Ambion) and reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen) with random hexamers. The resulting cDNA was used as a template for subsequent PCR amplification using primers specific to each mRNA (Table 2. 2 and 2. 3). The PCR products were visualized on agarose gel.

2. 9. SF9 cell culture

The SF9 cells were purchased from Sigma-Aldrich and grown in Grace's medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Cansera, Etobicoke, Ontario, Canada) as a monolayer at 27ºC. For transfection, cells were plated on MatTek glass-bottom culture dishes with a glass surface of 14 mm (MatTek Corporation, Ashland, MA) at a density of 0.05 x 10^6 cells/35-mm dish. Cells were transfected using the Cellfectin reagent (Invitrogen, Burlington, Ontrario, Canada) by following the recommendation of the manufacturer. Live imaging on the confocal microscope was performed 2 to 3 days posttransfection.

2. 10. Live imaging of PKC Apl II translocation

For live cell imaging, SF9 cells expressing mRFP-PKC Apl II and eGFP-5-HT_{1Ap}, -5-HT_{2Ap}, or -5-HT_{7Ap} or *Aplysia* sensory neurons expressing eGFP-PKC Apl II were examined using a Zeiss laser-scanning microscope (Zeiss, Oberkochen, Germany) with a 40x or 63x oil immersion objective. During imaging, 5-HT was added to the dish after 30 s, and a series of 7-12 confocal images were recorded for each dish at time intervals of 30 s. For *Aplysia* neurons, images were also obtained from cells outside the time-lapse image by taking images before and after 5-HT treatment.

5-HT, 1-butanol, U-73122, pirenperone, and genistein were purchased from Sigma-Aldrich. Spiperone and SB 200646 were purchased from Tocris

Bioscience. The drugs were used at the following concentrations: 10-20 μM 5-HT (10 μM for SF9 cells and 10-20μM for SNs), 1% 1-butanol, 10 μM U-73122, 100 μM genistein, 100 μM (or 200 μM in one experiment in SF9 cells) spiperone, 100 μM SB200646. 1,2-dioctanoyl-sn-glycero-3-phosphate (DiC-PA) was purchased from Avanti Polar Lipids (Alabaster, AL). DiC-PA was dissolved in dimethyl sulfoxide (DMSO), and diluted with Grace's medium before the experiments, and used at a final concentration of 2.5-5 μg/mL. In SF9 cells, 1-butanol, pirenperone, SB 200646 were present in the medium at least 5 min prior to the addition of 5-HT to the dish. Incubation with spiperone prior to 5-HT was varied from 5 to 30 min. U-73122 was present in the medium at least 1 min prior to the addition of 5-HT to the dish. In *Aplysia* SNs for PKC Apl II translocation experiments, spiperone and pirenperone were added to the dish at least 5 min prior to 5-HT. For experiments with genistein treatment, genistein or vehicle (1% DMSO) was added to the dish at least 30 min prior to 5-HT.

2. 11. Measurement and analysis of PKC Apl II translocation

The images obtained were analyzed using NIH Image J software. For SF9 cells, individual analysis of PKC Apl II translocation for each cell was performed by tracing three rectangles at random locations both at the plasma membrane and in the cytoplasm. The translocation ratio was calculated as the average intensity (membrane)/average intensity (cytoplasm) (Im/Ic) normalized to the degree of translocation before the addition of pharmacological agents (Post/Pre). For

statistical analysis, the average of images taken at 60, 90, 120, and 150 sec after the addition of pharmacological agents was normalized to the average before the addition of the agents. For *Aplysia* neurons, because the distribution of fluorescence on neuronal membranes was less uniform than that in SF9 cells, a different quantitative technique was used that allowed unbiased measurement of the entire membrane. The intensity at he plasma membrane of the entire cell was divided by the intensity in the cytoplasm (Im/Ic), and the translocation after 5 min of 5-HT was calculated by normalizing to before 5-HT (Post/Pre). The Student's t test or one-way analysis of variance was used to compare translocation ratios in SF9 cells and in *Aplysia* neurons. All data are presented as means $\pm$ SEM.

2. 12. Electrophysiology

Both SN and MN were impaled with sharp, glass microelectrodes (tip resistence; 15-30 MΩ) when backfilled with 2M K-acetate. Electrodes were attached to an Axoclamp 900A running Axoscope 4 for data acquisition (Axon Instruments), and bridge-balanced prior to cell entry and every couple minutes during an experiment, with positive pulses in the SN and negative pulses in the MN. A stable recording of the MN was performed before starting experiments to confirm electrophysiologically that the MN is in fact an LFS (Chitwood et al., 2001). This also allowed for the recording of any MN-recorded postsynaptic potentials (PSPs) that may be generated by the subsequent impalement of the SN. Throughout the experiment, the input resistance of the MN was measured with hyperpolarizing pulses (-0.5 nA, 500 ms), and trials with a change of greater than

25% prior to 5-HT addition were not included. Both the SN and MN were held at -80 mV with negative current injection by using a very slow voltage clamp (tau 2 s) that serves to maintain the membrane potential without affecting the PSP waveform. Action potentials (APs) in the SN were evoked with depolarizing pulses (cell specific current, 50 ms) in order to generate PSPs. PSPs were evoked at 0.05 Hz (once every 20 s) to depress the synapse. After 40 PSPs, 5-HT was added and at least 10 more PSPs were evoked at 0.05 Hz.

5-HT was added with rapid perfusion of the approximately 2 mL bath with 10 mL of 10 μM 5-HT. The bath inlet and outlet were attached to 10 mL syringes allowing manual perfusion for bath change in approximately 30 sec. Spiperone or genistein was included with the 5-HT perfusion to maintain drug concentrations. Application of spiperone (100 μM) or genistein (100 μM) occurred 10 min prior to the start of recording. Synaptic strength was measured as PSP amplitude or as PSP rise-rate (mV/ms) when initial or facilitated PSPs exceeded postsynaptic AP threshold (change in PSP amplitude and rise-rate were linear when compared simultaneously at sub-threshold PSP amplitudes). Data from individual trials was normalized to the amplitude of the first PSP, and facilitation was calculated by using the following equation:

Facilitation = 100 x (Ave. PSP #41-43/Ave. PSP #38-40)

Statistical analysis was conducted using unpaired Student's t-tests between the control (5-HT in ASW) and the drug group.

Table 2. 1. List of PCR primers for cloning of 5-HT receptors

cDNA	Sense primer (5'-3')	Antisense primer (5'-3')
$5HT_{2Ap}.1$	GTCATCGTCCACGAACTACAG	CACCACGATTGCACGCATCAT
$5HT_{2Ap}.2$ Outer Inner	 ATGATCAGGAGGTCGACGA GATTCAGGAGGTGGTTCTGGA	 AGGATGTTGACGGTGAAGAAG ACAGAGTTGTCTCGCCTCA
$5HT_{2Ap}.3$	CACTGGTCATCCTGGCTATCT	TCCTCGTCTGACAAAGTCTC
$5HT_{2Ap}.4$	CGGAGTACCAGGTGAATCACA	AGATAGCCAGGATGACCAGTG
$5HT_{2Ap}.5$	AGAGGCGTGAGTTCGATTC	GTCTTCTGAATCACCTGCAC
$5HT_{4Ap}.1$	CACTACAGCCATTGCAAGAG	TGGGACGCCACTAGTCATTC
$5HT_{4Ap}.2$	TCCATCTTCCACCTGTCCTG	CTCTTGCAATGGCTGTAGTG
$5HT_{4Ap}.3$	CGAAATATGACGTCAGTGGG	CAGGACAGGTGGAAGATGGA
$5HT_{7Ap}$	CTGACAACAGTCGGCTGTAACA	AGGACCAGCAACACAGTAGGA

Table 2. 2. List of primers for single cell RT-PCR for 5-HT receptors

mRNA	Sense primer (5'-3')	Antisense primer (5'-3')
Sensorin	AGCAACATGCCTTCCAGAG	CGTCCAGAAGTATAGGCTGT
MIP	TTACCGATCGCTACTCGATG	GTCCAGAGCCAATTGTTCAG
$5\text{-}HT_{2Ap}$	GTGCAGGTGATTCAGAAGACA	AGATAGCCAGGATGACCAGTG
$5\text{-}HT_{4Ap}$	CATCTGTGTTCCCATACTGAC	CAGGACAGGTGGAAGATGGA
$5\text{-}HT_{7Ap}$	TGGAGATGACGACGGATCAA	CTCTGCAGAATGTACTGACG
$5\text{-}HT_{1aAp}$	CATCTACGAGATCAGCATCC	TCGTCGGAATCATGTCTCCA
$5\text{-}HT_{1bAp}$	TACAGCGTTGTCAGTGACTG	CGTTACAACCGTTGGTGAAG

Note: MIP = Mytilus inhibitory peptide related precursor

Table 2. 3. List of primers for single cell RT-PCR for PLCs

PLC	Sense primer (5'-3')	Antisense primer (5'-3')
delta	TGCAGACCCTACATTCAGTG	AGAACGTATCCGACTCCTC
gamma-1	CTGCAACAGCTCTTTGCATG	CTATTCCTTCTGGTCTCCCAG
gamma-2	TCAGTTGAGTGCATCGTGCTG	TGATGTAGGAACCAGCGTTC
beta	GGACAAGTTGGATCTCAGTG	AGTGCACATGGTATAGCCGTG
beta-4	ACGTACGTAGAAGTGGACATG	ATGCCGATAGCCTGCTTGAAG
epsilon	CAGACTTCATCCTCCACTC	GATTGGCTGACAAGCTACTC

CHAPTER THREE

Mechanisms Regulating ApTrkl, a Trk-like Receptor in *Aplysia* Sensory Neurons

3. 1. Introduction

In mammals, there is accumulating evidence for the involvement of receptor tyrosine kinases (RTKs), especially the tropomyosin-related kinase (Trk) family of RTKs (Trk receptors), in long-term synaptic plasticity and behavioral memory. BDNF/TrkB signaling is implicated in long-term potentiation (LTP) in the mammalian hippocampus (Patterson et al., 1992; Korte et al., 1995; Patterson et al., 1996; Kang et al., 1997; Korte et al., 1998; Pozzo-Miller et al., 1999), as well as in hippocampus-dependent learning and memory (Mu et al., 1999; Hall et al., 2000; Mizuno et al., 2000; Alonso et al., 2002). In *Aplysia*, tyrosine kinase activities are involved in long-term memory (LTM) for sensitization of the gill-withdrawal reflex and 5-HT-induced long-term facilitation (LTF), the most commonly studied form of synaptic plasticity in *Aplysia*, through downstream activation of extracellular signal-related kinase (ERK) (Purcell et al., 2003). A Trk-like receptor in *Aplysia* (ApTrkl) is expressed in sensory neurons (Ormond et al., 2004). 5-HT activates ApTrkl, leading to activation of ERK, and ApTrkl is required for cell-wide LTF when 5-HT is applied to the cell soma (Ormond et al., 2004). Although ApTrkl is clearly implicated in 5-HT-induced signaling, how 5-

HT activates ApTrkl is not clear, especially because the extracellular domain of ApTrkl shows no homology with mammalian Trk receptors.

Regulation of Trk receptors through trafficking has been reported. TrkB is internalized by electric stimulation of hippocampal neurons and tyrosine kinase activity is required for the internalization (Du et al., 2003). Nerve growth factor (NGF) induces internalization of TrkA (Ehlers et al., 1995; Grimes et al., 1996). It is suggested that the functional consequence of internalization of growth factor receptors may be to initiate signal transduction (Sorkin and Waters, 1993).

5-HT is known to act through multiple G protein-coupled receptors (GPCRs) in sensory neurons (Barbas et al., 2003). In mammalian cells, transactivation of RTKs by GPCRs is documented for epidermal growth factor receptor (EGFR), TrkA, and platelet-derived growth factor receptor (PDGFR) (Daub et al., 1996; Lee and Chao, 2001; Kotecha et al., 2002). One mechanism of transactivation is for the GPCR to increase the levels of the ligand for RTK. For example, upon GPCR activation, the EGFR is activated by the metalloproteinase-dependent cleavage of proHB-EGF (Prenzel et al., 1999). In *Aplysia*, the neuropeptide sensorin is released from sensory neurons after repeated applications of 5-HT and involved in the activation of ERK (Hu et al., 2004a), similar to the activation by ApTrkl, suggesting that sensorin may be a ligand for ApTrkl. Moreover, K252a, an inhibitor of ApTrkl, blocked many of sensorin's actions (Hu et al., 2004a; Ormond et al., 2004). In other cases, transactivation is ligand-independent, and Src tyrosine kinases are involved in the transactivation of EGFR and TrkA (Daub et al., 1997; Luttrell et al., 1997; Maudsley et al., 2000; Lee and Chao, 2001). The D4 dopamine receptor transactivates PDGFR and leads to ERK

activation, possibly through the recruitment of β-arrestin and Src or through other components of the ERK signaling pathway (Ferguson, 2003). Thus, it is possible that activation of ApTrkl is a result of 5-HT GPCR-mediated transactivation, either by recruitment of a ligand or by activation of intracellular tyrosine kinases such as Src.

To explore the regulation of ApTrkl, we examined the localization and activation of overexpressed ApTrkl and several variants including extracellular TrkC chimeric ApTrkl constructs with or without intracellular mutations (kinase-dead or signaling tyrosine-deficient). The results revealed several mechanisms of ApTrkl trafficking that may be related to its regulation. Examination of the endogenous receptor suggested a novel transactivation mechanism involving signaling downstream of GPCRs, but sensorin was not required for the activation of ApTrkl by 5-HT.

3. 2. Results

3. 2. 1. Overexpression of wild-type ApTrkl leads to a large intracellular pool of the receptor and activation at the plasma membrane

We examined the localization and activation of the receptor after overexpressing ApTrkl constructs (Fig. 3. 1. A). All experiments were done in sensory neurons of *Aplysia*. To test for the role of endogenous ligands, we compared a wild-type (WT) ApTrkl construct to one in which we had replaced

most of the extracellular domain with the extracellular domain of TrkC (Ormond et al., 2004). We also used a number of constructs with mutations in the intracellular domain to test additional hypotheses. We used constructs either with no changes (-WT), with a K-R mutation that abrogated kinase activity (-KR) or with a Y-W mutation that abrogated the only conserved signaling tyrosine in ApTrkl (-YW) (Ormond et al., 2004). Finally, all constructs contained a red fluorescent protein tag (mRFP) inserted into the non-conserved carboxy-terminal domain to monitor localization of ApTrkl inside the cell. The localization of the receptor could then be monitored in conjunction with the activation state of the receptor using a phospho-specific antibody to the signaling tyrosine of ApTrkl (P1 antibody directed against NPx[pY] site in ApTrkl) (Ormond et al., 2004). We defined three areas for localization of the receptors: plasma membrane, juxtamembrane, and cytoplasm (Fig. 3. 1. B; see the Chapter Two Mehodology). Occasionally, a concentration of staining was observed in a juxtanuclear compartment; as this staining was quite variable and could have been a result of the concentration of the protein in the ER or Golgi after overexpression, this compartment was not included in the quantification.

Overexpression of constructs either containing the WT extracellular domain (WT-WT) or the extracellular domain of TrkC (TrkC-WT) led to constitutive phosphorylation of the receptor, suggesting that overexpression of the receptor leads to dimerization and activation of the receptors (Fig. 3. 2. A). This appeared to be independent of any endogenous ligand, as there were no differences in activation when the extracellular domain was switched with the extracellular domain of TrkC (Fig. 3. 2. A). This activation occluded any

additional actions of possible putative ligands, as phosphorylation of the receptor was not further increased by 5-HT or for the TrkC-WT receptor by NT3 (data not shown). The phosphorylated receptor was enriched in the plasma membrane and the juxtamembrane compartment; in contrast, the total receptor was more enriched in an intracellular pool (Fig. 3. 2). At this laser intensity, no staining was observed in non-injected cells; therefore, the signal observed is largely a result of the overexpressed receptor. Thus, from this experiment it can be concluded that when overexpressed, the receptor is much more activated when present in the plasma membrane or the juxtamembrane compartment. However, most of the receptor is localized in an intracellular pool, where it is largely inactive, even when overexpressed.

3. 2. 2. Kinase activity, but not the tyrosine phosphorylation at the NPxY site, is required for the presence of a large intracellular pool of ApTrkl

When kinase-dead (KR) constructs (WT-KR, TrkC-KR) were expressed, there was no immunoreactivity with the phospho-specific antibody (Fig. 3. 3. A). This result demonstrated that kinase activity is required for the phosphorylation of ApTrkl at the NPxY signaling site. Moreover, it demonstrates that our antibody is phospho-specific even when Aptrkl is overexpressed, as the antibody does not recognize the overexpressed non-phosphorylated receptor. One possible mechanism for the transactivation of RTKs by GPCRs is that GPCRs activate an intracellular kinase (such as Src) that then directly phosphorylates the signaling tyrosines of RTKs, inducing further downstream signaling. If this is true, we

would expect 5-HT to increase the staining of the overexpressed kinase-dead ApTrkl with the phospho-specific antibody to the NPxY site, even if ApTrkl has no intrinsic kinase activity. However, no detectable immunoreactivity was observed when 5-HT was added to the cells expressing either WT-KR or TrkC-KR receptor (data not shown).

There was a significant difference in the localization of the ApTrkl KR receptor compared with the WT. The TrkC-KR receptor was significantly more enriched in the plasma membrane relative to either the cytoplasm or the juxtamembrane compared with TrkC-WT or -YW ($P < .001$, Tukey's post hoc test; Fig. 3. 3. C). Thus, the ratio of the receptor in the plasma membrane to that in the intracellular pool was greatly increased in the absence of kinase activity. This suggests that when the kinase is active in the plasma membrane, it induces internalization; in contrast, the KR receptor is retained in the membrane because it is always inactive.

Surprisingly, overexpression of the YW receptors showed immunoreactivity with the P1 antibody (Fig. 3. 3. B). It should be noted that the level of immunoreactivity with P1 for the YW receptors was much less than that for the WT receptors (approximately fivefold based on the anti-P1/mRFP ratio). The P1 antibody is directed at this site that is mutated (Y-W), and we observed no immunoreactivity in previous experiments overexpressing this receptor in a heterologous system (Ormond et al., 2004), suggesting that this immunoreactivity was not derived from the overexpressed receptor. Moreover, the lack of immunoreactivity with the KR mutant rules out any cross-reactivity due to the level of overexpression. Thus, the most likely explanation for this

immunoreactivity is hetero-dimerization with the endogenous receptor and immunoreactivity due to phosphorylation of the endogenous receptor at this site; unlike the KR receptor, the YW receptor is not kinase dead; indeed it was still competent to partially signal ERK activation in heterologous cells (Ormond et al., 2004). Unlike the KR receptor, the YW receptor was not enriched in the plasma membrane (Fig. 3. 3). Thus, although kinase activity is required for the presence of the receptor in the intracellular pool, this is not a result of kinase activity being required for the phosphorylation at the NPxY site.

3. 2. 3. 5-HT induces kinase activity- and ligand-independent internalization of ApTrkl

Interestingly, 5-HT treatment led to a decrease in relative plasma membrane enrichment in the KR constructs ($P < .01$ for WT-KR, $P < .05$ for TrkC-KR; Fig. 3. 4. A and B), suggesting there was 5-HT-induced internalization of the receptor independent of kinase activity. Because this was observed in both WT-KR and TrkC-KR, it is also suggested that 5-HT-induced internalization is ligand independent. However, internalization of the receptor was not observed for the WT constructs with 5-HT treatment (data not shown). It is possible that the kinase activity-dependent internalization occluded the 5-HT-induced internalization.

We then hypothesized that 5-HT-induced internalization occurs through activation of 5-HT GPCRs, and examined this hypothesis by using methiothepin, an antagonist for most 5-HT GPCRs in *Aplysia* (Barbas et al., 2003; Dumitriu et

al., 2006). The TrkC-KR chimera was used in order to rule out the involvement of extracellular ligand binding or direct binding of 5-HT to ApTrkl. When methiothepin (100 μM) was applied together with 5-HT (20 μM, 5 min), it blocked the 5-HT-induced internalization of the receptors ($P < .01$ for control and 5-HT, $P < .05$ for 5-HT and 5-HT+methiothepin, Tukey's post hoc test; Fig. 3. 4. C and D). Therefore, the results indicate that the kinase activity-independent internalization induced by 5-HT occurs through activation of 5-HT GPCRs.

3. 2. 4. Activation of endogenous ApTrkl

Because overexpression of the receptor occluded ligand-dependent activation of the receptor, we returned to the measurement of the endogenous receptor by a ligand in an attempt to determine the mechanism of receptor activation. To measure endogenous receptor activation required much higher laser power, resulting in higher background signals. Nevertheless, similar to the previous results (Ormond et al., 2004), 5-HT treatment for either 5 min or 1 hr increased activation of the receptor (Fig. 3. 5. A and C). However, it should be noted that unlike when the receptor was overexpressed, the activated endogenous receptor was mainly observed in the internal pools, and little, if any, phospho-specific staining was observed in the plasma membrane (Fig. 3. 5). Although the increases measured were small, it should be pointed out that after treating ApTrkl with dsRNA, none of the background signal was removed, but there was complete elimination of the signal that was increased with 5-HT (Ormond et al., 2004).

Thus, we assume that in the absence of 5-HT, there is little or no activation of the receptor and the signal observed is background staining.

To determine if the receptor is activated by 5-HT through transactivation of GPCRs, the effect of methiothepin (100 μM) was examined. Surprisingly, the addition of methiothepin alone was sufficient to activate the receptor to the same level as 5-HT, and there was no additive effect when 5-HT and methiothepin were added together ($P < .05$ for control and all other groups, Tukey's post hoc test; Fig. 3. 5. A). Thus, for the activation of ApTrkl, methiothepin acts as an agonist, similar to 5-HT. This differs from its normal activity and contrasts with the results shown earlier where methiothepin blocked the ability of 5-HT to internalize the KR receptors.

It was shown that repeated 5-HT applications to sensory-to-motor neuron culture leads to the release of a neuropeptide sensorin from sensory neurons and that sensorin is involved in LTF and activation of ERK (Hu et al., 2004a). Moreover, K252a, an inhibitor of ApTrkl, blocked the ability of sensorin to activate ERK (Hu et al., 2004a; Ormond et al., 2004). To examine the possibility that sensorin is involved in the activation of ApTrkl, either as a ligand or by some other unknown mechanism, sensory neuron cultures were incubated with sensorin peptide (100 ng/mL) for either 5 min or 1 hr. Incubation for 5 min increased the activation of ApTrkl ($P < .01$), although this activation was transient and not observed after incubation for 1 hr (Fig. 3. 5. B). Because sensorin could activate ApTrkl, albeit transiently, we next asked whether 5-HT activation of ApTrkl was mediated through sensorin using an antibody to sensorin that can block most

actions of exogenously added sensorin (Hu et al., 2004a). The sensorin antibody (Sen ab; 400 ng/mL) did not block the 5-HT-induced activation of ApTrkl ($P < .05$ for control both from 5-HT and from 5-HT+Sen ab, Tukey's post hoc test; Fig. 3. 5. C). Therefore, sensorin is not required for the 5-HT-induced activation of ApTrkl. Since the ability of sensorin to produce LTF appears to first require PKA activation by 5-HT (Hu et al., 2004a), we asked if priming with 5-HT for 5 min before the sensorin incubation for 1 hr will activate ApTrkl. However, this treatment did not activate ApTrkl (data not shown).

3. 3. Discussion

3. 3. 1. Regulation of overexpressed ApTrkl

Overexpression of the wild-type (WT) ApTrkl constructs leads to activation of the receptors, mainly in the plasma membrane, most likely through self-dimerization of overexpressed receptors, whereas more receptors were localized in an intracellular pool (Fig. 3. 2). It is not clear why phosphorylation of the receptor is enhanced in the plasma membrane and juxtamembrane areas compared with the internal pool. Possible explanations are; (1) differences in the lipid composition of pH in the membrane, causing conformational changes that increase dimerization; (2) changes in levels of tyrosine phosphatases in different compartments; or (3) the presence of different binding partners that either activate or inhibit dimerization in different compartments.

Overexpression of the kinase-dead (KR) ApTrkl constructs showed more localization of the receptors in the plasma membrane, although there was no activation (Fig. 3. 3. A and C). This is consistent with constitutive receptor internalization of ApTrkl that is dependent on kinase activity. Consistent with this, tyrosine kinase activity is required for the internalization of TrkB by electric stimulation of hippocampal neurons (Du et al., 2003). Internalization is not a result of phosphorylation of the signaling site (NPxY) because mutations at this site did not affect localization of the receptor.

Interestingly, 5-HT treatment led to internalization of the KR receptor, as indicated by the decrease in the relative enrichment of the receptor in the plasma membrane (Fig. 3. 4. A and B). This suggests that there is a kinase activity-independent mode of internalization, which is not caused by a ligand-binding or direct binding of 5-HT to ApTrkl because the TrkC-KR chimera was capable of internalization. 5-HT is known to activate endocytosis in general, and endocytosis of proteins like *Aplysia* cell-adhesion molecule (apCAM) (Bailey et al., 1992). This effect was mediated by GPCRs since it was blocked by methiothepin. It was surprising that there was no observed internalization of the WT receptor; however the effect of 5-HT may have been occluded by the kinase-dependent internalization. Alternatively, 5-HT may stimulate insertion of the receptor as well, and although for the WT there was no overall change, the higher expression of the KR receptor in the plasma membrane allowed us to visualize the internalization.

Given the higher level of activation of the overexpressed receptor in the plasma membrane, it is surprising that activation of the endogenous receptor is

observed mainly in the internal pool, even after a 5-min treatment with 5-HT. This could be a result of a combination of activity-dependent and 5-HT-dependent internalization of the activated receptor or because of GPCR-mediated activation of the internal pool of the receptor. However, GPCR-mediated activation must still require kinase activity of ApTrkl; otherwise, it would have been detected by transactivation of the KR receptor.

3. 3. 2. Activation of endogenous ApTrkl

Surprisingly, blocking 5-HT GPCRs with the antagonist methiothepin did not prevent 5-HT-induced activation of endogenous ApTrkl; instead, methiothepin itself activated ApTrkl to the same level as 5-HT (Fig. 3. 5. A). Methiothepin acts as an inverse agonist for 5-HT receptors (5-HT_{1A} and 5-HT_7 receptors) (McLoughlin and Strange, 2000; Krobert et al., 2006), and orthologues of these receptors exist in *Aplysia* (Angers et al., 1998; Barbas et al., 2002; Cohen et al., 2003). As an inverse agonist, methiothepin binds to the same site of 5-HT and causes a conformational change that favors the inactive state of the receptor. However, it is possible that this conformation may be competent to signal through other pathways. Indeed, methiothepin induces heterologous down-regulation of receptors, and this down-regulation does not depend on the normal signaling pathway of 5-HT receptors (Krobert et al., 2006).

The receptor for sensorin has not been isolated. While sensorin could transiently activate endogenous ApTrkl, we have not been able to show sensorin activation of heterologously expressed ApTrkl (data not shown). Moreover,

antibodies to sensorin, which are able to block most actions of sensorin (Hu et al., 2004a), did not block 5-HT-induced activation of ApTrkl. Thus, we favor a model in which sensorin may activate a GPCR (like most other neuropeptides) that can also couple to ApTrkl. It is not clear if this is the major action of the sensorin receptor or if this is required for sensorin activation of ERK.

Figure 3. 1

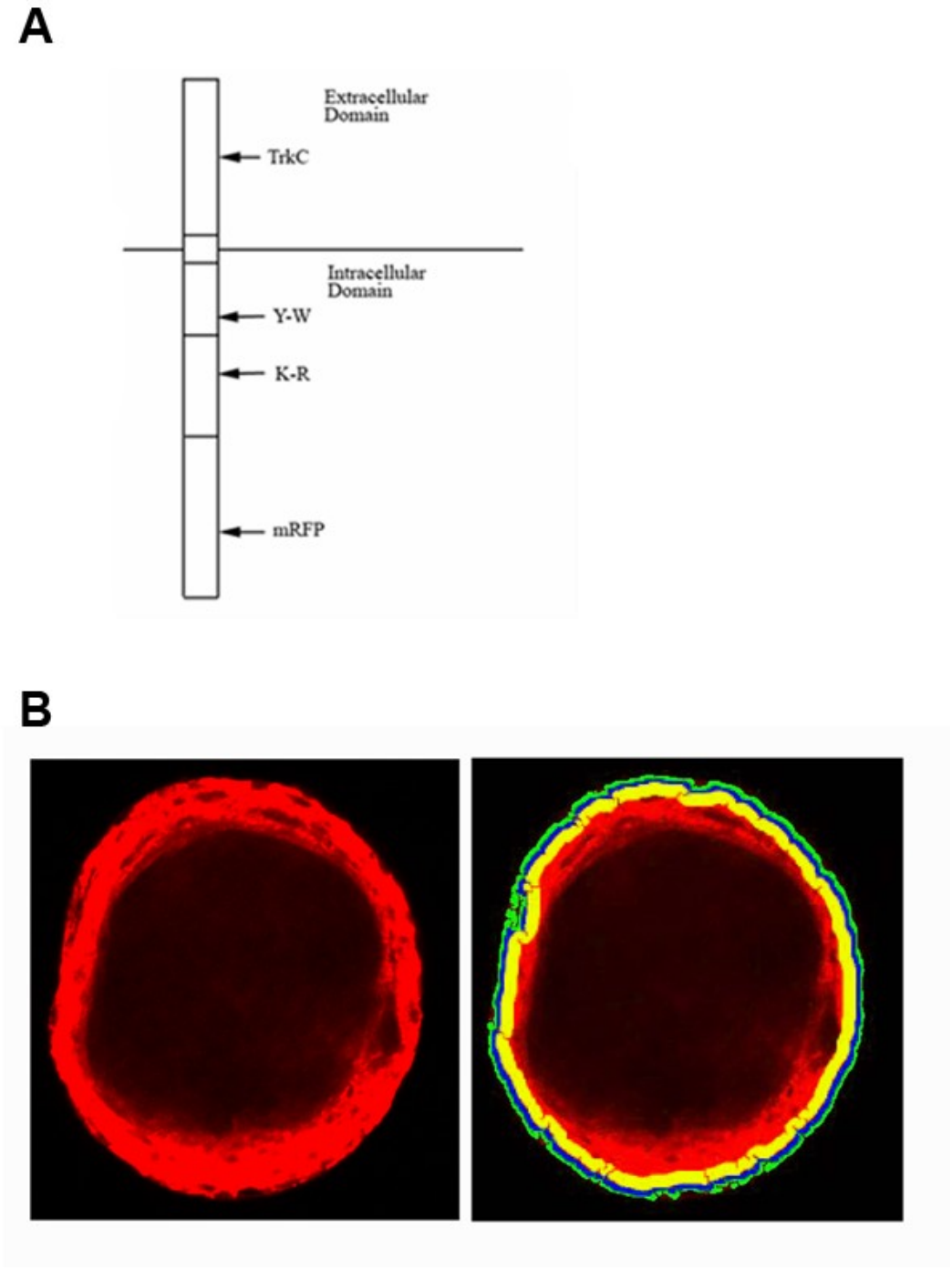

A
Extracellular Domain
TrkC
Intracellular Domain
Y-W
K-R
mRFP
B

Figure 3. 2

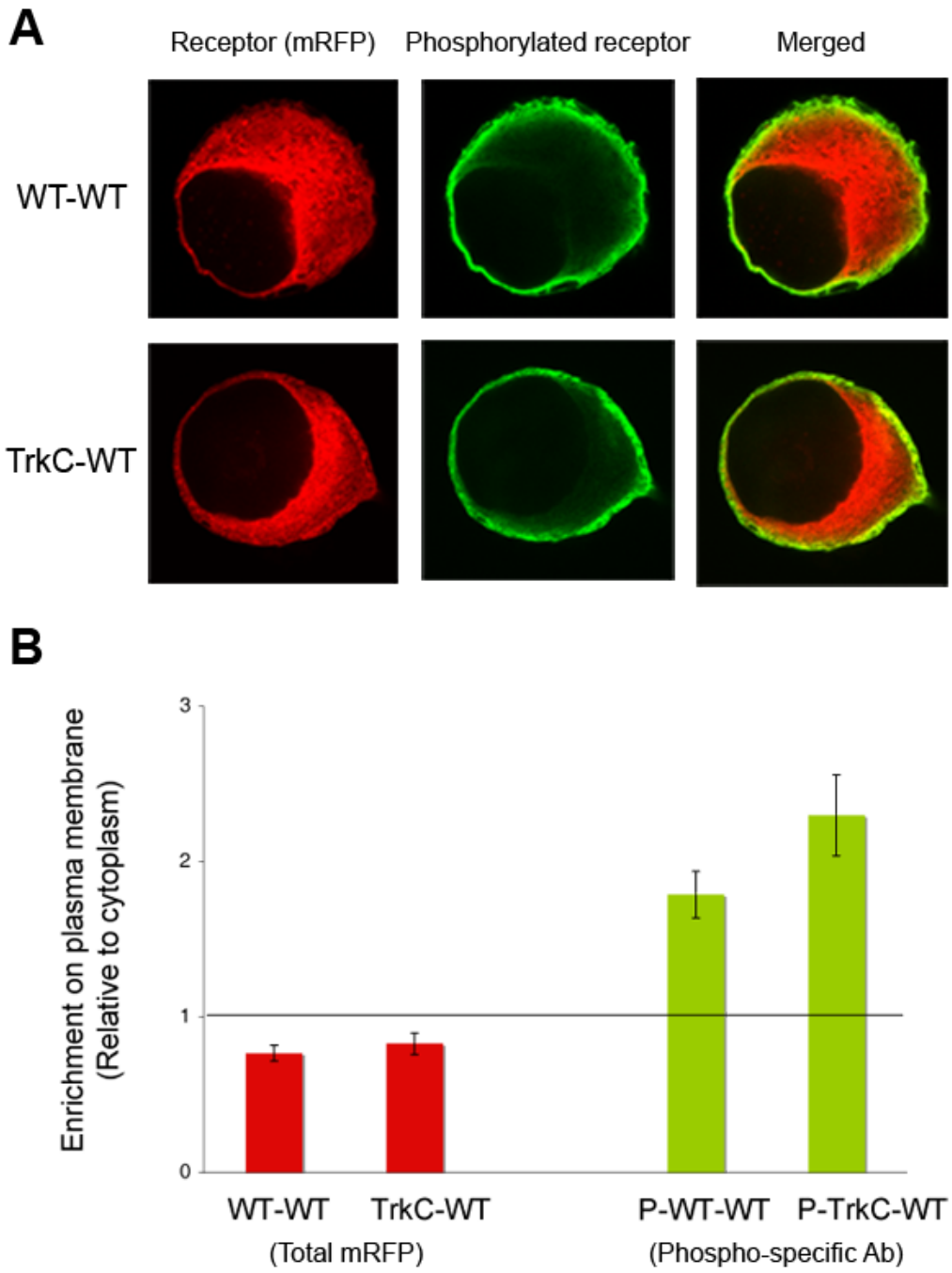

A
Receptor (mRFP)
Phosphorylated receptor
Merged
WT-WT
TrkC-WT
B
Enrichment on plasma membrane
(Relative to cytoplasm)
3
2
1
0
WT-WT
TrkC-WT
P-WT-WT
P-TrkC-WT
(Total mRFP)
(Phospho-specific Ab)

Figure 3. 3

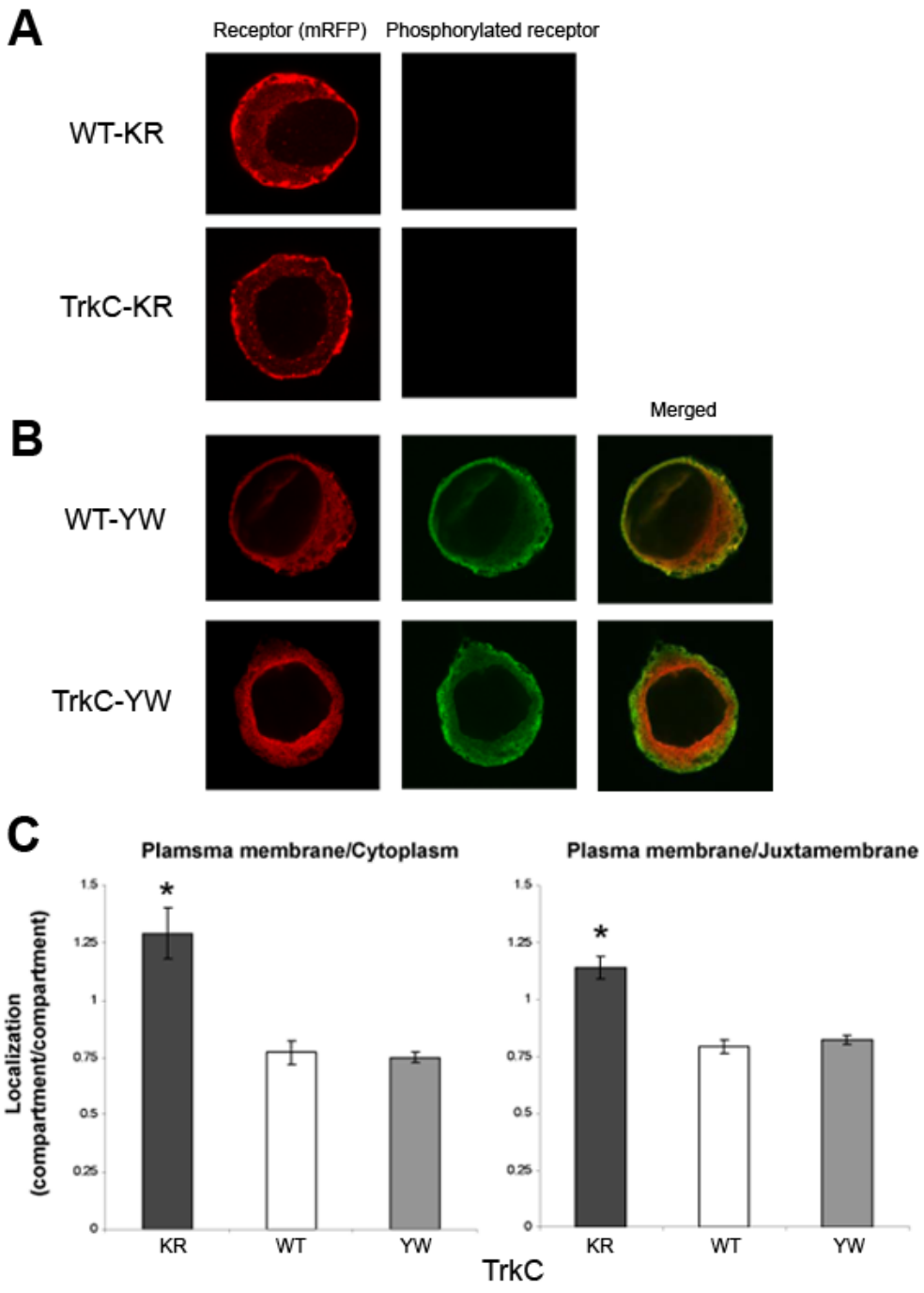

A
Receptor (mRFP)
Phosphorylated receptor
WT-KR
TrkC-KR
B
Merged
WT-YW
TrkC-YW
C
Plamsma membrane/Cytoplasm
Plasma membrane/Juxtamembrane
Localization (compartment/compartment)
1.5
1.25
1
0.75
0.5
0.25
0
*
KR
WT
YW
TrkC

Figure 3. 4

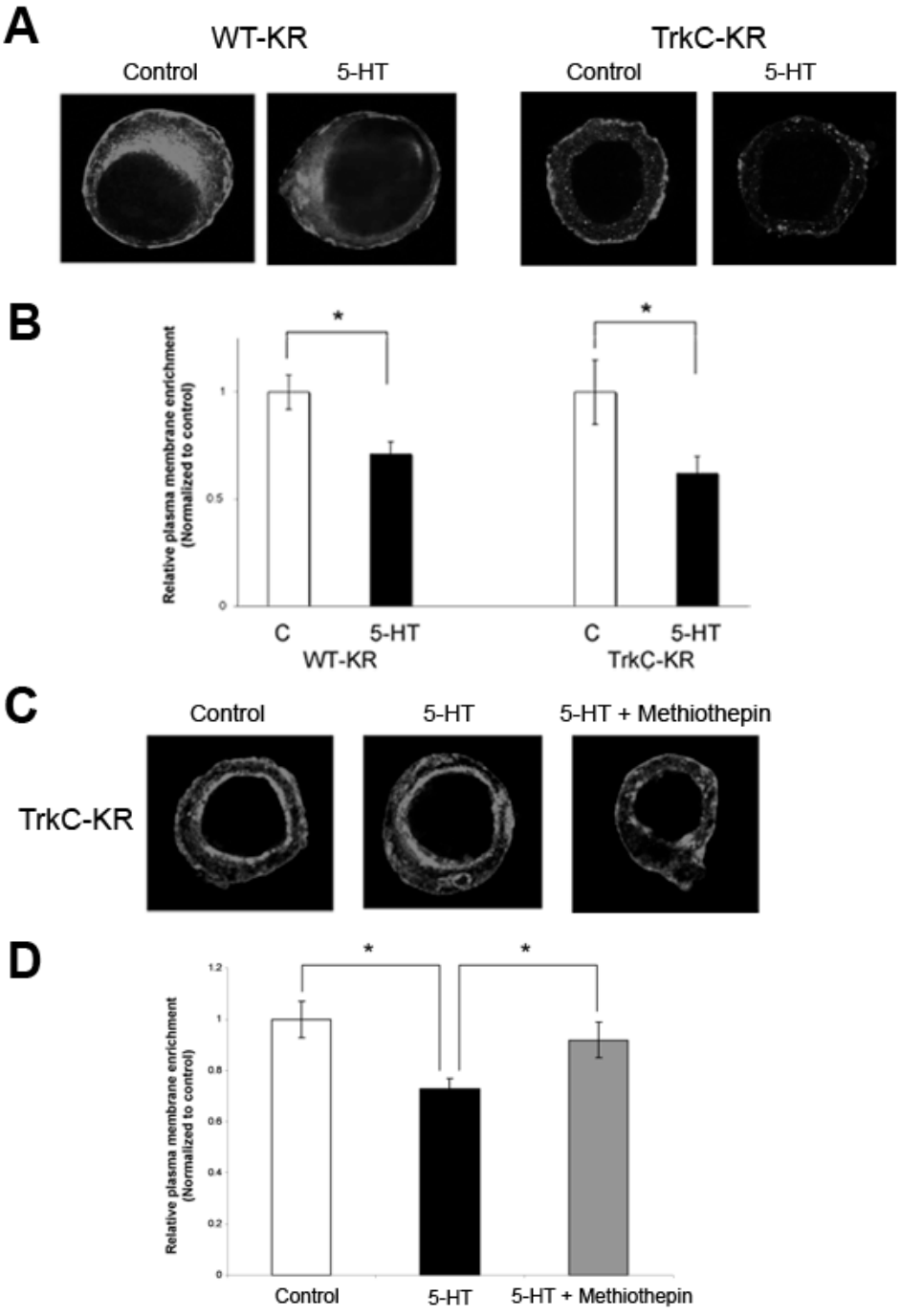

A
WT-KR
Control
5-HT
TrkC-KR
Control
5-HT
B
Relative plasma membrane enrichment (Normalized to control)
1
0.5
0
*
*
C
5-HT
WT-KR
C
5-HT
TrkC-KR
C
Control
5-HT
5-HT + Methiothepin
TrkC-KR
D
Relative plasma membrane enrichment (Normalized to control)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
5-HT
5-HT + Methiothepin

Figure 3. 5

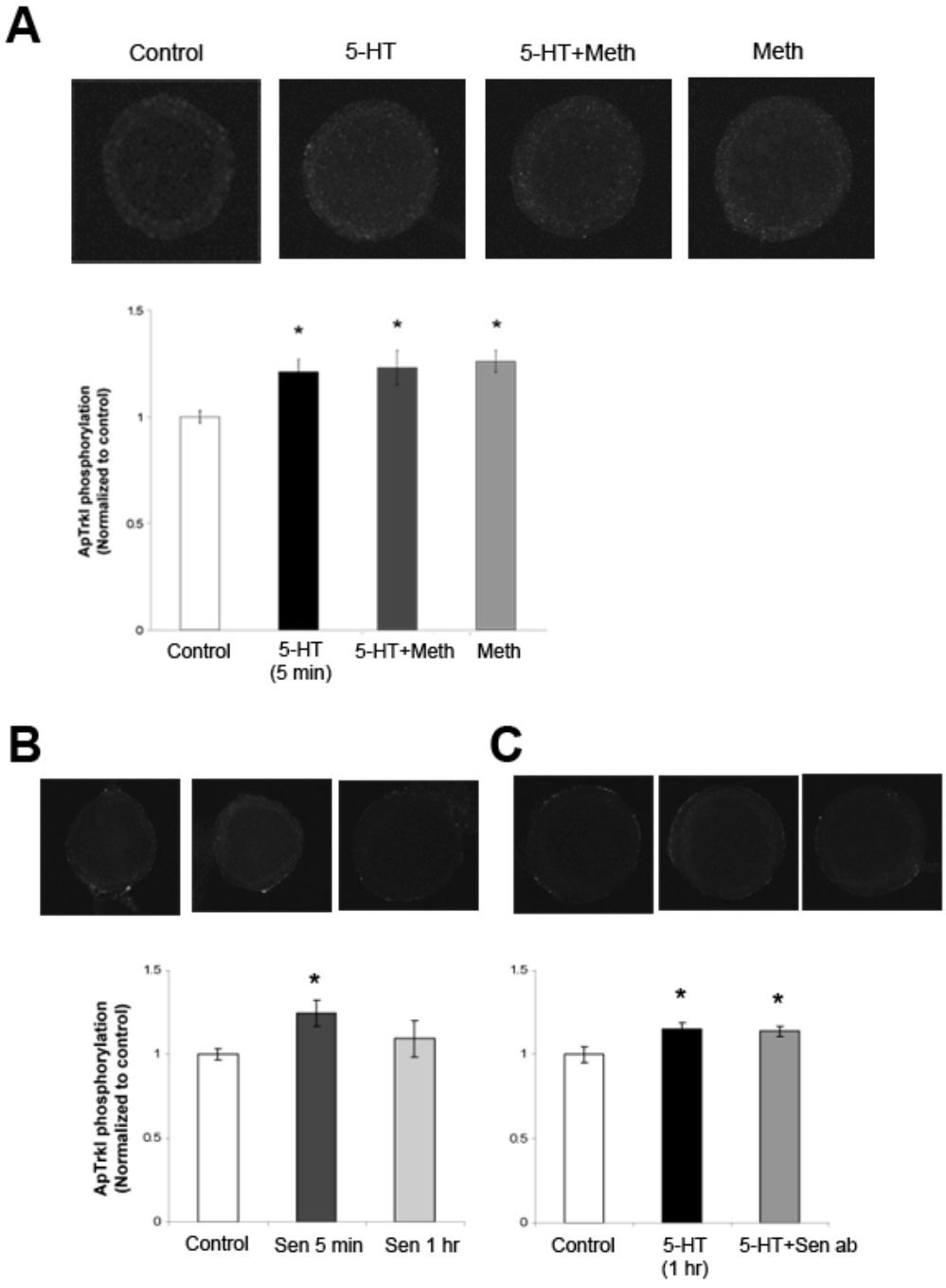

A
Control
5-HT
5-HT+Meth
Meth
ApTrkI phosphorylation (Normalized to control)
1.5
1
0.5
0
*
*
*
Control
5-HT (5 min)
5-HT+Meth
Meth
B
C
ApTrkI phosphorylation (Normalized to control)
1.5
1
0.5
0
*
Control
Sen 5 min
Sen 1 hr
1.5
1
0.5
0
*
*
Control
5-HT (1 hr)
5-HT+Sen ab

Figure 3. 6

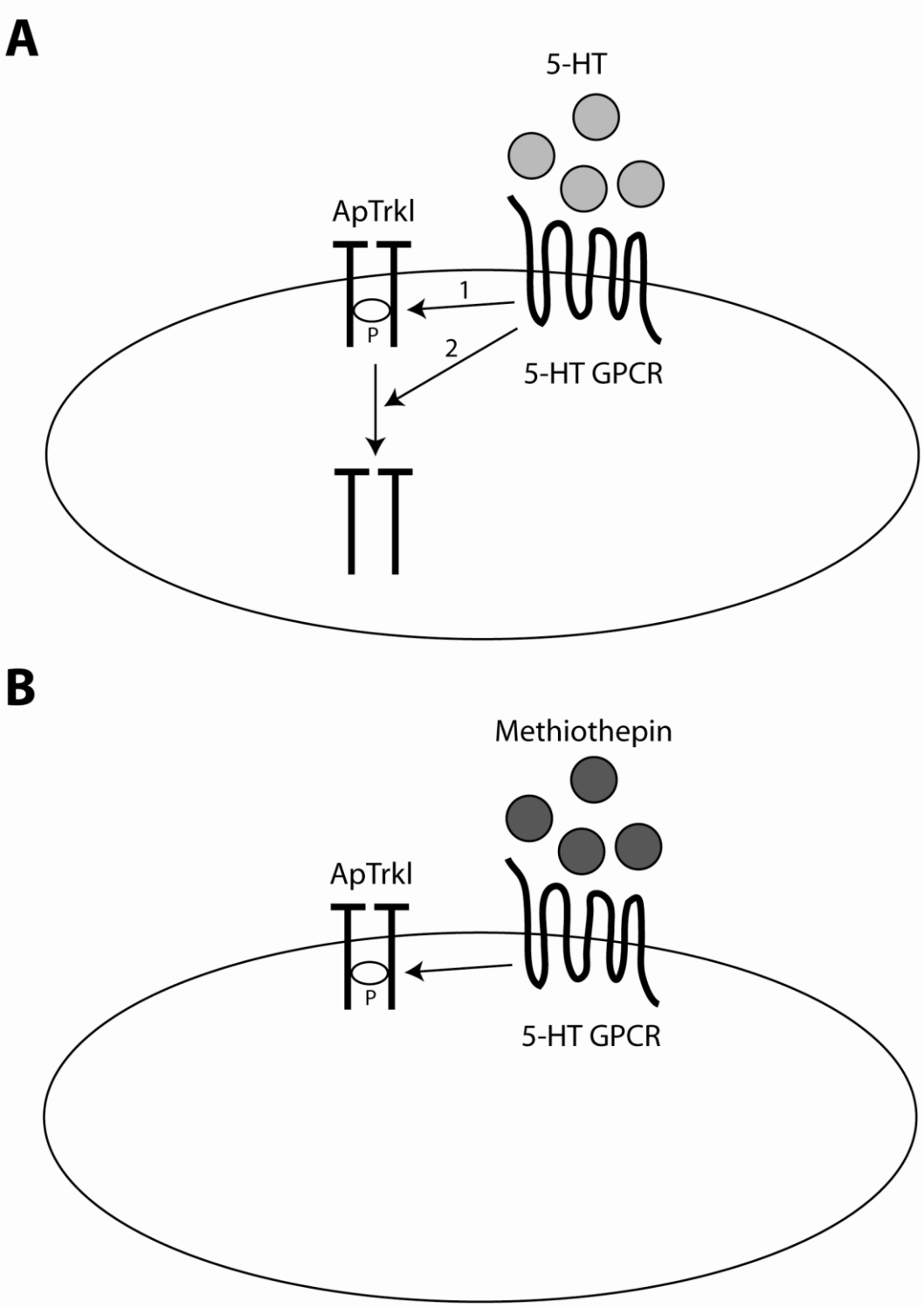


Figure Legends

Figure 3. 1. Chimeric ApTrkl constructs and definition of the plasma membrane, juxtamembrane, and cytoplasm. **A:** Schematic picture of chimeric ApTrkl constructs with extracellular TrkC illustrating the placement of mutations and the site of mRFP insertion. The Y-W (tyrosine to tryptophan) mutation is in the NPxY signaling motif of the juxtamembrane domain, K-R (lysine to arginine) is in the kinase domain, and mRFP is inserted in the middle of the C-terminal extension, leaving a possible PDZ interaction site at the carboxy-terminus intact. **B:** Quantification of ApTrkl localization. Definitions of intracellular boundaries used in this study are demonstrated. The plasma membrane, shown in green, was defined as 2-5 pixels away from the outline of each neuron, as identified by IPPlot (Scanalytics, Inc.; see the Chapter Two Methodology 2. 5). The juxtamembrane, shown in blue, was defined as 7-12 pixels from the outline of the neuron, and the cytoplasm, shown in yellow, was defined as 15-25 pixels from the outline of the neuron. Quantification was done automatically using the IPLab program (Scanalytics, Inc.) in the same way for all the neurons quantified (see the Chapter Two Methodology 2. 5 for details).

Figure 3. 2. Overexpression of wild-type ApTrkl leads to a large intracellular pool of the receptor and activation at the plasma membrane. **A:** WT- and TrkC-WT constructs were injected into sensory neurons. The neurons were fixed (1 or 2 days later) and immunostained with an antibody specific for the phosphorylated

ApTrkl. Confocal images were taken near the center of the cell. The representative figure shows localization of the receptors (mRFP) distributed throughout the cell and not enriched near the membrane, whereas the phosphorylated receptors (green fluorescence) were more localized at the plasma and juxtamembrane relative to the cytoplasm. **B:** Quantification of **A**. Enrichment of the receptors on the plasma membrane relative to the cytoplasm was measured for the total and phosphorylated receptors (WT-WT, n = 26; TrkC-WT, n = 13).

Figure 3. 3. Kinase activity, but not tyrosine phosphorylation at the NPxY site, is required for the presence of a large intracellular pool of ApTrkl. **A:** WT-KR and TrkC-KR constructs were injected into sensory neurons. The neurons were fixed (1 or 2 days later) and immunostained with an antibody specific for the phosphorylated ApTrkl. At this laser power (similar to that used for the other overexpressed receptors), phosphorylation was not detectable using the phospho-specifc antibody to ApTrkl. The representative image shows enrichment of the overexpressed receptor (mRFP) on the plasma membrane. **B:** Overexpression of WT- and TrkC-YW constructs shows localization and phosphorylation patterns similar to the WT constructs (Fig. 3. 2. A). That is, more receptors were localized internally, whereas the phosphorylated receptors were more localized at the plasma membrane, although the level of phosphorylation was much smaller in the YW constructs. **C:** Quantification and comparison of mRFP at the plasma membrane relative to the cytoplasm (left) and the juxtamembrane (right) in TrkC-KR (n = 15), -WT (n = 13), and -YW (n = 38) constructs. TrkC-KR was significantly more localized at the plasma membrane than were the other

constructs for both the cytoplasmic ratio (Tukey's post hoc test after one-way ANOVA, $P < .001$ for TrkC-KR compared with either TrkC-WT or TrkC-YW) and the juxtamembrane ratio (Tukey's post hoc test after one-way ANOVA, $P < .001$ for TrkC-KR with either TrkC-WT or TrkC-YW).

Figure 3. 4. 5-HT induces kinase activity- and ligand-independent internalization of ApTrkl. **A:** 5-HT treatment (5 min) induced internalization of the KR mutant receptors at the plasma membrane, which was not through the involvement of a ligand because this was observed both for WT- and TrkC-KR constructs. **B:** Quantification of **A**. Relative plasma membrane enrichment of the receptors (compared with the cytoplasm) with or without 5-HT treatment (control) was calculated and normalized to the control group. For the WT constructs: Control (C), n = 12; 5-HT, n = 14; $P < .01$ (Student's t test). For the TrkC constructs: Control (C), n = 15; 5-HT, n = 12; $P < .05$ (Student's t-test). **C:** Cells overexpressing TrkC-KR were treated either with 5-HT or with 5-HT and methiothepin for 5 min. Addition of methiothepin blocked 5-HT-induced internalization of TrkC-KR receptors. **D:** Quantification of **C**. Relative plasma membrane enrichment of the receptors (compared with the cytoplasm) shows that 5-HT-induced internalization of TrkC-KR was blocked by the addition of methiothepin (Control, n = 18; 5-HT, n = 15; 5-HT + Methiothepin, n = 19); Tukey's post hoc test after one-way ANOVA, $P < .01$ between Control and 5-HT, $P < .05$ between 5-HT and 5-HT + Methiothepin.

Figure 3. 5. Activation of endogenous ApTrkl. **A:** Phosphorylation of endogenous ApTrkl was examined for control (n = 62), treatment with 5-HT (n = 61), treatment with 5-HT and methiothepin (5-HT+Meth; n = 36), or treatment with methiothepin only (Meth; n = 45). All treatments were done for 5 min. Top: Staining for the phosphorylated ApTrkl. Note that the endogenous staining was much smaller than the staining for the overexpressed receptors (Fig. 3. 2. A). Bottom: Quantification of ApTrkl phosphorylation normalized to control; Tukey's post hoc test, $P < .05$ between control and all other groups. **B:** Treatment with sensorin peptide (Sen). Top: Staining for the phosphorylated ApTrkl. Phosphorylation of ApTrkl was examined for control (left; n = 68), treatment with Sen for 5 min (middle; n = 38), or treatment with Sen for 1 hr (right; n = 59). Bottom: Quantification of ApTrkl phosphorylation normalized to control; Student's t-test, $P < .01$ between control and Sen 5 min. **C:** Treatment with sensorin antibody (Sen ab). Top: Staining for the phosphorylated ApTrkl. Phosphorylation of ApTrkl was examined for control (left; n = 35), treatment with 5-HT for 1 hr (middle; n = 46), or treatment with 5-HT and Sen ab for 1 hr (right; n = 73). Bottom: Quantification of ApTrkl phosphorylation normalized to control; Tukey's post hoc test after one-way ANOVA, $P < .05$ for both between control and 5-HT and between control and 5-HT+Sen ab. The antibody was either applied 0-5 min before 5-HT application or preincubated for 1 hr before 5-HT application (and stayed with 5-HT). As there was no difference between these conditions, data from both experiments were pooled in 5-HT+Sen ab.

Figure 3. 6. Model for ApTrkl regulation through 5-HT GPCRs. **A:** 5-HT transactivates ApTrkl through 5-HT GPCRs (1). 5-HT also induces internalization of ApTrkl from the plasma membrane independent of activation of ApTrkl (2). **B:** Methiothepin can transactivate ApTrkl through 5-HT GPCRs occluding the effect of 5-HT.

CHAPTER FOUR

Identification of 5-HT GPCRs that can activate PKC Apl II

4. 1. Introduction

Aplysia is a powerful model system to reveal molecular events that control synaptic plasticity underlying learning and memory. There are three simple forms of non-associative learning studied in *Aplysia*; sensitization, habituation, and dishabituation of the gill-, tail-, or siphon-withdrawal reflex. Experimentally, sensitization of the withdrawal reflex is achieved by giving an aversive shock to a part of the body such as the tail, which produces a stronger reflex to a previously innocuous stimulation (Pinsker et al., 1973). Habituation is, on the other hand, achieved when the stimulus is repeatedly presented, which decreases the reflexive response. Dishabituation takes place when a stronger or noxious stimulation is applied after once habituating the animal, which restores a stronger response. These behaviors can be replicated in culture of sensory neuron (SN) to motor neuron (MN) synapses. The cellular analog for sensitization is synaptic facilitation induced by the application of 5-HT to a SN-MN synapse, and habituation can be replicated in culture as synaptic depression induced by low frequency stimulation (Castellucci et al., 1970; Pinsker et al., 1970). This synaptic depression can be reversed by 5-HT application, called reversal of synaptic depression, and is thought to underlie dishabituation (Hochner et al., 1986). Interestingly, the mechanism for the reversal of depression is distinct from that for

the facilitation at previously non-depressed synapses (sensitization), as the former is mediated by protein kinase C (PKC) while the latter is dependent on protein kinase A (PKA) (Braha et al., 1990; Goldsmith and Abrams, 1991; Ghirardi et al., 1992).

PKC is a family of serine/threonine kinases that mediate a wide variety of cellular processes including synaptic plasticity. In vertebrates, there are four families of PKC isoforms: classical PKCs (cPKCs; α, β, and γ), novel type I PKCs (nPKCs; ε and η), novel type II PKCs (δ and θ), and atypical PKCs (aPKCs; ζ and ι) (Sossin, 2007). The cPKCs are activated by Ca^{2+} and diacylclycerol (DAG), while the nPKCs are activated by DAG alone and thus Ca^{2+}-independent and the aPKCs are activated by neither Ca^{2+} nor DAG (Sossin, 2007). In *Aplysia*, three isoforms of PKCs have been identified; PKC Apl I (cPKC), PKC Apl II (nPKC), and PKC Apl III (aPKC) (Kruger et al., 1991; Bougie et al., 2009). Among these PKCs, Ca2+-independent PKC Apl II is required for 5-HT-induced reversal of synaptic depression (Manseau et al., 2001). PKC Apl II is translocated by 5-HT in *Aplysia* SNs (Zhao et al., 2006). Previous experiments in a heterologous system, SF9 cells, demonstrated that PKC Apl II is translocated by phosphatidic acid (PA), which is produced by phospholipase D (PLD), as well as by DAG produced by phospholipase C (PLC) (Zhao et al., 2006; Farah et al., 2008). Moreover, translocation of PKC Apl II in *Aplysia* SNs was inhibited by the application of the either PLC or PLD inhibitor (Farah et al., 2008), suggesting that there are multiple signaling pathways leading to the activation of PKC Apl II. However, little is known about the coupling of 5-HT G protein-coupled receptors (GPCRs) to PKC Apl II activation. In particular, 5-HT

GPCRs that are coupled to the PLC or PLD pathway have not been identified in *Aplysia*. The requirement of both the PLC and PLD pathways brings into question whether multiple GPCRs are required to activate PKC Apl II.

5-HT is a major facilitatory neurotransmitter in *Aplysia* and a crucial mediator of synaptic facilitation. Despite its critical role in synaptic plasticity, the receptors for 5-HT have not been well examined in *Aplysia*. In particular, 5-HT GPCRs are known to be critically involved in facilitating synaptic plasticity by inducing activation of second messengers such as PKA and PKC. In vertebrates, 5-HT_1 and 5-HT_5 receptors are negatively coupled to adenylyl cyclase (AC) leading to the inhibition of PKA, while 5-HT_4, 5-HT_6, and 5-HT_7 receptors lead to the activation of PKA through the increased production of cAMP (Raymond et al., 2001; Barbas et al., 2003). 5-HT_2 receptors activate PLC, resulting in the generation of DAG and inositol triphosphate (IP_3) (Conn and Sanders-Bush, 1986). DAG is bound to the plasma membrane and activates PKC, and IP_3 leads to the release of Ca^{2+} from intracellular stores (Nichols, 2004). In *Aplysia*, there were only two 5-HT GPCRs that had been identified at the time the current study was started, called 5-HT_{ap1} and 5-HT_{ap2} that are both negatively coupled to AC (Angers et al., 1998; Barbas et al., 2002).

The current study was aimed to find 5-HT GPCRs that are coupled to PKC Apl II activation. To this end, the first part was to identify all 5-HT GPCRs, which led to the successful cloning of the orthologues for the mammalian AC-coupled 5-HT_4 (5-HT_{4Ap}) and 5-HT_7 (5-HT_{7Ap}) and PLC-coupled 5-HT_2 (5-HT_{2Ap}). A receptor almost identical to 5-HT_{7Ap} was also cloned and reported from *Aplysia Kurodai* (Lee et al., 2009). However, our cloning was an independent

attempt from *Aplysia Californica*. The second part was to find the receptor(s) involved in the translocation of PKC Apl II.

4. 2. Results

4. 2. 1. Molecular cloning and structural analysis of 5-HT_{2Ap}, 5-HT_{4Ap}, and 5-HT_{7Ap}

In order to identify all 5-HT receptors other than previously cloned 5-HT_{ap1} and 5-HT_{ap2}, we performed comprehensive bioinformatics screening for both vertebrate and invertebrate 5-HT receptors. As a result, we found *Aplysia* genomic DNA sequences homologous to *Lymnaea* 5-HT_2, putative *Lottia* 5-HT_4, and *Helisoma* 5-HT_7 receptors. We first obtained putative *Aplysia* sequences for 5-HT_{2Ap}, 5-HT_{4Ap} and 5-HT_{7Ap} based on the homology to *Lymnaea* 5-HT_2, *Lottia* 5-HT_4, and *Helisoma* 5-HT_7 receptor sequences, respectively (see the Chapter Two Methodolgy 2.6 for details). For isolating all these receptors, PCR reactions were performed from *Aplysia* nervous system cDNA using oligonucleotide primers derived from the putative sequences.

For isolating 5-HT_{2Ap}, five overlapping clones (5-HT_{2Ap}.1, 5-HT_{2Ab}.2, 5-HT_{2Ap}.3, 5-HT_{2Ap}.4, and 5-HT_{2Ap}.5) covering 2,976 bp of the 5-HT_{2Ap} sequence were amplified and cloned by overlap PCR (Fig. 4. 1. A). Alternative splicing of 660 bp was found in the clone 5-HT_{2Ap}.2, which corresponds to the third intracellular loop. Thus, spliced and non-spliced (full-length) forms of 5-HT_{2Ap} exist in the *Aplysia* nervous system. However, there was difficulty in

reconstituting the full-length receptor, and we were only successful in cloning the spliced form, which we call 5-HT_{2Ap}. The spliced region of the receptor shares some homology with *Lymnaea* 5-HT_2 and contains several repetitive amino acid sequences (Fig. 4. 1. C). 5-HT_{2Ap} contains an open reading frame (ORF) that encodes a protein of 992 amino acids with a predicted molecular weight of 108 kDa (Fig. 4. 1. B). 5-HT_{2Ap} has a very long N-terminal of more than 1.2k bp before the first transmembrane domain and also a long third intracellular loop, both of which are not well conserved. Because of almost no conservation in the 5'UTR, we based our 5'UTR and starting methionine assignment on a stop codon prior to the 5'UTR. The homology within the seven putative transmembrane domains and adjacent sequences is: 78% to *Lymnaea* 5-HT_2, 62% to *Lottia* 5-HT_2, 40% to rat 5-HT_{2A}, and 38% to rat 5-HT_{2B} and 5-HT_{2C}. Phylogenetic analysis with other invertebrate and mammalian 5-HT receptors revealed that the cloned 5-HT_{2Ap} belongs within the PLC-coupled 5-HT_2 family of receptors (Fig. 4. 3). Ten potential N-glycosylation sites were found in the extracellular N-terminus, and five predicted sites for PKC phosphorylation and four sites for PKA were found in the third intracellular loop. One additional PKA and two additional PKC sites were found in the C-terminal intracellular tail. In the spliced region in the third intracellular loop, there were one PKA and three PKC sites. For predicting PKA and PKC sites, we used the GPS 2.1 software (The CUCKOO Workgroup) with high threshold.

For isolating 5-HT_{4Ap}, three overlapping clones (5-HT_{4Ap}.1, 5-HT_{4Ap}.2, and 5-HT_{4Ap}.3) covering 1,152 bp of the 5-HT_{4Ap} sequence were amplified and cloned by overlap PCR (Fig. 4. 2. A). The putative ORF of 5-HT_{4Ap} encodes a

protein of 384 amino acids with a predicted molecular weight of 43 kDa (Fig. 4. 2. B). The homology within the seven putative transmembrane domains and adjacent sequences is: 68% to *Lottia* 5-HT_4 and 44% to human 5-HT_4. Phylogenetic analysis with other invertebrate and mammalian 5-HT receptors revealed that the cloned 5-HT_{4Ap} belongs within the AC-coupled 5-HT_4 family of receptors (Fig. 4. 3). Two potential N-glycosylation sites were found in the extracellular N-terminus. However, there was no predicted PKA or PKC site using the same criteria mentioned above.

5-HT_{7Ap} was obtained by single PCR reaction, and the putative 1,455 bp ORF encodes a protein of 485 amino acids with a predicted molecular weight of 54 kDa (Fig. 4. 2. C and D). The homology within the seven putative transmembrane domains and adjacent sequences is: 77% to *Helisoma* 5-HT_7, 43% to *Drosophila* 5-HT_7, and 42% to rat 5-HT_7. Phylogenetic analysis with other invertebrate and mammalian 5-HT receptors revealed that the cloned 5-HT_{7Ap} belongs within the AC-coupled 5-HT_7 family of receptors (Fig. 4. 3). Six potential N-glycosylation sites were found in the extracellular N-terminus. Two predicted PKA and four PKC sites were found in the third intracellular loop. One additional PKA site was found in the C-terminal intracellular tail.

All three receptors have seven putative transmembrane domains characteristic of GPCRs and display several key features common to mammalian and invertebrate 5-HT receptors. In particular, the tripeptide DRY at the interface of the third transmembrane domain and the second intracellular loop required for G protein coupling are conserved for 5-HT_{4Ap} and 5-HT_{7Ap}. Interestingly, instead of DRY, ERY appears at the end of the third transmembrane domain, which is

conserved in *Lymnaea* 5-HT_2. For all the receptors, the NPXXY motif required for receptor desensitization and internalization in the seventh transmembrane domain is conserved.

4. 2. 2. Expression of endogenous 5-HT receptors in *Aplysia* neurons and production of eGFP-tagged constructs

To examine the expression of all *Aplysia* 5-HT receptors identified, single cell RT-PCR analysis was performed separately from SNs and LFS MNs, which are involved in synaptic facilitation as well as behavioral sensitization and dishabituation. This included the previously cloned 5-HT_{ap1} and 5-HT_{ap2}, which are negatively coupled to AC, and we call 5-HT_{1aAp} and 5-HT_{1bAp}, respectively, for the purpose of consistency with other receptors. The results showed that 5-HT_{2Ap}, 5-HT_{7Ap}, and 5-HT_{1aAp} are expressed in both SNs and MNs (Fig. 4. 4. A). For 5-HT_{2Ap}, both the spliced and non-spliced form of receptors were expressed in SNs (data not shown). 5-HT_{1bAp} is slightly expressed only in MNs, and 5-HT_{4Ap} is not expressed in both neuron types (Fig. 4. 4. A). Since PKC Apl II is translocated in SNs but not in MNs (Zhao et al., 2006), we decided to further examine the receptors that are expressed in SNs; 5-HT_{2Ap}, 5-HT_{7Ap}, and 5-HT_{1aAp}, which we call 5-HT_{1Ap} from the following sections.

In order to image the overexpressed receptors, eGFP-tagged constructs were produced by replacing the N-terminal of the receptors with eGFP (Fig. 4. 4. B). The long N-terminal seems to interfere with high expression of the receptor in 5-HT_{2Lym}, and the N-terminal truncated mutant, Δ*N*-5-HT_{2Lym}, expresses high

levels of the receptor protein (Gerhardt et al., 1996). However, N-terminus secondary structure of a GPCR is suggested to be required for targeting into the endoplasmic reticulum (ER), and therefore replacing its N-terminal with eGFP might not lead to successful surface expression of the receptor. This problem was solved by adding an artificial signal sequence at the N-terminus, which led to efficient surface expression of the cannabinoid CB_1 receptor (McDonald et al., 2007). Therefore, the signal sequence of the neuropeptide sensorin was placed in front of eGFP at the 5' end in order to induce insertion of the receptors into ER (Fig. 4. 4. B). The resulting constructs, eGFP-$5HT_{1Ap}$, -$5HT_{2Ap}$, and -$5HT_{7Ap}$, were expressed in SF9 cells and *Aplysia* SNs. Strong plasma membrane localization of the receptor was observed in eGFP-$5HT_{1Ap}$ and -$5HT_{7Ap}$ expressed cells, while more internal cytoplasmic localization was observed in eGFP-$5HT_{2Ap}$ expressed cells (Fig. 4. 4. C), suggesting that there is constitutive internalization of 5-HT_{2Ap} when overexpressed.

4. 2. 3. Translocation of PKC Apl II in SF9 cells by 5-HT_{2Ap} and 5-HT_{7Ap}

In order to determine the ability of 5-HT receptors to translocate PKC Apl II, we used a SF9 cell system where there believed to be no endogenous 5-HT receptors. In fact, PKC Apl II cannot be translocated by 5-HT without the expression of receptors in SF9 cells (Fig. 4. 5). Translocation of PKC Apl II in this system is a good assay to determine the coupling of the receptors to PKC and has been successfully used in the previous studies (Zhao et al., 2006; Farah et al., 2008). SF9 cells were coexpressed with either eGFP-5-HT_{1Ap}, eGFP-5-HT_{2Ap}, or

eGFP-5-HT_{7Ap} and mRFP-PKC Apl II, and the ability of translocating PKC Apl II was examined by the addition of 5-HT. Overexpression of 5-HT_{2Ap} and 5-HT_{7Ap} led to a significant translocation of PKC Apl II after 5-HT treatment compared to pre-treatment ($P < .05$; Fig. 4. 5). Overexpression of 5-HT_{1Ap}, however, did not translocate PKC Apl II (Fig. 4. 5).

It was somewhat surprising that not only 5-HT_{2Ap}, but also 5-HT_{7Ap} was able to translocate PKC Apl II. One possibility is that while 5-HT_{2Ap} translocates PKC Apl II by the PLC pathway through the production of DAG, 5-HT_{7Ap} may be able to translocate PKC Apl II by the PLD pathway through the production of PA. To test this, we used the PLC inhibitor U-73122 and the PLD inhibitor 1-butanol. 5-HT-induced translocation of PKC Apl II in 5-HT_{2Ap} overexpressed cells was blocked by the application of U-73122, although 1-butanol did not block the translocation ($P < .01$ between control and U-73122, Tukey's post hoc test; Fig. 4. 6. A). On the other hand, 5-HT-induced translocation of PKC Apl II in 5-HT_{7Ap} overexpressed cells was completely blocked by the addition of either U-73122 or 1-butanol ($P < .05$ between control and U-73122, $P < .01$ between control and 1-butanol, Tukey's post hoc test; Fig. 4. 6. A). Although the difference did not reach significance, the level of translocation was decreased in 5-HT_{2Ap} overexpressed cells with 1-butanol treatment, suggesting that 5-HT_{2Ap} might also be able to signal through PLD. We have previously shown that low concentrations of DAG and PA, which do not normally cause the translocation of PKC Apl II on their own, can synergistically translocate PKC Apl II when combined (Farah et al., 2008). If 5-HT_{2Ap} only activates PLC but not PLD, addition of low concentration of PA would synergize with DAG produced by PLC and cause increase in the

translocation of PKC Apl II. Indeed, addition of 2.5-5 μg/mL of DiC8-PA (a cell-permeable PA), which corresponds to the highest concentration at which no translocation is observed (Farah et al., 2008), significantly increased the level of 5-HT-induced PKC Apl II translocation in 5-HT_{2Ap} overexpressed cells ($P < .05$; Fig. 4. 6. B). The same treatment did not further increase the translocation in 5-HT_{7Ap} overexpressed cells (Fig. 4. 6. B), likely because the synergism is already present. Taken together, these results suggest that the translocation of PKC Apl II by 5-HT_{2Ap} requires PLC activation, whereas that by 5-HT_{7Ap} requires both PLC and PLD activation. However, it is unlikely that 5-HT_{7Ap} is directly coupled to PLC (Lee et al., 2009). There is endogenous DAG in SF9 cells (Farah et al., 2008). Due to the synergism, low concentrations of endogenous DAG may be sufficient in the presence of PA activated by 5-HT_{7Ap}. This constitutive DAG may still be blocked by U-73122. Therefore, we suggest a model in which 5-HT_{2Ap} translocates PKC Apl II by the PLC pathway, whereas 5-HT_{7Ap} does so by the PLD pathway in synergism with endogenous DAG (Fig. 4. 7).

4. 2. 4. Requirement of 5-HT_{2Ap} for PKC Apl II translocation in SNs

Previously, it was reported that reversal of synaptic depression was blocked by spiperone (Dumitriu et al., 2006), which is known to be an antagonist for 5-HT_2 receptors in mammals. Methiothepin, however, failed to block reversal of synaptic depression, although it was partially inhibited (Dumitriu et al., 2006). Methiothepin has been shown to be an effective antagonist for 5-HT_{apAC1} (Lee et

al., 2009). However, methiothepin is known to block 5-HT GPCRs other than AC-coupled receptors (Angers et al., 1998; Barbas et al., 2002; Cohen et al., 2003). In fact, we have previously observed that the translocation of PKC Apl II is blocked by methiothepin in SNs (data not shown), suggesting that methiothepin might also antagonize the receptors coupled to PLC. Moreover, 5-HT_{apAC1} was not shown to be required for reversal of synaptic depression at highly depressed synapses (Lee et al., 2009). Therefore, we hypothesized that reversal of synaptic depression is mediated through 5-HT_{2Ap} by coupling to PKC Apl II, which is blocked by spiperone. In order to test this hypothesis, we first examined if spiperone blocks 5-HT-induced PKC Apl II translocation in SF9 cells expressing 5-HT_{2Ap}. Surprisingly, spiperone failed to block the translocation (Fig. 4. 8. A), suggesting that spiperone is not an effective antagonist for 5-HT_{2Ap}. Moreover, spiperone did not block 5-HT-induced translocation of PKC Apl II in SNs nor 5-HT-induced reversal of depression at SN-MN synapses (Fig. 4. 8. A, B and C). Inconsistent with the previous study, our data suggests that spiperone is not coupled to PKC Apl II nor required for reversal of synaptic depression. The discrepancy between the previous and our studies might have come from the different protocols: we used cultured SN-MN synapses and SNs were from pleural ganglia, while Dumitriu et al. (2006) used preparation from desheathed ganglia and SNs from abdominal ganglia.

We tested other potential antagonists for 5-HT_{2Ap} and found that pirenperone and SB 200646 blocked the translocation of PKC Apl II mediated by 5-HT_{2Ap} ($P < .05$ for both antagonists compared to 5-HT alone) (Fig. 4. 8. D). However, there was still significant translocation of PKC Apl II even with the

addition of these antagonists ($P < .01$ for all three groups), suggesting that these antagonists cannot completely block the 5-HT_{2Ap} signaling. Contradictory to our hypothesis, one of the antagonists, pirenperone failed to block the translocation of PKC Apl II in SNs (Fig. 4. 8. D). Instead, these results imply that 5-HT_{2Ap} is not required for PKC Apl II translocation in SNs.

4. 2. 5. Involvement of tyrosine kinase signaling in PKC Apl II translocation and reversal of synaptic depression

The vertebrate homolog of PKC Apl II, PKC-epsilon of nPKCs can be translocated by PLC-epsilon (Oestreich et al., 2009). PLC-epsilon is activated by Ras (Sossin and Abrams, 2009), which can be activated by receptor tyrosine kinases. ApTrkl is a Trk-like receptor in *Aplysia* that is expressed in SNs and activated by 5-HT, and has a site leading to the activation of Ras (Ormond et al., 2004). In fact, translocation of PKC Apl I, mediating intermediate-term facilitation and memory (ITF and ITM), was shown to be blocked by inhibiting tyrosine kinase activities (Shobe et al., 2009). Therefore, it is an intriguing possibility that the translocation of PKC Apl II is mediated in part through tyrosine kinase activities, in particular, through ApTrkl, which has been shown to be regulated by 5-HT GPCRs (Chapter Three).

Interestingly, genistein, a general tyrosine kinase inhibitor, significantly decreased 5-HT-induced translocation of PKC Apl II is SNs ($P < .05$; Fig. 4. 9. A and B). Moreover, genistein also inhibited 5-HT induced reversal of depression at SN-MN synapses ($P < .05$; Fig. 4. 9. C and D). However, inhibition of the

translocation and reversal of synaptic depression by genistein was not complete and there was still a significant effect ($P < .01$; for both translocation and reversal of synaptic depression). PKC Apl II is only translocated in SNs but not in MNs (Zhao et al., 2006). If PLC-epsilon is a crucial mediator for PKC Apl II translocation, absence of the translocation in MNs might be explained by the lack of PLC-epsilon in MNs. Using single cell RT-PCR, we looked at expression of different isoforms of PLCs available from genomic database; delta, gamma-1, gamma-2, beta, beta-4, and epsilon. Delta, beta and beta-4 isoforms of PLC were expressed in both SNs and MNs (Fig. 4. 9. E). However, PLC-epsilon was not expressed in MNs although it was expressed in SNs (Fig. 4. 9. E). While PLC-gamma-1 and PLC-gamma-2 were not expressed in MNs neither, ApTrkl does not have a conserved site for them (Ormond et al., 2004). Therefore, we suggest another model for the translocation of PKC Apl II where PKC Apl II is translocated by PLC-epsilon, which is activated by ApTrkl through Ras after transactivation of ApTrkl by 5-HT GPCRs (Fig. 4. 10).

4. 3. Discussion

4. 3. 1. Isolation of 5-HT_{2Ap}, 5-HT_{4Ap}, and 5-HT_{7Ap}

In the present study, we isolated three 5-HT receptors from *Aplysia Californica* that belong to the GPCR family. Evolutionary analysis revealed that the cloned receptors, 5-HT_{2Ap}, 5-HT_{4Ap}, and 5-HT_{7Ap} are the mammalian family of PLC-coupled 5-HT_2, AC-coupled 5-HT_4 and 5-HT_7 receptors, respectively.

During our study, cloning of AC-coupled 5-HT_{apAC1} was reported from *Aplysia Kurodai* (Lee et al., 2009). 5-HT_{apAC1} is almost identical to 5-HT_{7Ap} except for a couple of amino acid differences, which might have come from different *Aplysia* species. They showed that 5-HT_{apAC1} is pharmacologically different from the mammalian 5-HT_7 receptor family, possibly due to the evolutionary divergence of 5-HT receptors between vertebrates and invertebrates (Lee et al., 2009). Our evolutionary analysis, however, revealed that 5-HT_{7Ap} is still closer to the mammalian 5-HT_7 receptor than to any other *Aplysia* 5-HT receptors. This is also true to 5-HT_{2Ap} and 5-HT_{4Ap}, which are closer to the mammalian 5-HT_2 and 5-HT_4 receptors, respectively, than to any other *Aplysia* receptors. It is interesting to see that the 5-HT_4 receptor family is closer to dopamine receptors than to any other 5-HT receptor families.

Single cell RT-PCR results showed that 5-HT_{2Ap}, 5-H_{7Ap}, and previously reported 5-HT_{1aAp} (5-HT_{ap1}) are expressed in both SNs and MNs, whereas 5-HT_{1bAp} (5-HT_{ap2}) is slightly expressed only in MNs and 5-HT_{4Ap} is not expressed in SNs nor MNs. This is consistent with the finding that 5-HT_{apAC1} is expressed in both SNs and MNs (Lee et al., 2009). Although not examined at a single cell level, 5-HT_{1aAp} was shown to be expressed in all ganglia (Angers et al., 1998), and 5-HT_{1bAp} mostly in the abdominal ganglion (Barbas et al., 2002), where LFS MNs reside. The lack of 5-HT_{4Ap} in both SNs and MNs might indicate this receptor is not abundant in the CNS and explain why overlap PCR from three separate clones was required to get the entire receptor even though it is smaller in its molecular mass (43 kDa) compared to other receptors.

4. 3. 2. 5-HT receptors coupled to PKC Apl II activation

Since PKC Apl II is translocated in SNs but not in MNs (Zhao et al., 2006), we decided to test 5-HT_{1Ap} (5-HT_{1aAp}), 5-HT_{2Ap}, and 5-HT_{7Ap} that are expressed in SNs for their ability to translocate PKC Apl II. In SF9 cells, overexpression of either 5-HT_{2Ap} or 5-HT_{7Ap} led to the translocation of PKC Apl II after 5-HT treatment, while PKC Apl II was not translocated without the expression of receptors or with overexpression of 5-HT_{1Ap}. The translocation induced by 5-HT_{2Ap} is most likely through activation of PLC, since this receptor is a family of PLC-coupled receptors and the translocation was blocked by U-73122. Moreover, addition of exogenous PA to 5-HT further increased the translocation, suggesting the synergism of PA with DAG activated by 5-HT_{2Ap}. On the other hand, the 5-HT_7 receptor family is not known to be coupled to PLC, and indeed 5-HT_{apAC1} is not coupled to PLC (Lee et al., 2009). However, the translocation induced by 5-HT_{7Ap} was blocked by both U-73122 and 1-butanol. We suggest that U-73122 inhibits endogenous DAG that synergizes with PA activated by 5-HT_{7Ap}, and favor the model that there are multiple receptors coupled to PKC Apl II activation, at least one through the PLC pathway and another through the PLD pathway (Fig. 4. 7). One possibility is that PKC Apl II is activated by Epac, a cAMP activated guanine exchange factor (GEF), through downstream activation of PLC and PLD. Such activation mechanism by Epac has been reported for PKC-epsilon, the vertebrate orthologue of PKC Apl II (Hucho et al., 2005; Oestreich et al., 2009).

4. 3. 3. Requirement of 5-HT_{2Ap} for PKC Apl II translocation

Overexpression of either 5-HT_{2Ap} or 5-HT_{7Ap} was sufficient to mediate translocation of PKC Apl II induced by 5-HT in SF9 cells. In order to examine the requirement of the receptors for the translocation of PKC Apl II, we used an antagonist approach. Since 5-HT_{apAC1} is not required for reversal of synaptic depression, 5-HT_{7Ap} is unlikely to be required for the translocation of PKC Apl II. Also because of the difficulty in finding a specific antagonist to 5-HT_{7Ap} (methiothepin, a known antagonist to the mammalian 5-HT_7 receptor and an effective antagonist to 5-HT_{apAC1}, is also known to block other 5-HT receptors in *Apysia*), we decided to test for 5-HT_{2Ap}. Spiperone, an antagonist to the mammalian 5-HT_2 receptor, has been shown to block reversal of synaptic depression (Dumitriu et al., 2006). However, in our experiments spiperone failed to block reversal of synaptic depression nor did it block translocation of PKC Apl II. Indeed, spiperone did not block the translocation of PKC Apl II mediated by the overexpression of 5-HT_{2Ap} in SF9 cells, suggesting that spiperone does not act as an antagonist to 5-HT_{2Ap}. Consistently, spiperone is not an effective antagonist to 5-HT_{2Lym} (Gerhardt et al., 1996). The reason why spiperone blocked reversal of synaptic depression in the previous study but not in this study is not clear. The previous study recorded reversal of synaptic depression from the desheathed abdominal ganglion (Dumitriu et al., 2006), while we recorded from the cultured pleural SN-MN synapse. Different SNs from abdominal and pleural ganglia might have different 5-HT receptor compositions, and the receptor(s) which spiperone blocks might not be present in pleural SNs. Since spiperone is known to block

dopamine receptors in mammals, it is possible that dopamine receptors, not 5-HT receptors, are required for reversal of synaptic depression. The concentrations of 5-HT used in these experiments are quite high and may begin to activate dopaminergic receptors.

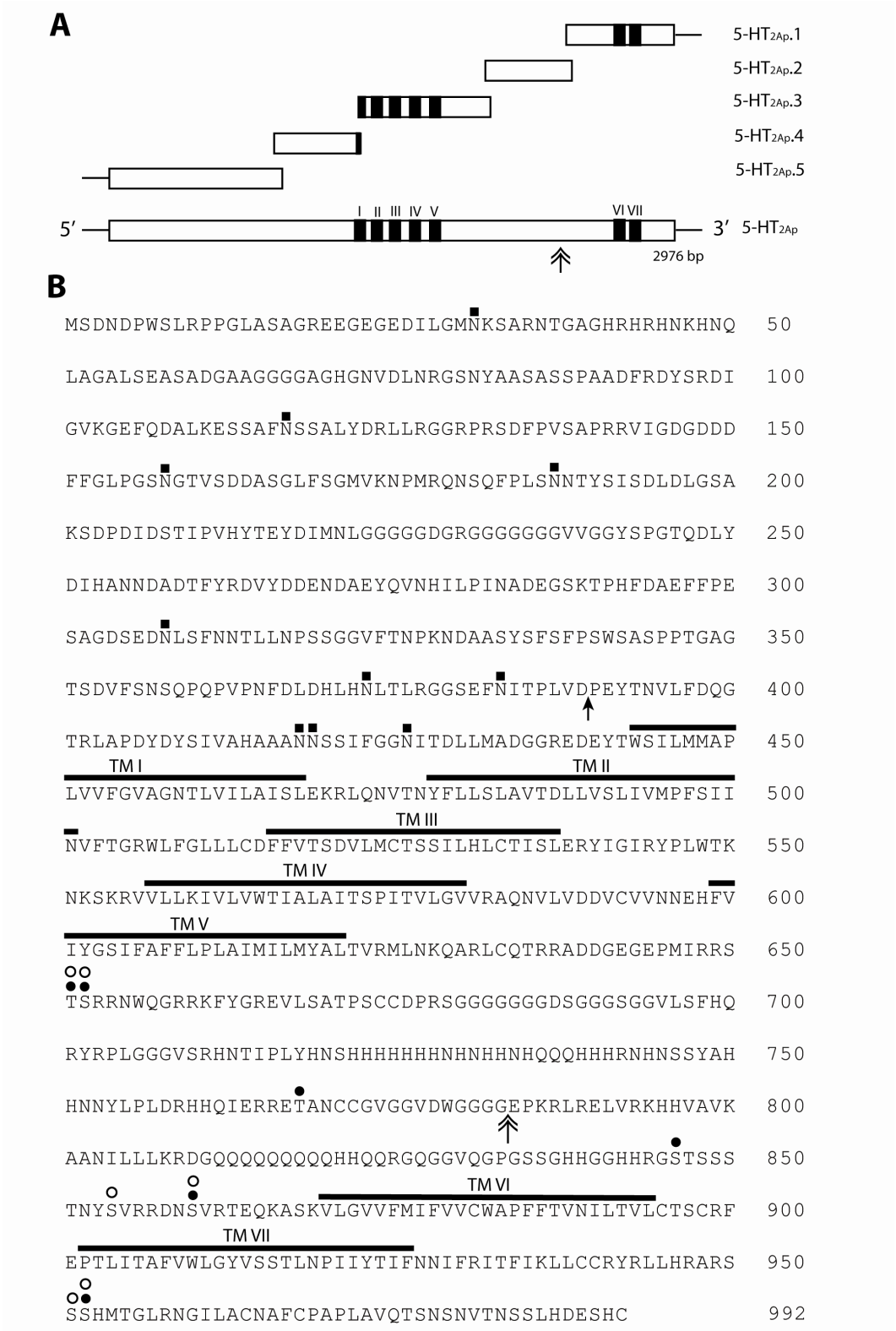
Among several antagonists tested, pirenperone and SB 200646 blocked the translocation of PKC Apl II mediated by 5-HT_{2Ap} in SF9 cells. However, pirenperone did not block the translocation of PKC Apl II in *Aplysia* SNs, suggesting that 5-HT_{2Ap} is not required for the translocation of PKC Apl II in neurons. There may be a flexibility in the system and the blockade of 5-HT_{2Ap} may be compensated by 5-HT_{7Ap}. Alternatively, since the translocation of PKC Apl II was still observed, although small, in SF9 cells overexpressing 5-HT_{2Ap} even with the application of pirenperone, it is possible that the portion or signaling of 5-HT_{2Ap} that is not blocked by pirenperone is enough to cause the translocation of PKC Apl II in neurons. It is important to confirm the data using a different approach such as knockdown of the receptors by siRNA or dsRNA.

4. 3. 4. Role for tyrosine kinase signaling in PKC Apl II activation

Genistein, a general tyrosine kinase inhibitor, inhibited translocation of PKC Apl II as well as reversal of synaptic depression, suggesting a role for tyrosine kinase signaling in PKC Apl II activation. ApTrkl is an *Aplysia* Trk-like receptor that is activated by 5-HT and contains a site that leads to the activation of Ras (Ormond et al., 2004), and one of the PLC isoforms, PLC-epsilon has a Ras binding domain and is activated by Ras (Sossin and Abrams, 2009). PKC Apl II is

only translocated in SNs but not in MNs (Zhao et al., 2006), and we showed that PLC-epsilon is not expressed in MNs although it is expressed in SNs. Therefore, the lack of translocation of PKC Apl II in MNs might be explained by the lack of PLC-epsilon in MNs. It is possible that PKC Apl II is activated by different mechiansms, and we suggest a model where PKC Apl II is activated by PLC-epsilon, which is activated by ApTrkl through Ras (Fig. 4. 9). ApTrkl is transactivated by 5-HT through GPCRs (Chapter Three), and we have preliminary data suggesting that ApTrkl colocalizes with 5-HT_{1Ap} and 5-HT_{7Ap} but not with 5-HT_{2Ap}. Overexpression of 5-HT_{1Ap} might not be able to translocate PKC Apl II in SF9 cells because ApTrkl is not expressed in SF9 cells (Ormond et al., 2004). This opens up a possibility that all three receptors, 5-HT_{1Ap}, 5-HT_{2Ap}, and 5-HT_{7Ap} might somehow be coupled to the activation of PKC Apl II through distinct mechanisms, giving more flexibility to the system.

Figure 4. 1


A
5-HT2Ap.1
5-HT2Ap.2
5-HT2Ap.3
5-HT2Ap.4
5-HT2Ap.5
I II III IV V
VI VII
5′
3′ 5-HT2Ap
2976 bp
B
MSDNDPWSLRPPGLASAGREEGEGEDILGMNKSARNTGAGHRHRHNKHNQ 50
LAGALSEASADGAAGGGGAGHGNVDLNRGSNYAASASSPAADFRDYSRDI 100
GVKGEFQDALKESSAFNSSALYDRLLRGGRPRSDFPVSAPRRVIGDGDDD 150
FFGLPGSNGTVSDDASGLFSGMVKNPMRQNSQFPLSNNTYSISDLDLGSA 200
KSDPDIDSTIPVHYTEYDIMNLGGGGGDGRGGGGGGGVVGGYSPGTQDLY 250
DIHANNDADTFYRDVYDDENDAEYQVNHILPINADEGSKTPHFDAEFFPE 300
SAGDSEDNLSFNNTLLNPSSGGVFTNPKNDAASYSFSFPSWSASPPTGAG 350
TSDVFSNSQPQPVPNFDLDHLHNLTLRGGSEFNITPLVDPEYTNVLFDQG 400
TRLAPDYDYSIVAHAAANNSSIFGGNITDLLMADGGREDEYTWSILMMAP 450
TM I
TM II
LVVFGVAGNTLVILAISLEKRLQNVTNYFLLSLAVTDLLVSLIVMPFSII 500
TM III
NVFTGRWLFGLLLCDFFVTSDVLMCTSSILHLCTISLERYIGIRYPLWTK 550
TM IV
NKSKRVVLLKIVLVWTIALAITSPITVLGVVRAQNVLVDDVCVVNNEHFV 600
TM V
IYGSIFAFFLPLAIMILMYALTVRMLNKQARLCQTRRADDGEGEPMIRRS 650
TSRRNWQGRRKFYGREVLSATPSCCDPRSGGGGGGGDSGGGSGGVLSFHQ 700
RYRPLGGGVSRHNTIPLYHNSHHHHHHHNHNHHNHQQQHHHRNHNSSYAH 750
HNNYLPLDRHHQIERRETANCCGVGGVDWGGGGEPKRLRELVRKHHVAVK 800
AANILLLKRDGQQQQQQQQQHHQQRGQGGVQGPGSSGHHGGHHRGSTSSS 850
TM VI
TNYSVRRDNSVRTEQKASKVLGVVFMIFVVCWAPFFTVNILTVLCTSCRF 900
TM VII
EPTLITAFVWLGYVSSTLNPIIYTIFNNIFRITFIKLLCCRYRLLHRARS 950
SSHMTGLRNGILACNAFCPAPLAVQTSNSNVTNSSLHDESHC 992

C

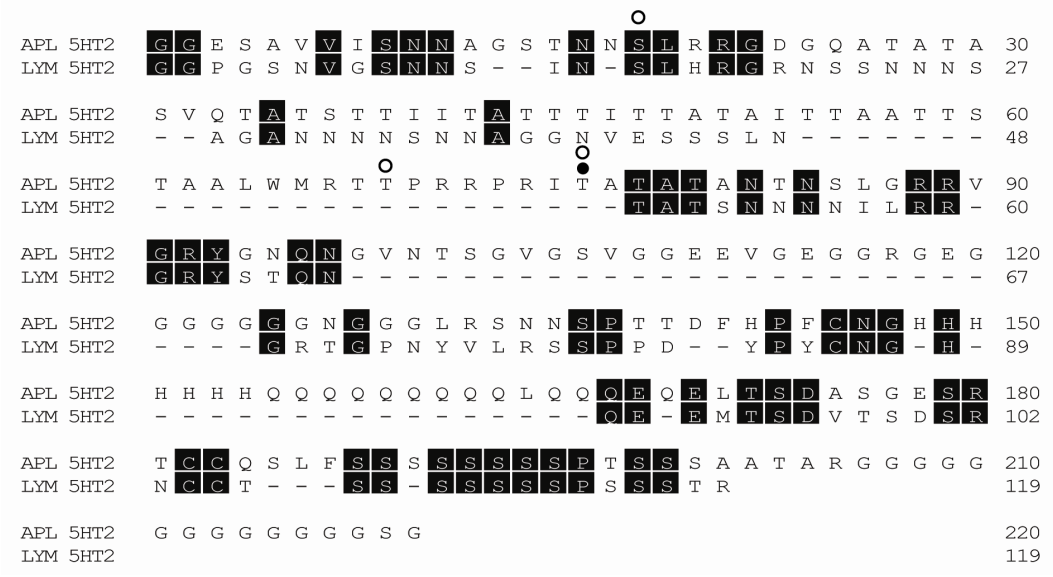

APL 5HT2 GGESAVVISNNAGSTNNSLRRGDGQATATA 30
LYM 5HT2 GGPGSNVGSNNS---INNSLHRGRNSSNNNS 27
APL 5HT2 SVQTATSTTIITATTTITTATAITTAATTS 60
LYM 5HT2 --AGANNNNSNNAGGNVESSSLN------- 48
APL 5HT2 TAALWMRTTPRRPRITATATANTNSLGRRV 90
LYM 5HT2 ----------------------TATSNNNILRR- 60
APL 5HT2 GRYGNQNGVNTSGVGSVGGEEVGEGGRGEG 120
LYM 5HT2 GRYSTQN----------------------- 67
APL 5HT2 GGGGGGNGGGLRSNNSPTTDFHPFCNGHHH 150
LYM 5HT2 ----GRTGPNYVLRSSPPD--YPYCNG-H- 89
APL 5HT2 HHHHQQQQQQQQLQQQEQELTSDASGESR 180
LYM 5HT2 ----------------QE-EMTSDVTSDSR 102
APL 5HT2 TCCQSLFSSSSSSSSPTSSSAATARGGGGG 210
LYM 5HT2 NCCT---SS-SSSSSSPSSSTR 119
APL 5HT2 GGGGGGGGSG 220
LYM 5HT2 119

Figure 4. 2

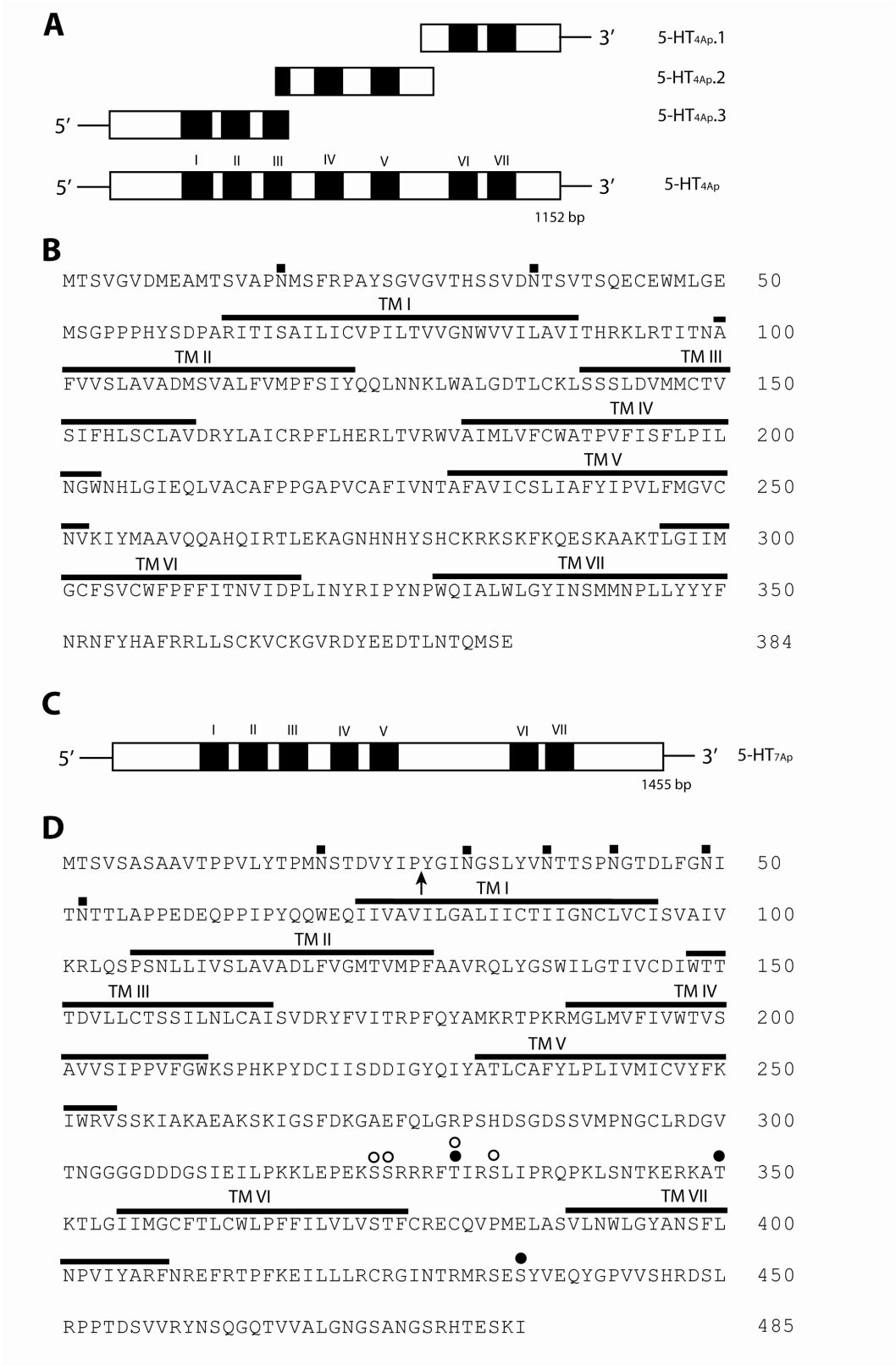

A
3′
5-HT4Ap.1
5-HT4Ap.2
5′
5-HT4Ap.3
I II III IV V VI VII
5′
3′
5-HT4Ap
1152 bp
B
MTSVGVDMEAMTSVAPNMSFRPAYSGVGVTHSSVDNTSVTSQECEWMLGE 50
TM I
MSGPPPHYSDPARITISAILICVPILTVVGNWVVILAVITHRKLRTITNA 100
TM II
TM III
FVVSLAVADMSVALFVMPFSIYQQLNNKLWALGDTLCKLSSSLDVMMCTV 150
TM IV
SIFHLSCLAVDRYLAICRPFLHERLTVRWVAIMLVFCWATPVFISFLPIL 200
TM V
NGWNHLGIEQLVACAFPPGAPVCAFIVNTAFAVICSLIAFYIPVLFMGVC 250
NVKIYMAAVQQAHQIRTLEKAGNHNHYSHCKRKSKFKQESKAAKTLGIIM 300
TM VI
TM VII
GCFSVCWFPFFITNVIDPLINYRIPYNPWQIALWLGYINSMMNPLLYYYF 350
NRNFYHAFRRLLSCKVCKGVRDYEEDTLNTQMSE 384
C
I II III IV V VI VII
5′
3′
5-HT7Ap
1455 bp
D
MTSVSASAAVTPPVLYTPMNSTDVYIPYGINGSLYVNTTSPNGTDLFGNI 50
TM I
TNTTLAPPEDEQPPIPYQQWEQIIVAVILGALIICTIIGNCLVCISVAIV 100
TM II
KRLQSPSNLLIVSLAVADLFVGMTVMPFAAVRQLYGSWILGTIVCDIWTT 150
TM III
TM IV
TDVLLCTSSILNLCAISVDRYFVITRPFQYAMKRTPKRMGLMVFIVWTVS 200
TM V
AVVSIPPVFGWKSPHKPYDCIISDDIGYQIYATLCAFYLPLIVMICVYFK 250
IWRVSSKIAKAEAKSKIGSFDKGAEFQLGRPSHDSGDSSVMPNGCLRDGV 300
TNGGGGDDDGSIEILPKKLEPEKSSRRRFTIRSLIPRQPKLSNTKERKAT 350
TM VI
TM VII
KTLGIIMGCFTLCWLPFFILVLVSTFCRECQVPMELASVLNWLGYANSFL 400
NPVIYARFNREFRTPFKEILLLRCRGINTRMRSESYVEQYGPVVSHRDSL 450
RPPTDSVVRYNSQGQTVVALGNGSANGSRHTESKI 485

Figure 4. 3

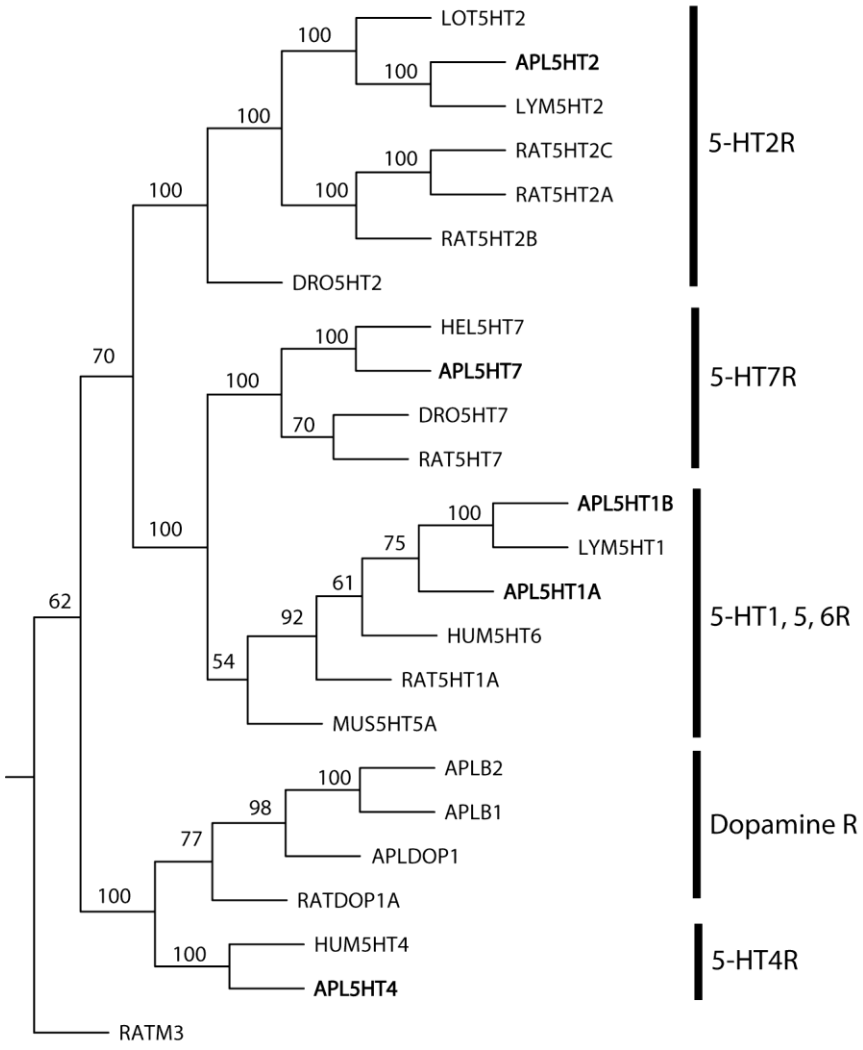

LOT5HT2
APL5HT2
LYM5HT2
RAT5HT2C
RAT5HT2A
RAT5HT2B
DRO5HT2
5-HT2R
HEL5HT7
APL5HT7
DRO5HT7
RAT5HT7
5-HT7R
APL5HT1B
LYM5HT1
APL5HT1A
HUM5HT6
RAT5HT1A
MUS5HT5A
5-HT1, 5, 6R
APLB2
APLB1
APLDOP1
RATDOP1A
Dopamine R
HUM5HT4
APL5HT4
5-HT4R
RATM3
100
100
100
100
100
100
100
70
100
70
100
75
61
92
54
62
100
98
77
100
100

Figure 4. 4

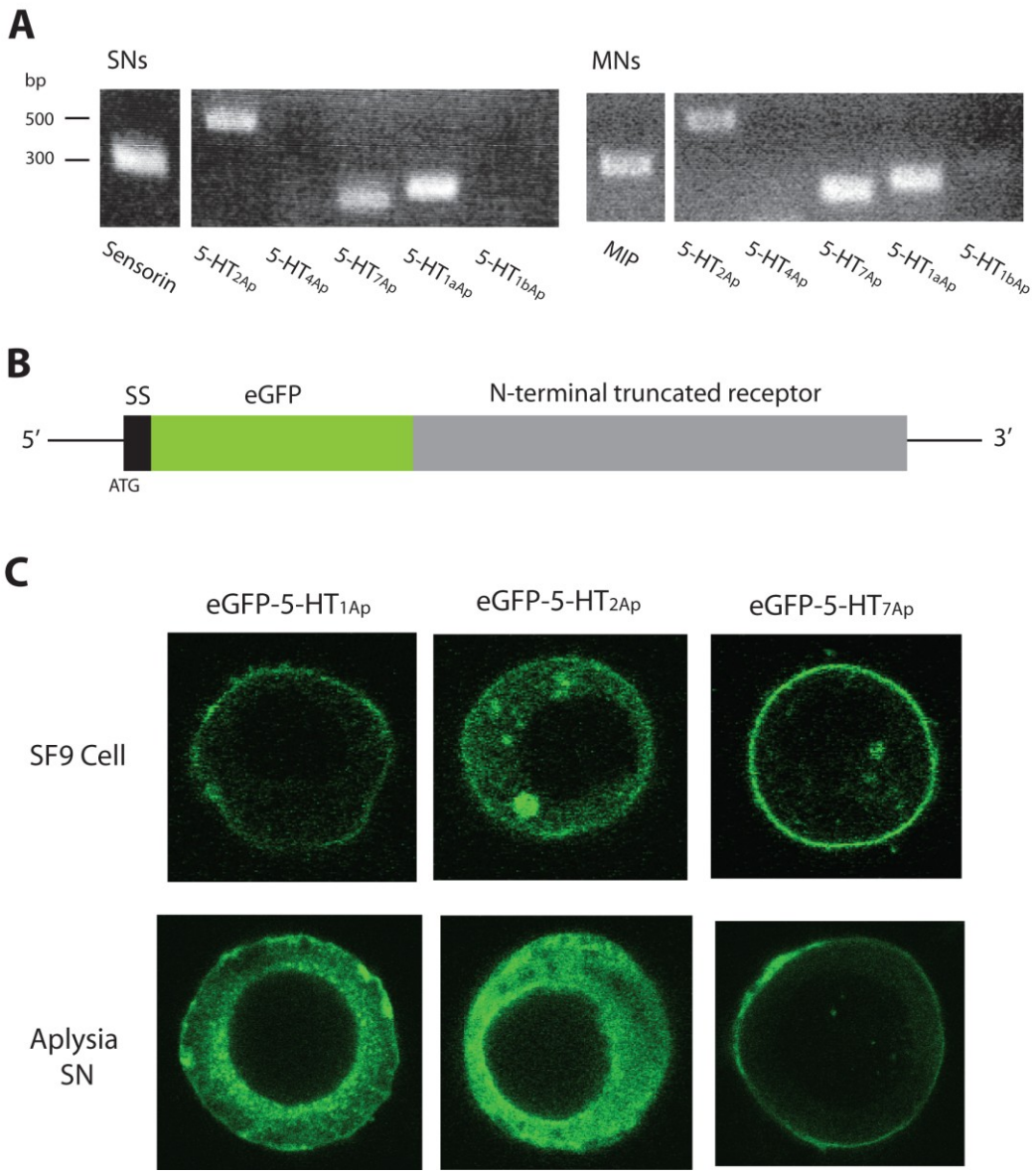

A
SNs
MNs
bp
500
300
Sensorin
5-HT2Ap
5-HT4Ap
5-HT7Ap
5-HT1aAp
5-HT1bAp
MIP
5-HT2Ap
5-HT4Ap
5-HT7Ap
5-HT1aAp
5-HT1bAp
B
SS
eGFP
N-terminal truncated receptor
5′
3′
ATG
C
eGFP-5-HT1Ap
eGFP-5-HT2Ap
eGFP-5-HT7Ap
SF9 Cell
Aplysia SN

Figure 4. 5

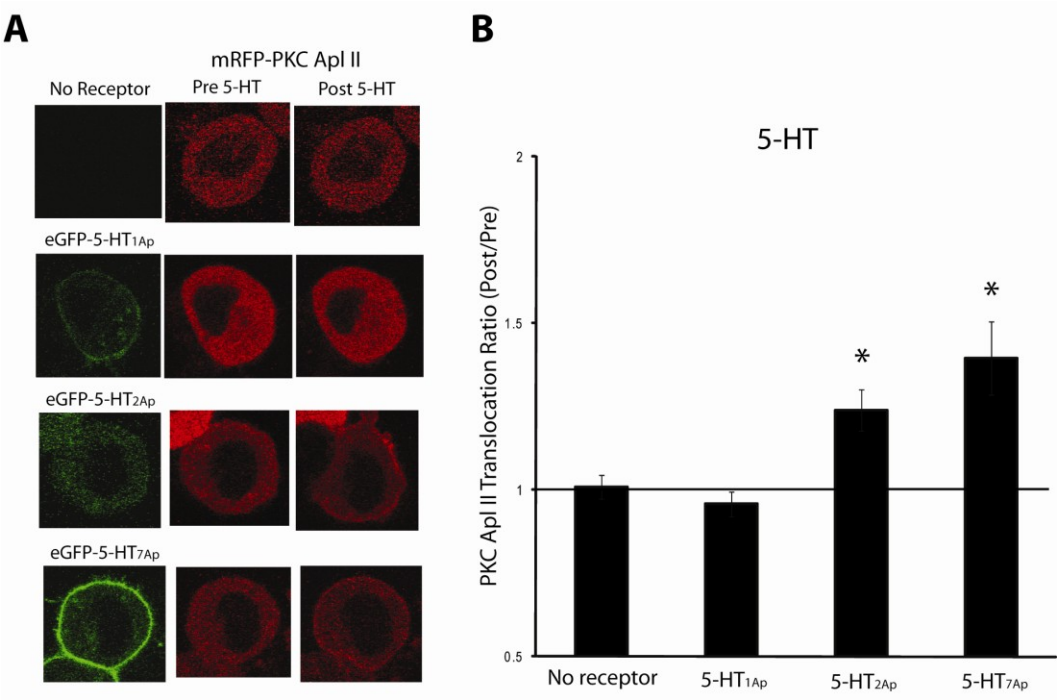

A
mRFP-PKC ApI II
No Receptor
Pre 5-HT
Post 5-HT
eGFP-5-HT1Ap
eGFP-5-HT2Ap
eGFP-5-HT7Ap
B
5-HT
PKC ApI II Translocation Ratio (Post/Pre)
2
1.5
1
0.5
*
*
No receptor
5-HT1Ap
5-HT2Ap
5-HT7Ap

Figure 4. 6

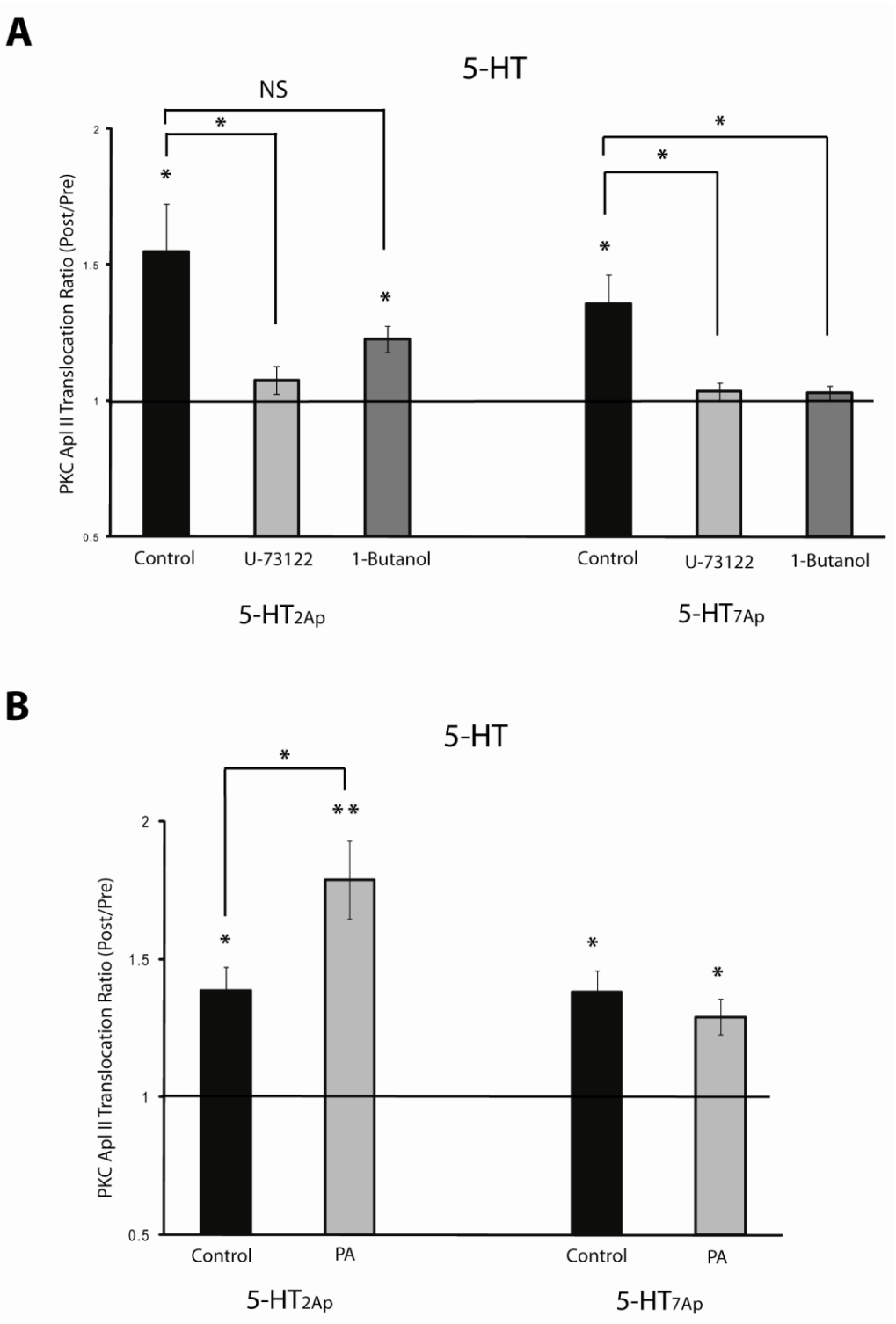

A
5-HT
NS
*
*
*
*
*
*
*
2
1.5
1
0.5
PKC Apl II Translocation Ratio (Post/Pre)
Control
U-73122
1-Butanol
Control
U-73122
1-Butanol
5-HT2Ap
5-HT7Ap
B
5-HT
*
**
*
*
*
2
1.5
1
0.5
PKC Apl II Translocation Ratio (Post/Pre)
Control
PA
Control
PA
5-HT2Ap
5-HT7Ap

Figure 4. 7

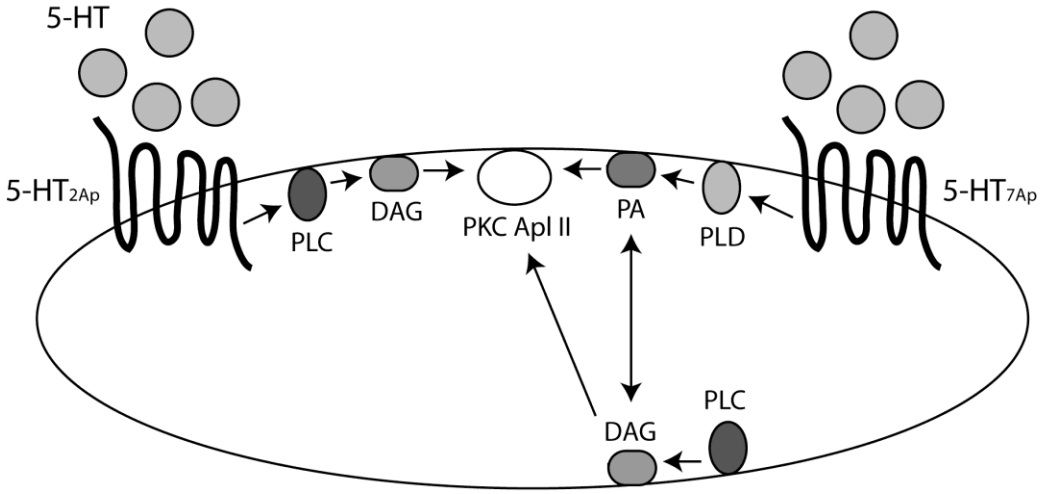
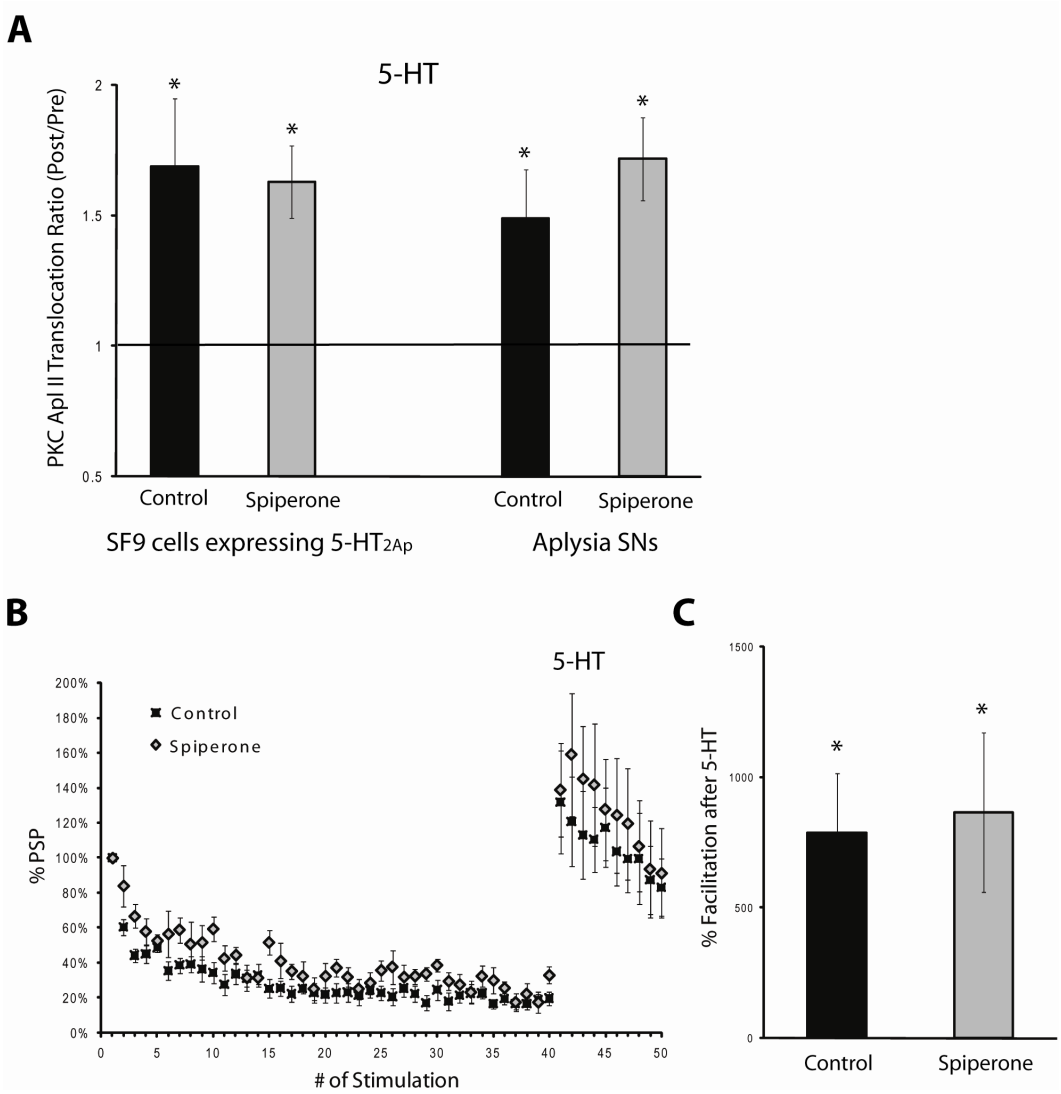


Figure 4. 8


A
5-HT
PKC Apl II Translocation Ratio (Post/Pre)
2
1.5
1
0.5
*
*
*
*
Control
Spiperone
Control
Spiperone
SF9 cells expressing 5-HT2Ap
Aplysia SNs
B
5-HT
Control
Spiperone
% PSP
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
5
10
15
20
25
30
35
40
45
50
# of Stimulation
C
% Facilitation after 5-HT
1500
1000
500
0
*
*
Control
Spiperone

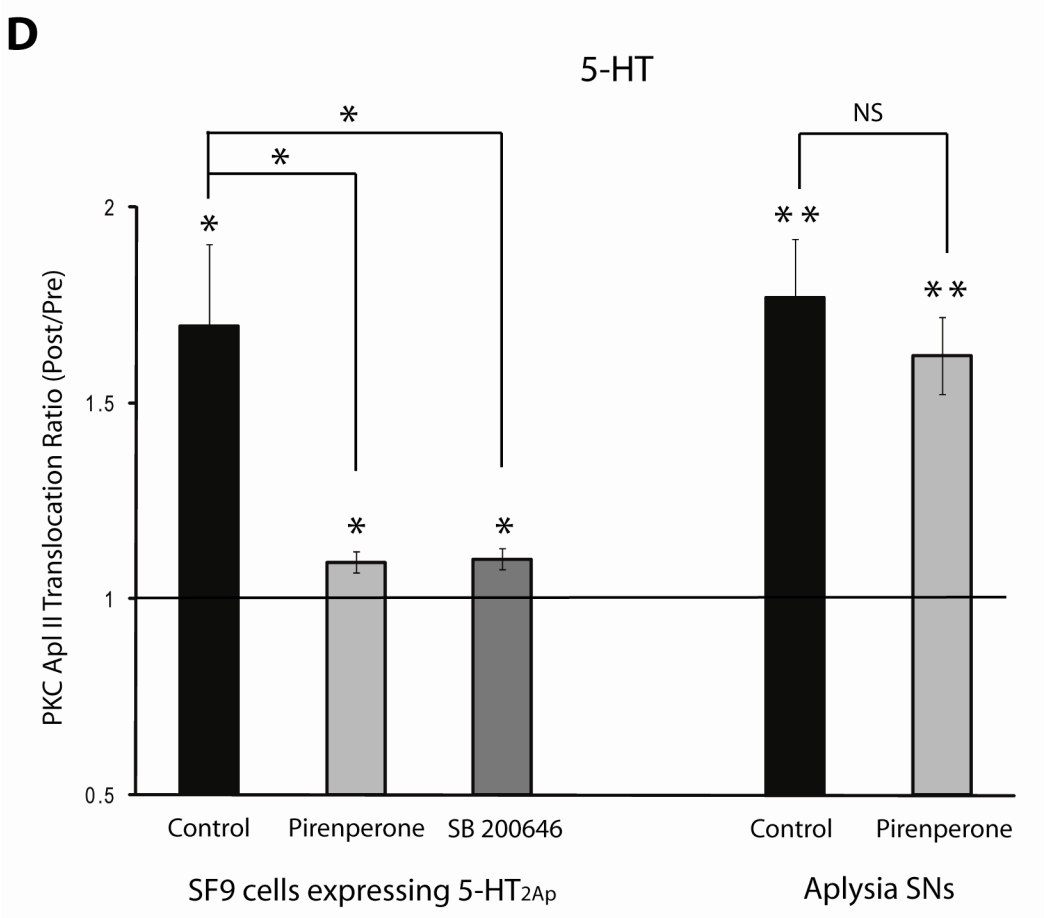

D
5-HT
NS
PKC Apl II Translocation Ratio (Post/Pre)
2
1.5
1
0.5
Control
Pirenperone
SB 200646
Control
Pirenperone
SF9 cells expressing 5-HT2Ap
Aplysia SNs

Figure 4. 9

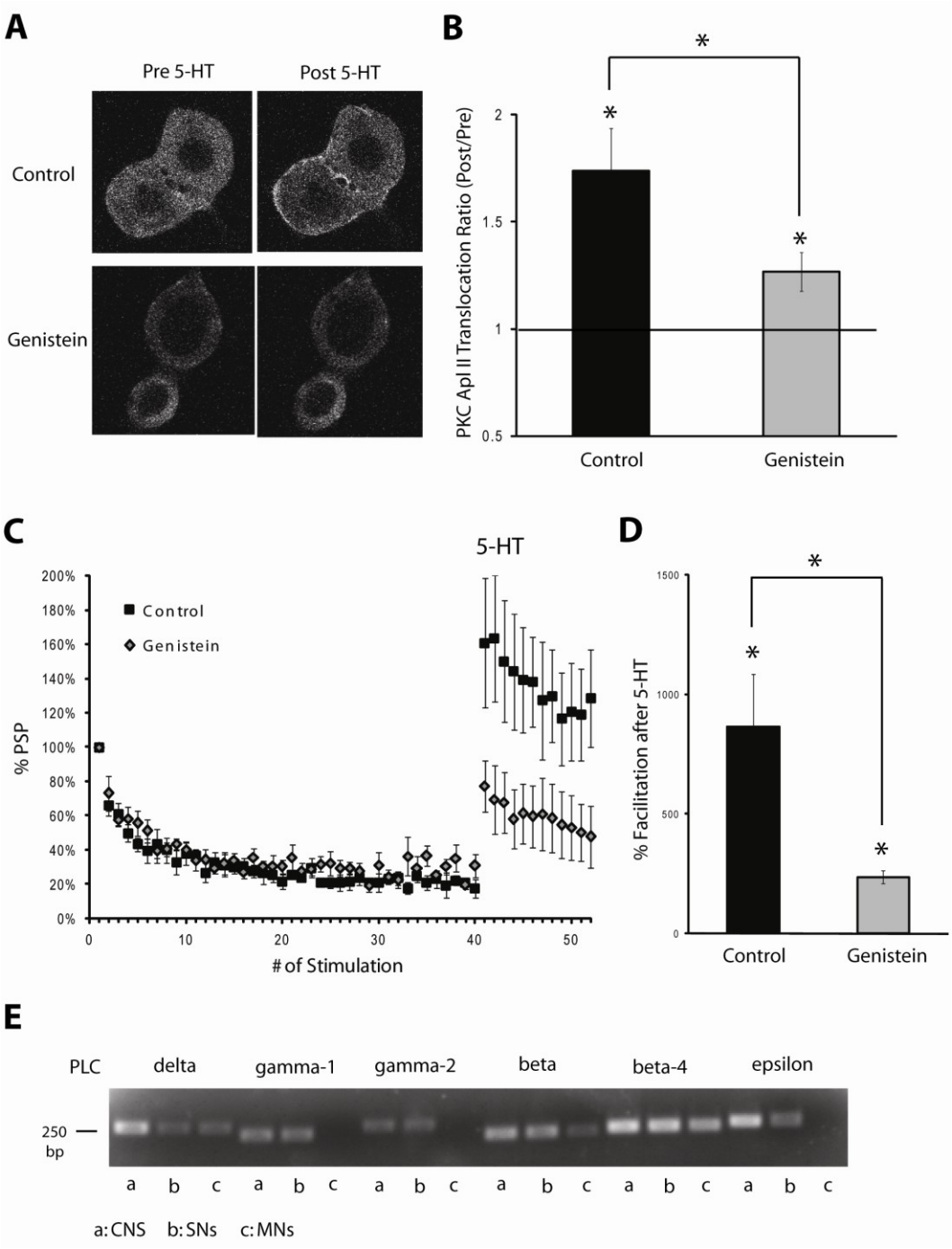

A
Pre 5-HT
Post 5-HT
Control
Genistein
B
PKC Apl II Translocation Ratio (Post/Pre)
2
1.5
1
0.5
Control
Genistein
C
5-HT
Control
Genistein
% PSP
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
10
20
30
40
50
# of Stimulation
D
% Facilitation after 5-HT
1500
1000
500
0
Control
Genistein
E
PLC
delta
gamma-1
gamma-2
beta
beta-4
epsilon
250 bp
a b c a b c a b c a b c a b c a b c
a: CNS b: SNs c: MNs

Figure 4. 10

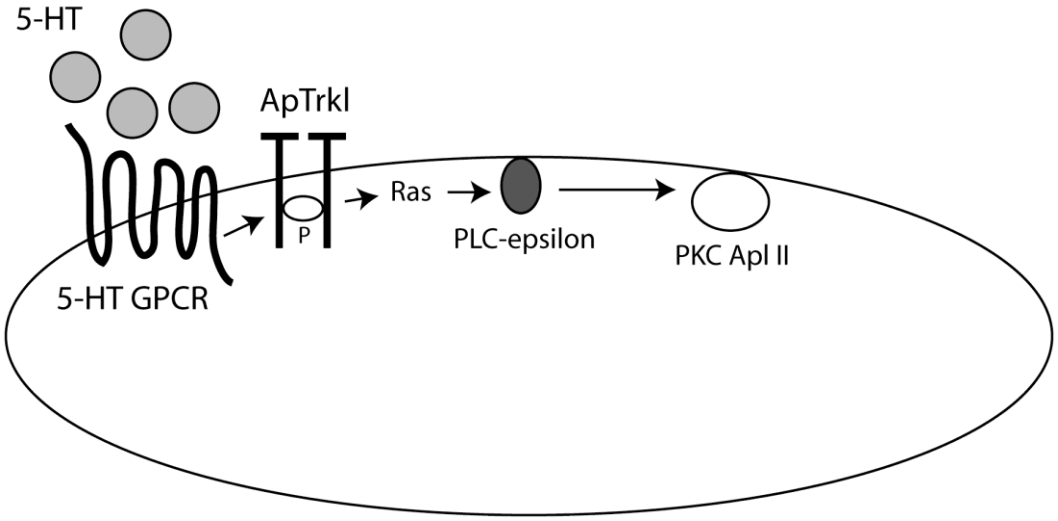


Table 4. 1. Effects of various drug treatments on PKC Apl II translocation and reversal of synaptic depression

Drug 5-HT +	PKC Apl II translocation			Reversal of depression	
	SF9 cells overexpressing		Aplysia SNs	SN-MN	Ganglia
	5-HT_{2Ap}	5-HT_{7Ap}			
U-73122	Block	Block	Block (Ref. 1)	—	—
1-butanol	No change	Block	Block (Ref. 1)	—	—
PA	Increase	No change	—	—	—
Spiperone	No change	—	No change	No change	Block (Ref. 2)
Pirenperone	Block	—	No change	—	—
SB 200646	Block	—	—	—	—
Genistein	—	—	Block	Block	—

Note: — (Not reported), Ref. 1 (Farah et al., 2008), Ref. 2 (Dumitriu et al., 2006)

Figure Legends

Figure 4. 1. Molecular cloning of 5-HT_{2Ap}. **A:** Schematic representation of the cloned 5-HT_{2Ap}. Five overlapping cDNA fragments (5-HT_{2Ap}.1 to 5-HT_{2Ap}.5) were PCR amplified from a CNS cDNA library, and the reconstituted full-length cDNA is schematized at the bottom. The putative transmembrane domains are represented by black boxes. **B:** Deduced amino acid sequence of 5-HT_{2Ap}. Amino acids are given in single letter code. Seven transmembrane domains are indicated (TM I to TM VII). Potential N-linked glycosylation sites (closed rectangle) and predicted phosphorylation sites for PKA (closed circle) and PKC (open circle) are indicated. Single arrowhead indicates the position of the truncation in eGFP-$5HT_{2Ap}$, and double arrowheads indicate the position of splicing. **C:** Alignment of the deduced amino acid sequence of the spliced region of 5-HT_{2Ap} with 5-HT_{2Lym}. Identical residues are shown in black.

Figure 4. 2. Molecular cloning of 5-HT_{4Ap} and 5-HT_{7Ap}. **A and C:** Schematic representation of the cloned 5-HT_{4Ap} (A) and 5-HT_{7Ap} (C). Three overlapping cDNA fragments (5-HT_{4Ap}.1 to 5-HT_{4Ap}.3) were PCR amplified from a CNS cDNA library, and the reconstituted full-length cDNA is schematized at the bottom (A). The putative transmembrane domains are represented by black boxes. **B and D:** Deduced amino acid sequence of 5-HT_{4Ap} (B) and 5-HT_{7Ap} (D). Amino acids are given in single letter code. Seven transmembrane domains are indicated (TM I to TM VII). Potential N-linked glycosylation sites (closed rectangle) and

predicted phosphorylation sites for PKA (closed circle) and PKC (open circle) are indicated. Single arrowhead indicates the position of the truncation in eGFP-$5HT_{7Ap}$.

Figure 4. 3. Phylogenetic analysis of 5-HT receptors. The surrounding trans membrane regions (without non-consensus N- and C-terminals) were aligned with Clustal-W and 100 replicates were generated and analyzed with the Phylip programs. Bootstrap numbers are given in each node and represent the percentage of total trees that give the tree shown. The abbreviations are as follwed: LOT (*Lottia*), APL (*Aplysia*), LYM (*Lymnaea*), DRO (*Drosophila*), HEL (*Helisoma*), HUM (Human), MUSS (Mouse), DOP (Dopamine). RATM3 (muscarinic acetylcoline receptor) was included to represent an outlyer.

Figure 4. 4. Expression of 5-HT receptors in *Aplysia* neurons and construction of eGFP-tagged receptors. **A:** Single cell RT-PCR analysis for 5-HT receptors from *Aplysia* sensory neurons (SNs) and LFS motor neurons (MNs). Sensorin and MIP (Mytilus inhibitory peptide related precursor) were used as a positive control for SNs and MNs, respectively. **B:** Schematic representation of an eGFP-tagged receptor construct. N-terminal of a receptor was truncated and replaced by eGFP following the signal sequence (SS) of sensorin. **C:** Overexpression of eGFP-tagged receptors (eGFP-5-HT_{1Ap}, eGFP-5-HT_{2Ap}, and eGFP-5-HT_{7Ap}) in SF9 cells and *Aplysia* SNs.

Figure 4. 5. Translocation of PKC Apl II in SF9 cells by 5-HT_{2Ap} and 5-HT_{7Ap}. SF9 cells were transfected with mRFP-PKC Apl II (No receptor), or cotransfected with either eGFP-5-HT_{1Ap}, eGFP-5-HT_{2Ap}, or eGFP-5-HT_{7Ap} and mRFP-PKC Apl II. **A:** Confocal microscope images of SF9 cells showing eGFP-tagged receptors and mRFP-PKC Apl II pre and post 5-HT (10 μM, 120 sec) treatment. **B:** Quantification of PKC Apl II translocation ratio (Post/Pre 5-HT). Post 5-HT is an average of 60, 90, 120, and 150 sec after 5-HT treatment (No receptor, n = 9; 5-HT_{1Ap}, n = 6; 5-HT_{2Ap}, n = 11; 5-HT_{7Ap}, n = 7). * denotes $P < .05$ (Student's paired t-test).

Figure 4. 6. Involvement of PLC and PLD pathways in PKC Apl II translocation by 5-HT_{2Ap} and 5-HT_{7Ap}. **A:** Effects of PLC and PLD inhibitors on 5-HT-induced PKC Apl II translocation in SF9 cells. U-73122 (10 μM) or 1-Butanol (1%) was used as a PLC or PLD inhibitor, respectively, and applied pre and during 5-HT (10 μM) treatment in SF9 cells expressing 5-HT_{2Ap} (Left: Control, n = 6; U-73122, n = 11; 1-Butanol, n = 8) or 5-HT_{7Ap} (Right: Control, n = 11; U-73122, n = 7; 1-Butanol, n = 12). U-73122 treatment significantly blocked 5-HT-induced translocation in SF9 cells cells expressing 5-HT_{2Ap}; One-way ANOVA, $F_{(2,22)} = 7.02$, $P = .004$; Tukey's post hoc test, $P < .01$ between Control and U-73122. Both U-73122 and 1-Butanol treatments blocked the translocation in cells expressing 5-HT_{7Ap}; One-way Anova, $F_{(2,27)} = 7.31$, $P = .003$; Tukey's post hoc test, $P < .05$ between Control and U-73122, $P < .01$ between Control and 1-Butanol. * on a bar denotes $P < .05$ (Student's paired t-

test). **B:** Effects of PA on 5-HT-induced PKC Apl II translocation. DiC8-PA (PA, 2.5-5 μg/mL) was applied in addition to 5-HT (10 μM) in SF9 cells expressing 5-HT_{2Ap} (Left: Control, n = 17; PA, n = 23) or 5-HT_{7Ap} (Right: Control, n = 15; PA, n = 13). Addition of PA increased 5-HT-induced translocation in SF9 cells expressing 5-HT_{2Ap}; Student's unpaired t-test, $P < .05$. * and ** on a bar denote $P \leq .001$ and $P < .0001$ (Student's paired t-test), respectively.

Figure 4. 7. Model of PKC Apl II translocation by 5-HT_{2Ap} and 5-HT_{7Ap}. In this model, 5-HT_{2Ap}, after 5-HT treatment, leads to the translocation of PKC Apl II through coupling to the PLC-DAG pathway. On the other hand, 5-HT_{7Ap} activates the PLD-PA pathway, which synergizes with endogenous DAG, to induce the translocation of PKC Apl II.

Figure 4. 8. Effects of spiperone and 5-HT_{2Ap} antagonists on PKC Apl II translocation. **A:** Effects of spiperone on 5-HT-induced PKC Apl II translocation. Spiperone (100-200 μM) was applied pre and during 5-HT (10-20 μM) treatment to SF9 cells expressing 5-HT_{2Ap} (Left: Control, n = 10; Spiperone, n = 9) or to SNs (Right: Control, n = 6; Spiperone, n = 5). * on a bar denotes $P < .05$ (Student's paired t-test). **B:** Effect of spiperone on 5-HT-induced reversal of synaptic depression. 5-HT (10 μM) was added after 40^{th} postsynaptic potential (PSP). Spiperone (100 μM) was applied pre and during 5-HT treatment (Control, n = 6; Spiperone, n = 6). PSP was normalized to the first PSP. **C:** Quantification of facilitation after 5-HT treatment. % Facilitation = 100 x (Ave. PSP #41-

43/Ave. PSP #38-40). * on a bar denotes P < .05 (Student's paired t-test between PSP #40 and PSP #41). **D:** Effects of pirenperone on 5-HT-induced PKC Apl II translocation. (Left) Pirenperone (100 μM) or SB 200646 (100 μM) was applied pre and during 5-HT (10 μM) treatment to SF9 cells expressing 5-HT_{2Ap} (Control, n = 12; Pirenperone, n = 10; SB 200646, n = 9), and blocked 5-HT-induced translocation of PKC Apl II; Student's unpaired t-test, P < .05 for both compared to Control. (Right) Pirenperone (100 μM) was applied pre and during 5-HT (20 μM) treatment to SNs (Control, n = 11; Pirenperone, n = 11), and did not block 5-HT-induced translocation of PKC Apl II. * and ** on a bar denote P < .01 and P < .001, respectively (Student's paired t-test).

Figure 4. 9. Involvement of tyrosine kinase signaling in PKC Apl II translocation and reversal of synaptic depression. **A:** Confocal microscope image of SNs pre and post 5-HT (10 μM, 5 min) in the presence or absence of genistein (100 μM). **B:** Quantification of PKC Apl II translocation after 5-HT addition normalized to Pre 5-HT. Genistein significantly decreased 5-HT-induced translocation of PKC Apl II (Control, n = 16; Genistein, n = 18); Student's unpaired t-test, P < .05. * on a bar denotes P < .01 (Student's paired t-test). **C:** Effect of genistein on 5-HT-induced reversal of synaptic depression. 5-HT (10 μM) was added after 40^{th} postsynaptic potential (PSP). Genistein (100 μM) was applied pre and during 5-HT treatment (Control, n = 6; Genistein, n = 6). PSP was normalized to the first PSP. **D:** Quantification of facilitation after 5-HT treatment. % Facilitation = 100 x (Ave. PSP #41-43/Ave. PSP #38-40). * on a bar denotes P

< .05 (Student's paired t-test between PSP #40 and PSP #41). **E:** Single cell RT-PCR analysis for different isoforms of PLCs from *Aplysia* neurons. Primers were generated based on *Aplysia* genomic database and RT-PCR was performed from *Aplysia* CNS (a), SNs (b), and LFS MNs (c).

Figure 4. 10. Model of PKC Apl II translocation by ApTrkl through PLC-epsilon. In this model, after transactivation by 5-HT through GPCRs, ApTrkl leads to the translocation of PKC Apl II through coupling to PLC-epsilon. ApTrkl might be able to activate PLC-epsilon through activation of Ras.

CHAPTER FIVE

Conclusion

5. 1. Overview

In the chapter one, I presented a comprehensive literature review up to the current knowledge of molecular mechanisms underlying learning and memory in *Aplysia*, and roles for tyrosine kinases and PKCs in synaptic plasticity in both vertebrates and *Aplysia*. A significant amount of literature emphasizes the crucial role for TrkB signaling in learning and memory from molecular to behavioral levels in vertebrates. Tyrosine kinase cascades are also implicated in LTF and sensitization in *Aplysia*. Previous study for ApTrkl was introduced and several hypotheses regarding its regulation by 5-HT were raised, which led to the study in the chapter three. Although a role for novel PKCs in synaptic plasticity in vertebrates is not well known, novel PKC Apl II plays an important role in reversal of synaptic depression in *Aplysia*. PKC Apl II is activated and translocated to the plasma membrane by 5-HT through activation of PLC and PLD. However, 5-HT GPCRs that are coupled to PKC Apl II activation have not been identified, which led to the study in the chapter four.

In the chapter three, regulation of ApTrkl was examined for its activation and internalization by overexpression and/or 5-HT. Overexpression of ApTrkl led to the constitutive activation of ApTrkl that was dependent on the kinase activity. Overexpressed ApTrkl was localized mostly at the cytoplasm, while kinase-dead

(KR) ApTrkl was localized at the plasma membrane, suggesting there is constitutive internalization of ApTrkl by overexpression that is dependent on the kinase activity. However, KR ApTrkl was internalized when 5-HT was applied, the effect of which was inhibited by methiothepin, an antagonist to 5-HT GPCRs, suggesting there is kinase activity-independent form of internalization through 5-HT GPCRs. Both forms of internalization did not require a functional extracellular domain and thus they were independent of a ligand. Surprisingly, methiothepin, instead of blocking activation of endogenous ApTrkl, activated ApTrkl to the same level as 5-HT, suggesting that methiothepin acts as an agonist and induces transactivation through a novel coupling of the 5-HT GPCRs to ApTrkl. A possible ligand to RTKs in *Aplysia*, sensorin, was not required for 5-HT-induced ApTrkl activation, although it was able to transiently activate ApTrkl.

In the chapter four, we isolated 5-HT GPCRs from *Aplyisa Californica*; 5-HT_{2Ap}, 5-HT_{4Ap}, and 5-HT_{7Ap}, and demonstrated that 5-HT_{2Ap} and 5-HT_{7Ap} were both able to translocate PKC Apl II in SF9 cells, suggesting that both receptors are coupled to PKC Apl II activation. The translocation of PKC Apl II by 5-HT_{2Ap} was blocked by the PLC inhibitor, whereas the translocation induced by 5-HT_{7Ap} was blocked by both the PLC and PLD inhibitors. Moreover, synergism with PA was observed for the translocation induced by 5-HT_{2Ap} but not by 5-HT_{7Ap}. It was suggested that although 5-HT_{2Ap} translocates PKC Apl II through the PLC pathway, 5-HT_{7Ap} does so through the PLD pathway, which synergizes with endogenous DAG. Spiperone, an antagonist for the mammalian 5-HT_2 receptor and previously shown to block reversal of synaptic depression, was not an

effective antagonist for 5-HT_{2Ap}. Moreover, in our study spiperone did not block 5-HT-induced reversal of synaptic depression. Pirenperone acted as an antagonist for 5-HT_{2Ap} in SF9 cells but failed to block 5-HT-induced translocation of PKC Apl II in *Aplysia* SNs. Tyrosine kinases were shown to be involved in 5-HT-induced translocation of PKC Apl II as well as 5-HT-induced reversal of synaptic depression. PLC-epsilon, which can bind to Ras, was not expressed in MNs, in which PKC Apl II cannot be translocated, and ApTrkl is the only identified Trk-like receptor that is activated by 5-HT. Therefore, we made a model where PKC Apl II is translocated by PLC-epsilon, which is activated by ApTrkl after transactivation by 5-HT GPCRs.

5. 2. Contribution and significance of the studies

The main objectives of the studies were: (1) To elucidate the mechanism of ApTrkl regulation by 5-HT (Chapter Three): (2) To identify 5-HT GPCRs that are coupled to PKC Apl II activation (Chapter Four). The following sections discuss the findings in terms of their contribution and significance to the current knowledge in the field.

5. 2. 1. Mechanisms for ApTrkl regulation by 5-HT

ApTrkl plays an important role in long-term synaptic plasticity as it is required for the activation of ERK and LTF induced by 5-HT (Ormond et al.,

2004). ApTrkl is activated by 5-HT through some unknown mechanism. To elucidate the mechanism of ApTrkl activation by 5-HT is a critical step to understand the signal transduction induced by 5-HT that is important for long-term synaptic changes. Our overexpression system allowed us to visualize the receptors at high levels and manipulate their function by expressing the constructs with various modifications. The former was particularly important because the levels of activation of endogenous ApTrkl were low even with the addition of 5-HT. For the latter, making an extracellular chimera made it possible to examine the involvement of a ligand in the regulation of ApTrkl. As well, constructs with a mutation at the signaling tyrosine site or the kinase domain allowed us to examine their role in the regulation of ApTrkl.

We have shown that internalization of overexpressed ApTrkl constructs does not require ligand-binding to ApTrkl, as observed by the constitutive internalization of TrkC-WT and the 5-HT-induced internalilzation of TrkC-KR, which was blocked by the 5-HT GPCR antagonist methiothepin. These results thus suggest that regulation of ApTrkl does not involve ligand-binding, unlike mammalian Trk receptors, and instead there is a novel mechanism mediated by 5-HT GPCRs. It distinguishes ApTrkl from mammalian Trk receptors, suggesting ancient function of Trk-like receptors. In fact, Trk-like receptors are conserved in most invertebrate species but have been lost in mammals (Sossin, 2006; Wilson, 2009). In particular, the function of the extracellular domain to bind a ligand seems specific to Trk receptors. In *Aplysia*, BDNF and an unidentified TrkB-like receptor play a role for 5-HT-induced LTF and LTM for sensitization (Purcell et al., 2003; Sharma et al., 2006). A Trk receptor in *Aplysia*, ApTrk, has been

presented, which is closer to mammalian Trks and activated by an *Aplysia* orthologue of neurotrophin (Kassabov et al., 2007).

5. 2. 2. Novel ApTrkl transactivation by 5-HT GPCRs

Instead of blocking the effect of 5-HT, the 5-HT GPCR antagonist methiothepin induced the activation of ApTrkl to the same extent as 5-HT, and occluded the effect of 5-HT since the addition of methiothepin to 5-HT did not further increase the activation. While it suggests that the action of 5-HT on increasing ApTrkl activation is through GPCRs, the action of methiothepin acting as an agonist has not been reported in both vertebrates and *Aplysia*. One possibility is that methiothepin induces a conformational change of GPCRs that can induce signaling through other pathways.

Traditional theory of receptor activation assumes that there are only two receptor states, active (R*) and inactive (R). However, evidence for differential effector activation by agonists is not consistent with this traditional theory. In particular, 5-HT_{2A} receptors in vertebrates have more than one signaling pathways and different agonist can activate these pathways in a distinct manner (Kurrasch-Orbaugh et al., 2003). Contrary to the traditional theory that does not allow for R* to activate effectors differentially in an agonist-dependent manner, the extended ternary complex model suggests that each agonist has an ability to promote its own specific receptor active state, which theoretically leads to unlimited number of receptor conformations, R^n* (Kenakin, 2003). The observation that diverse agonists to 5-HT_{2A} receptors show distinct patterns of activation of the PLC or

PLA_2 pathway favors the idea that there are R^n* conformational states rather than a restricted set of conformations (Kurrasch-Orbaugh et al., 2003).

Drugs acting at GPCRs can be divided into three classes, agonists, neutral antagonists, and inverse agonists (Strange, 2002). Agonists act by preferentially coupling to R* state, thereby increasing the effector activity, whereas neutral antagonists act by coupling equally well to both R and R* states, thereby not changing the effector activity (Berg et al., 1998). The concept of inverse agonism came from the experimental observation that certain drugs can reduce the basal activity of receptor systems in the absence of agonists (Strange, 2002). Inverse agonists are generally thought to act by preferentially coupling to R form, switching the R:R* equilibrium in favor of R and reducing the basal effector activity, and therefore switching the receptor to an inactive conformation that prohibits signaling (Strange, 2002).

Methiothepin is known to act as an inverse agonist for 5-HT_{1A} and 5-HT_7 receptors in vertebrates by decreasing the basal activity of these receptors (McLoughlin and Strange, 2000; Krobert et al., 2006), and orthologues of these receptors exist in *Aplysia* (Angers et al., 1998; Barbas et al., 2002; Cohen et al., 2003; Lee et al., 2009). Therefore, methiothepin seems to bind to the same site as 5-HT and causes a conformational change that favors the inactive state of the receptor. This conformational change, however, might open up other signaling pathways through which methiothepin could exert its effect as an agonist, although there has been so far no report for this kind of effect by methiothepin. Therefore, this study is the first report for methiothepin acting as an agonist,

suggesting a novel 5-HT GPCR signaling by methiothepin that can transactivate ApTrkl.

5. 2. 3. Role for sensorin in ApTrkl activation

Sensorin has a critical role in LTF underlying sensitization in *Aplysia*, and acts through a RTK that has not been identified yet (Hu et al., 2004a). Since ApTrkl was the first Trk-like receptor isolated in *Aplysia*, it was crucial to examine the possibility that ApTrkl is activated by sensorin, and if so, what the mechanism would be. Sensorin, released by 5-HT, might act as a ligand to ApTrkl. Or, sensorin might activate ApTrkl through some unknown mechanism not as a ligand. In the current study, although sensorin was able to activate ApTrkl after 5 min incubation, it was no longer able to do so after 60 min incubation. This contrasts with the effect of 5-HT, which can activate ApTrkl at least up to 90 min (Ormond et al., 2004), and suggests that additional signal(s) other than sensorin is required for the prolonged activation of ApTrkl. Unpublished data suggests that sensorin cannot activate ApTrkl in the heterologous cells, and the antibodies to sensorin failed to block 5-HT-induced activation of ApTrkl. These data led us to believe that sensorin is not a ligand to ApTrkl but able to transiently activate ApTrkl through some unknown mechanism. One possibility is that sensorin can activate a GPCR that can also couple to ApTrkl.

Therefore, the results indicate that sensorin is not crucial for ApTrkl activation, and it is unlikely that ApTrkl is the receptor for sensorin. Although the data were negative, it is important to know that there is likely to be a different

receptor for sensorin like most other neuropeptides, which might act on ApTrkl. Both sensorin and ApTrkl are required for the activation of ERK and LTF. It is unknown whether there are distinct pathways leading to these long-term effects or signaling pathways activated by sensorin and ApTrkl converge at some point.

5. 2. 4. Identification of novel *Aplysia* 5-HT GPCRs

5-HT is a major facilitatory neurotransmitter in *Aplysia* and known to mediate learning and memory processes such as sensitization and dishabituation through downstream activation of the second messenger signaling. In fact, the finding that 5-HT acts to produce the second messenger cAMP, which is an underlying mechanism for sensitization of the gill-withdrawal reflex, was one of the contributions for which Dr. Eric Kandel won the Nobel Prize in Physiology or Medicine in 2000 for his research on the physiological basis of memory storage in neurons. It has been almost 30 years since the discovery, and more and more mechanisms have been revealed downstream of 5-HT-activation of GPCRs such as activation of PKA and PKC as molecular mechanisms for memory processing in *Aplysia*. Yet, not much attention has been paid to 5-HT GPCRs and their involvement in synaptic plasiticy. Part of the reasons was a difficulty in cloning these receptors due to the lack of genomic information. However, recent advances in bioinformatics have made it easier and faster to clone genes.

In the current study, we took a different approach to clone the receptors. Instead of using degenerate primers like in most studies, we used primers based on the available *Aplysia* genomic sequences. The current study thus demonstrated,

for the first time, the use of *Aplysia* genomic information to successfully clone 5-HT GPCRs. The bioinformatics tool also made it possible to screen all existing 5-HT receptors in *Aplysia*, and the resulting receptors, 5-HT_{2Ap}, 5-HT_{4Ap}, and 5-HT_{7Ap} were believed to be the only receptors that had not been identified prior to the cloning. With the identification of all 5-HT GPCRs, it is now possible to examine why 5-HT induces activation of distinct molecular pathways leading to different types of synaptic plasiticity in *Aplysia*.

5. 2. 5. 5-HT GPCRs that are coupled to PKC Apl II activation

PKC Apl II is required for reversal of synaptic depression (Manseau et al., 2001), which is thought to underlie behavioral dishabituation. Although PKC Apl II is known to be activated and translocated to the plasma membrane by 5-HT (Zhao et al., 2006), it was not known through which 5-HT receptor(s) the action of 5-HT is mediated. We have shown that not only 5-HT_{2Ap}, which is a family of PLC-coupled receptors, but also 5-HT_{7Ap}, which is not likely to be coupled to PLC, could translocate PKC Apl II in SF9 cells. We have previously shown that both DAG, generated by PLC, and PA, generated by PLD, have a role in translocating PKC Apl II in SF9 cells, and an inhibitor to either PLC or PLD decreases the translocation of PKC Apl II in SNs (Farah et al., 2008). Indeed, our results suggest that the translocation of PKC Apl II by 5-HT_{2Ap} in SF9 cells requires activation of PLC, whereas the translocation by 5-HT_{7Ap} requires activation of both PLC and PLD. However, we favor the model that 5-HT_{7Ap} activates the PLD-PA pathway, which synergizes with endogenous DAG to

translocate PKC Apl II, and the PLC inhibitor U-73122 may block endogenous DAG, which may otherwise synergize with PA activated by 5-HT_{7Ap}. Although activation of the PLD pathway has been reported for the mammalian 5-HT_2 receptors (Mitchell et al., 1998; McGrew et al., 2002; Robertson et al., 2003), such signaling has not been reported for the 5-HT_7 receptors, and this study might be the first demonstration. Alternatively, 5-HT_{7Ap} may activate Epac through cAMP, leading to the activation of PLC and PLD (Hucho et al., 2005).

Pirenperone, shown to act as an antagonist to 5-HT_{2Ap}, failed to block 5-HT-induced translocation of PKC Apl II in SNs. This might be due to the flexibility in the system, where 5-HT_{7Ap} might be able to compensate for the lack of 5-HT_{2Ap} signaling. Inconsistent with the previous finding, spiperone did not block 5-HT-induced reversal of synaptic depression in our experiments, nor did it block the translocation of PKC Apl II. Indeed, spiperone was not an effective antagonist to 5-HT_{2Ap}. The descrepancy might have come from the use of different SNs: abdominal versus pleural SNs. Spiperone is known to act as an antagonist to dopamine receptors. Although 5-HT can induce reversal of synaptic depression in both neuron types, it may act on dopaminergic receptors in abdominal SNs because of a high concentration of 5-HT. It is not known if dopamine receptors can couple to PLC, and the possibility that these receptors are also coupled to PKC Apl II activation cannot be excluded.

5. 2. 6. Activation of PKC Apl II through tyrosine kinase signaling

We found that activities of tyrosine kinases are involved in 5-HT-induced translocation of PKC Apl II and reversal of synaptic depression in neurons. Tyrosine kinase activities are implicated in ITF and ITM for sensitization through the activation of PKC Apl I, a Ca^{2+}-dependent PKC, and they seem to be required upstream of ERK activation (Shobe et al., 2009). On the other hand, we suggest tyrosine kinase activities are coupled to PLC-epsilon to activate PKC Apl II, and this might be mediated by ApTrkl, which is transactivated by 5-HT through 5-HT GPCRs (Chapter Three). We have preliminary data that ApTrkl colocalizes with $5\text{-}HT_{1Ap}$ or $5\text{-}HT_{7Ap}$ in SF9 cells. ApTrkl is not expressed in SF9 cells (Ormond et al., 2004), which might be the reason why $5\text{-}HT_{1Ap}$ could not translocate PKC Apl II in this system although it might be able to activate PKC Apl II in neurons.

PKC Apl II is translocated in SNs but not in MNs (Zhao et al., 2006). Understanding why it is not translocated in MNs will give further insight into the mechanism of PKC Apl II activation. Moreover, it might help understand a difference between plasticity mediated pre- and postsynaptically, which has been a controversial issue in the field. Since $5\text{-}HT_{2Ap}$ and $5\text{-}HT_{7Ap}$ are expressed in both SNs and MNs, the lack of PKC Apl ll translocation in MNs is not likely due to the lack of 5-HT receptors coupled to PKC Apl II. Instead, it might be due to the lack of molecule(s) downstream of the receptors. Indeed, gamma-1, gamma-2, and epsilon family of PKCs were not expressed in MNs. In mammals, PLC-epsilon is able to activate PKC-epsilon, the mammalian orthologue of PKC Apl II (Oestreich et al., 2009). Moreover, PLC-epsilon can be activated by Ras, and ApTrkl has a site leading to Ras activation but does not have a site for PLC-gamma (Ormond et al., 2004). Therefore, PLC-epsilon seems a strong candidate

for being critically involved in PKC Apl II activation, and the lack of PKC Apl II translocation in MNs might be explained by the lack of PLC-epsilon.

5. 2. 7. Plasticity at cell body vs. synapse

Most of the results from the chapters three and four came from the experiments examining plasticity at the cell body of *Aplysia* SNs. However, considerable evidence in *Aplysia* indicates that application of 5-HT to the SN cell body or the SN-MN synapse leads to different forms of synaptic plasticity. Single application of 5-HT at the synapse, but not at the cell body, induces STF (Martin et al., 1997b; Casadio et al., 1999). Moreover, five applications of 5-HT to the synapse induce LTF that lasts 72 hrs accompanying structural changes such as increased varicosities, while the same treatment to the cell body only induces LTF at 24 hrs but not 72 hrs and does not accompany structural changes (Casadio et al., 1999).

ApTrkl is required for ERK activation and LTF induced by 5-HT (Ormond et al., 2004). However, although 5 min application of 5-HT is sufficient to activate ApTrkl at the SN cell body, it cannot induce LTF. Therefore, activation of ApTrkl itself is not capable of inducing long-term plasticity and likely to require additional signal(s) for long-term plasticity. Sensorin is released from SNs when contacted with MNs and regulates changes accompanying long-term changes in synaptic plasticity (Hu et al., 2004b). Although shown not to be required for 5-HT-induced activation of ApTrkl at the SN cell body, sensorin

might act as an additional signal required for ERK activation and LTF through the release from SN terminals, which might be able to activate local, synaptic ApTrkl.

PKC Apl II is translocated both at the SN cell body and synaptic regions (Farah et al., 2008), and mediates reversal of synaptic depression (Manseau et al., 2001). Although reversal of depression takes place at the SN-MN synapse, degree of translocation of PKC Apl II at the SN cell body seems to correlate with that of reversal of synaptic depression with drug treatments such as spiperone and genistein. It is thus suggested that action of PKC Apl II at the cell body is indicative of events taking place at the synapse.

5. 3. Future direction

5. 3. 1. Regulation of ApTrkl

Now that we believe to have all 5-HT receptors in *Aplysia*, it will be interesting to see which receptor(s) is coupled to ApTrkl. This can be done by examining colocalization of a 5-HT receptor with ApTrkl by overexpression in SF9 cells. We have premilinary data that ApTrkl is colocalized with 5-HT_{1Ap} or 5-HT_{7Ap} but not with 5-HT_{2Ap}. This has to be confirmed biochemically by performing co-immunoprecipitation experiments for ApTrkl and 5-HT receptors. Change in ERK activation can be used as a measure of downstream activation of ApTrkl by 5-HT receptors, which can be done using antibodies to phosphorylated ERK. It will be important to examine regulation or internalization of ApTrkl by 5-HT GPCRs. Also, we need to elucidate the mechanism of methiothepin-induced

novel coupling of 5-HT GPCRs to ApTrkl. Finally, we can test a new model, arising from the chapter four, that ApTrkl is required for the activation of PKC Apl II, which can be tested using a kinase-dead (KR) mutant or by knocking down the receptor using siRNA or dsRNA.

5. 3. 2. Activation of PKC Apl II

There are several things that can be done to complete and follow up the current study. (1) It is important to test the requirement of 5-HT_{2Ap} and 5-HT_{7Ap} for PKC Apl II translocation as well as for reversal of synaptic depression with a different approach than antagonists. This can be done by knocking down the receptors including 5-HT_{1Ap} with siRNA or dsRNA. Because of the possible involvement of ApTrkl in activating PKC Apl II and preliminary data suggesting the colocalization of ApTrkl with 5-HT_{1Ap} and 5-HT_{7Ap}, the role for 5-HT_{1Ap} in activating PKC Apl II in neurons cannot be excluded. (2) Requirement of PLC and PLD for the translocation of PKC Apl II by 5-HT_{7Ap} is a novel finding and thus its mechanism needs to be examined. We can use a cAMP inhibitor to test if it is mediated through cAMP-activated GEF such as Epac. (3) Testing the role of full length 5-HT_{2Ap} will be necessary to see if there is any function for the spliced region in the third intracellular loop of 5-HT_{2Ap}. (4) Cloning of PLC-epsilon will be a next step: we can then overexpress PLC-epsilon in MNs and see if that can induce the translocation of PKC Apl II.

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